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Time domains of hypoxia adaptation: Evolutionary insights and applications, volume II

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Time domains of hypoxia adaptation: Evolutionary insights and applications, volume II

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Influence of Exposure at Different Altitudes on the Executive Function of Plateau Soldiers—Evidence From ERPs and Neural Oscillations

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This study investigates the changes in soldiers' brain executive function at different altitude environments and their relationship with blood oxygen saturation. Stratified sampling was conducted in different altitude 133 active-duty soldiers who were stationed in Weinan (347 m, $n = 34$), Nyingchi (2,950 m, $n = 32$), Lhasa (3,860 m, $n = 33$), and Nagqu (4,890 m, $n = 34$) for 2 years. The Go/NoGo paradigm with event-related potentials (ERPs) and event-related oscillations (EROs) was used to explore the time and neural oscillation courses of response inhibition. Behavioral results revealed that at the 4,890-m altitude area, the soldiers had the highest false alarm rate, the longest reaction time, and the slowest information transmission rate. The electrophysiological results revealed that NoGo-N2 and N2d decreased with increasing altitude, with significant changes at 3,860 m; the amplitudes of NoGo-P3 and P3d in plateau groups were significantly more negative than the plain and changed significantly at 2,950 m. The results of correlation analysis showed that NoGo-P3 was negatively correlated with altitude ($r = -0.358$, $p = 0.000$), positively correlated with SpO_2 ($r = 0.197$, $p = 0.041$) and information translation rate (ITR) ($r = 0.202$, $p = 0.036$). P3d was negatively correlated with altitude ($r = -0.276$, $p = 0.004$) and positively correlated with ITR ($r = 0.228$, $p = 0.018$). N2d was negatively correlated with ITR ($r = 0.204$, $p = 0.034$). The power spectrum analysis of NoGo-N2 and NoGo-P3 showed that the power of δ and θ bands at the plateau area was significantly lower than the plain area and showed a significant step-by-step decrease; the α-band power increases significantly only in the area of 4,890 m. The effect of chronic hypoxia exposure at different altitudes of the plateau on the response inhibition of soldiers was manifested: 3,860 m was the altitude at which the brain response inhibition function decreased during the conflict monitoring stage, and 2,950 m was the altitude at which it dropped during the response inhibition stage. In addition, the soldier's brain's executive function was closely related to SpO_2, and a reduction in SpO_2 may lead to a decline in response inhibition.

Keywords: high altitude, hypobaric hypoxia, SpO_2, response inhibition, military personnel

INTRODUCTION

The plateau is in an important strategic military position. Hypobaric and hypoxia are the plateau environment's main characteristics. The brain is the most sensitive organ to hypobaric hypoxia exposure (Heinrich et al., 2019), which can have a significant impact on a variety of cognitive functions, such as long-term exposure that may lead to a decrease in response inhibition (Wang et al., 2014; Davis et al., 2015; Nation et al., 2017).

Neuroimaging studies have proved that high-altitude environment can have significant effects on the human brain. Functional magnetic resonance imaging (fMRI) studies have shown that the activation of the prefrontal cortex (PFC) and anterior cingulate cortex (ACC) is related to response inhibition (Yan et al., 2010). An analysis of the Go/NoGo task found that a significant neural network, including the right superior middle/inferior frontal gyrus, the right lower parietal lobe, and the medial frontal gyrus, was associated with response inhibition (Buchsbaum et al., 2005). Besides, in the study of chronic hypoxia using magnetic resonance imaging (MRI), it was found that a group of subjects born and living at high altitudes had structural changes in the inferior frontal gyrus, middle gyrus, and anterior cingulate cortex (Yan et al., 2011). From altitude on the anterior cingulate cortex and middle frontal gyrus, the response inhibition control-related cortex will be affected, indicating that the plateau's response inhibition control was affected. Response inhibition control is an essential part of cognitive ability and a necessary executive function aspect (Schlaghecken and Eimer, 2006). It focuses on limited cognitive resources on specific target tasks, suppresses some dominant responses, and reduces the impact of habitual behavior or other interference stimuli on target tasks (Palmwood et al., 2017). Then, it improves the efficiency of completing the target task and increases the probability of success. If the individual was affected by the disorder of inhibitory control, there might be behavior out of control and a series of impulsive behaviors. Event-related brain potentials (ERPs) reflect the sum of the brain's neuroelectric responses to specific events with temporal resolution on milliseconds' order (Vinet and Zhedanov, 2011). In Go/NoGo paradigms, the subjects are instructed to respond to repetitive Go stimuli and refrain from responding to infrequent NoGo stimuli (Cheng et al., 2019). There are two main electrophysiological components associated with response inhibition: N2 and P3 (Folstein and Van Petten, 2008; Pires et al., 2014; Fogarty et al., 2018).

Although some of the existing studies focus on the ERPs phase locked in the time domain, little research on the non-phase-locked nerve oscillation reflects the energy change in electroencephalogram (EEG) rhythm when the stimulus was locked. The ongoing information processing can reduce or block the brain waves' amplitude in the α and β rhythms, which is called event-related desynchronization (ERD). Studies have found a positive correlation between ERD intensity and cortical excitation (Daly et al., 2018). ERD activity indicates that the brain is in a state of activation or excitement. The α and β rhythms show increased concussion, called event-related synchronization (ERS) (Pfurtscheller et al., 1997). ERS activity indicates that the brain is in an inactive or resting state. Depending on the characteristics of work and the brain's shape, ERD and ERS can be induced by external or internal events and form a specific brain map distribution. Previous studies have confirmed that ERD and ERS phenomena appear in the process of cognitive information processing (Vázquez-Marrufo et al., 2017).

Therefore, is there no direct evidence that long-term exposure to high-altitude weather affects response inhibition control? If so, is there a regular pattern of the effect of different elevations on response inhibition control? A high-altitude environment can also lead to changes in blood oxygen saturation (SpO_2) (Brutsaert et al., 2000), and the partial pressure of oxygen decreases with the increase in height. The partial pressure of oxygen in the artery also decreases, leading to a decrease in the artery's SpO_2 (Bernardi et al., 2017). However, the relationship between SpO_2 and executive brain function at different elevations remains to be studied.

Therefore, in this study, the Go/NoGo paradigm with event-related potentials (ERPs) and event-related oscillations (EROs) was used to investigate the dynamic changes in brain response inhibition and neuron characteristics oscillation at different altitudes, as well as the relationship between physiological indicators and cognitive components. We hypothesized that the effects of varying altitude plateaus on soldiers' inhibitory control and non-attentive tasks show a progressively decreasing trend change with increasing altitude.

SUBJECTS AND METHODS

Participants

Self-compiled general demographic questionnaire was used to register the subject's demographic information, including name, gender, birth date, place of origin, place of residence before enlistment, and education level. A total of 133 soldiers, who were stationed in Weinan ($n = 34$), Nyingchi ($n = 32$), Lhasa ($n = 33$), and Nagqu ($n = 34$) for 2 years, were invited in this study. The mean age of the participants was 22.21 years ($SD = 0.27$). There were no statistical differences for age, body mass index (BMI), and education level among the four groups. More details for the information can be found in **Figure 1**.

Common inclusion criteria were the following: (1) male, high school education, right-handed (Annett, 1970), normal eye vision or corrected vision, and normal BMI; (2) no head trauma, no physical or mental illness; and (3) no alcohol, drugs, or narcotics within 1 week; and (4) informed consent to participate.

Inclusion criteria for the plateau group (Nyingchi, Lhasa, and Nagqu) were as follows: (1) birthplace was a low-altitude area (<500 m) after stationed in the current altitude area and (2) permanent residence in the current altitude area for 24 months. Exclusion criterion was the experience of going to the plateau before stationing or returning to the plains during stationing. The plain group's inclusion criteria (Weinan) were that the birthplace and stationing place are low altitude (<500 m). Exclusion criterion was an experience of going to the plateau.

The study was conducted following the Declaration of Helsinki, and all procedures were carried out with the adequate

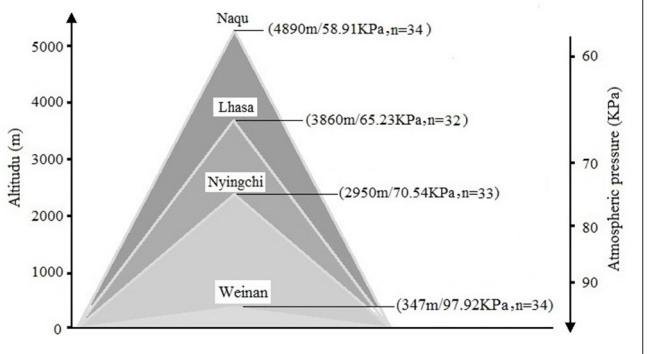

FIGURE 1 | Information on different altitude areas.

understanding and written informed consent of the subjects. The study protocol was approved by the Ethics Committee of the Fourth Military of Medical University.

General Demographic Information Statistics

Measurement of SpO_2 and HR

The participants' SpO_2 and heart rate (HR) were measured by an oxygen saturation meter (Kehui Covidien, American). The participants were asked to rest for at least 15 min before measuring. The subjects' right index finger was ensured to be dry and clean without accessories before testing. When calculating, the excessive sunlight affecting the fingers' temperature was avoided, and cold was prevented to ensure the fingers' warmth. When the subjects were breathing calmly, the portable fingertip oxygen saturation meter was used to clip the right hand's index finger. The values of SpO_2 and HR were read after the reading was stable for at least 10 s.

Stimuli and Procedures

Participants were seated in a comfortable experimental laboratory and exposed to limited sound and appropriate light. The stimuli were presented on a computer screen ∼75 cm away from the participant, with a visual angle of $4^\circ \times 4^\circ$. Visual stimuli included single and double triangles in the gray background, presented on a computer screen (light degree = 60 cd/m^2). There were four blocks with 60 Go (double triangles) and 40 NoGo (single triangle) stimuli for each. Before EEG recording, participants performed two practice blocks consisting of 40 Go and NoGo trials to ascertain that their operation was correct. The behavior data such as accuracy rate (AR), false alarm rate (FAR), and reaction time (RT) were collected using E-prime 3.0. Each stimulus was presented for 100 ms, with the mean interstimulus intervals (ISI) being 1,200 ms (randomly between 1,000 and 1,400 ms). During the experiment, participants were instructed to watch the center of the screen, relax, and minimize eye blinks or body movements.

Information transfer rate (ITR) was the standard measurement method of measuring communication and control systems. It refers to the amount of data transmitted per unit of time (Pierce and Epling, 1980). ITR has been widely used in the evaluation of the cognitive attention system. Wolpaw (2002) suggested that ITR be used to evaluate the brain–computer interface's performance. Thus, in this study, ITR was used to assess the effects of cognitive processing rate and hypoxia on soldiers' brains at different altitudes.

ITR is calculated as follows:

$$ITR = \left\{ \log_2 N + P \log_2 P + (1-P) \log_2 \frac{1-P}{N-1} \right\} / T$$

T is the reaction time (RT), N is the total number of stimuli, and P is the accurate rate (AR).

EEG Acquisition

Brain electric activity was measured from 34 channels using a modified 10–20 system electrode cap (Beijing Yiran Sunny Co. Ltd., www.yiransunny.com.cn). All EEGs were continuously sampled at 1,000 Hz with a left mastoid reference and a forehead ground and referenced offline to an averaged reference derivation by mathematically combining the left and right mastoid. The vertical electrooculogram (EOG) was recorded with electrodes placed above and below the left eye, and the horizontal EOG was recorded with the electrodes placed outboard of both eyes. All electrode impedances were maintained below 5 kΩ. EEG and EOG were amplified using a 0.05–100 Hz bandpass and continuously sampled at 1,000 Hz/channel for offline analysis. EEGs contaminated with artifacts due to amplifier clipping, bursts of electromyographic (EMG) activity, or peak-to-peak deflection exceeding ±75 μV were excluded from trials.

Event-Related Potential Analysis

Raw EEG data preprocessing was performed using EEGLAB software (Version R2013b, San Diego, USA), an open-source toolbox running MATLAB (Version R2013b, MathWorks, United States). The processing process consisted of bilateral mastoid reference and a bandpass filter with a low cut of 0.5 Hz and a high cut at 30 Hz. Eye movement artifacts were corrected using individual independent component analysis (ICA) by removing the corresponding components based on the particular activation curve (Jung et al., 2000; Li et al., 2006). Segments that contained excessive amplitudes (±75) were marked and rejected. The averaged epoch for ERP was 1,000 ms, including a 200-ms prestimuli baseline. The EEGs associated with each stimulus to which subjects correctly responded were overlapped and averaged for each condition.

The peak amplitudes (baseline to peak) of visual N2 and P3 waves were measured in 200–340 ms and 340–430 ms time windows. Furthermore, difference waves were computed by subtracting the average Go-ERP from the average NoGo-ERP for midline electrodes for each condition. As in previous studies (Liu et al., 2009; Zhang et al., 2011), the five electrodes were chosen for statistical analysis: Fz, FCz, Cz, CPz, and Pz. The amplitudes were evaluated by selecting the peak amplitude and computing the mean amplitude over a time window of ±50 ms. The latency onsets were measured with fractional peak latency.

TABLE 1 | Behavior results of participants for Go and NoGo stimulations.

Groups	Go			NoGo		
	Correct rate (%)	RT (ms)	ITR (bps)	False alarm rate (%)	RT (ms)	ITR (bps)
Weinan ($n = 34$)	58.33 ± 11.28	153.35 ± 44.31	19.49 ± 2.66	20.58 ± 9.73	143.63 ± 76.29	6.63 ± 5.92
Nyingchi ($n = 33$)	62.20 ± 13.72	174.73 ± 54.57	18.85 ± 3.23	21.97 ± 10.94	141.00 ± 71.78	7.27 ± 6.17
Lhasa ($n = 32$)	62.70 ± 13.23	192.50 ± 75.91	18.87 ± 6.69	24.48 ± 10.88	135.77 ± 65.56	5.07 ± 3.16
Naqu ($n = 34$)	56.27 ± 12.94	204.80 ± 83.73*	17.10 ± 10.27	31.75 ± 16.77**##	185.40 ± 54.82#&	12.44 ± 11.19**#&&

*vs. Weinan: *$p < 0.05$, **$p < 0.01$; vs. Nyingchi: #$p < 0.05$, ##$p < 0.01$; vs. Lhasa: &$p < 0.05$, &&$p < 0.01$.*

Event-Related Spectral Perturbation Analysis

The EEG data sampling rate was reduced to 500 Hz, and short-time Fourier transform (STFT) in Matlab was used to analyze EEG data's neural concussion. STFT divides the signal into many identical small-time intervals by window function and uses Fourier transform to explore each time interval to determine the time interval frequency and get a series of variation results of signals in the frequency domain. The baseline of (−200 0) ms corrects the oscillation energy value before stimulation. This study focused on the successful inhibition of N2 and P3 components in NoGo stimulation. STFT was used to calculate the power in five frequency bands: δ (1–4 Hz), θ (4–8 Hz), α (8–14 Hz), β (14–30 Hz), and γ (30–45 Hz).

Statistical Analysis

All the statistical analyses were performed by SPSS (23.0; SPSS, Inc., Chicago, IL, United States). Kolmogorov–Smirnov test was used to test the normal distribution of each group of data. Comparisons data from the four altitude groups were made using mixed analysis of variance (ANOVA) for normally distributed data. One-way analysis of variance with elevation (Weinan/Nyingchi/Lhasa/Naqu) as a between-subjects factor was conducted for behavior results and physiology indexes. Three-way analysis of variance with elevation (Weinan/Nyingchi/Lhasa/Naqu) as a between-subjects factor and condition (Go, NoGo, NoGo–Go), and electrodeposition (Fz, FCz, Cz, CPz, and Pz) as a within-subjects factor for ERP results. Three-way analysis of variance with elevation (Weinan/Nyingchi/Lhasa/Naqu) as a between-subjects factor and five frequencies bands (δ, θ, α, β, and γ) and electrodeposition (Fz, FCz, Cz, CPz, and Pz) as a within-subjects factor for event-related spectral perturbation (ERSP) results. A p-value of 0.05 was considered a significant consequence. The Bonferroni-corrected method was performed for *post-hoc* testing of significant main effects. Effect size in all ANOVA analyses was reported by partial eta-squared (η^2), where 0.05 represents a small effect, 0.10 represents a medium effect, and 0.20 represents a large effect (Faul et al., 2007).

RESULTS

Behavior Results and Physiology Measures

The behavioral outcomes can be seen in **Table 1**. For the Go stimuli, the RT was significantly increased in the Naqu group compared with the plain area ($p < 0.01$). Meanwhile, the false alarm rate and ITR were markedly enhanced in the Naqu group than in the plain group for the NoGo stimuli ($p < 0.01$). The false alarm rate, RT, and ITR in the Naqu group increased than that in the Nyingchi or Lhasa groups ($p < 0.05$ or 0.01) for the NoGo stimuli.

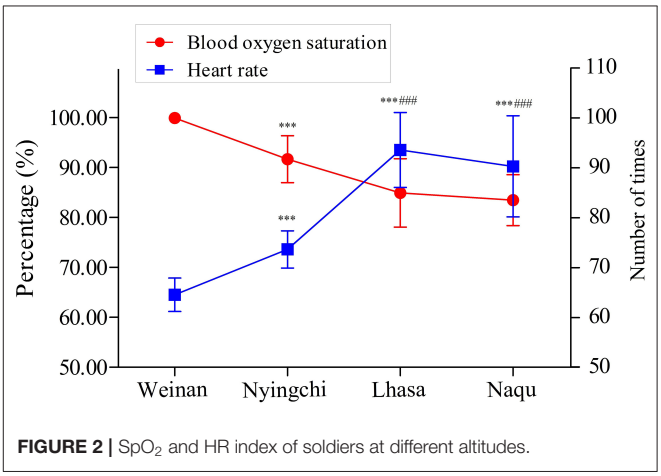

FIGURE 2 | SpO_2 and HR index of soldiers at different altitudes.

The SpO_2 and HR of soldiers from four areas are shown in **Figure 2**. The SpO_2 percentage was significantly decreased in plateau groups (Nyingchi, Lhasa, and Naqu) compared with the plain group ($p < 0.001$). On the contrary, the soldiers' HR in the three plateau groups markedly increased than that in the plain group ($p < 0.001$). In addition, the SpO_2 percentage and HR of soldiers in Lhasa and Naqu presented the same changes as Nyingchi ($p < 0.001$). However, no significant difference was found between Lhasa and Naqu.

ERP Results

The Amplitude and Peak Latency of N2

N2 Amplitude

A 4(altitude) × 3(trial type) × 5(electrode distribution) mixed analysis of variance was conducted for the peak amplitudes of N200. The main effect of the electrodes was significant [$F_{(4, 1,455)} = 9.352$, $p < 0.000$, $MSE = 86.49$, $\eta^2 = 0.025$]. *Post-hoc* analysis revealed that the amplitude of N2 were larger in electrode Fz showing the minimum amplitude ($M = 2.443$ μV, $SE = 0.273$ μV) and electrode CPz showing the maximum amplitude ($M = 4.600$ μV, $SE = 0.259$ μV).

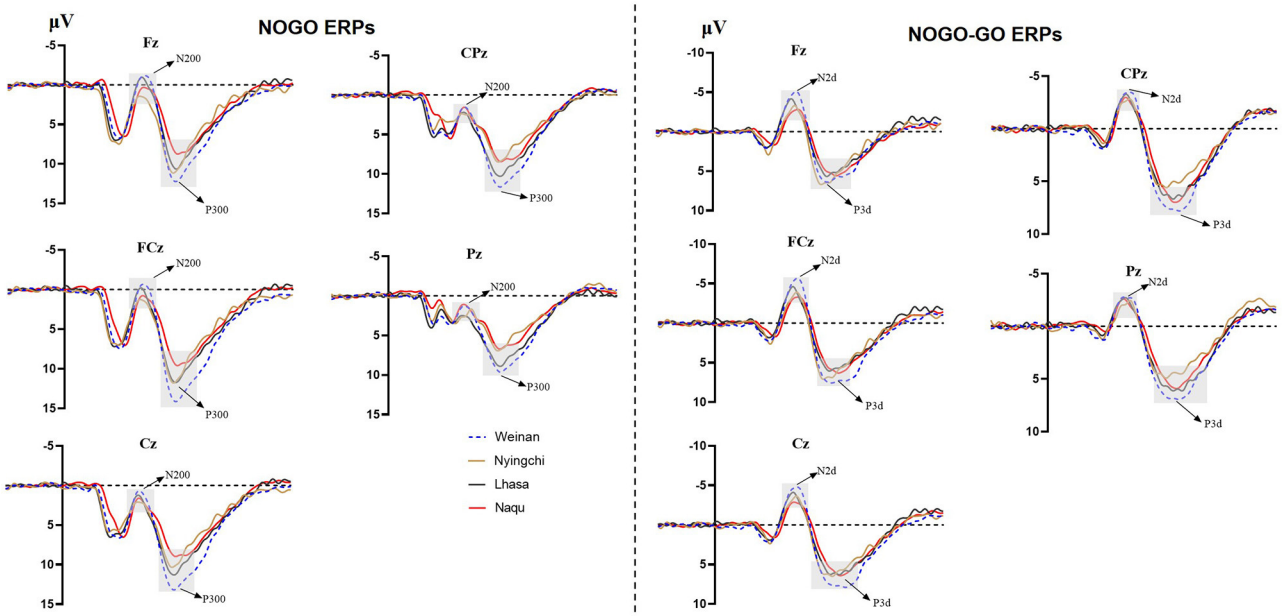

FIGURE 3 | Total average ERP waveform at different altitudes.

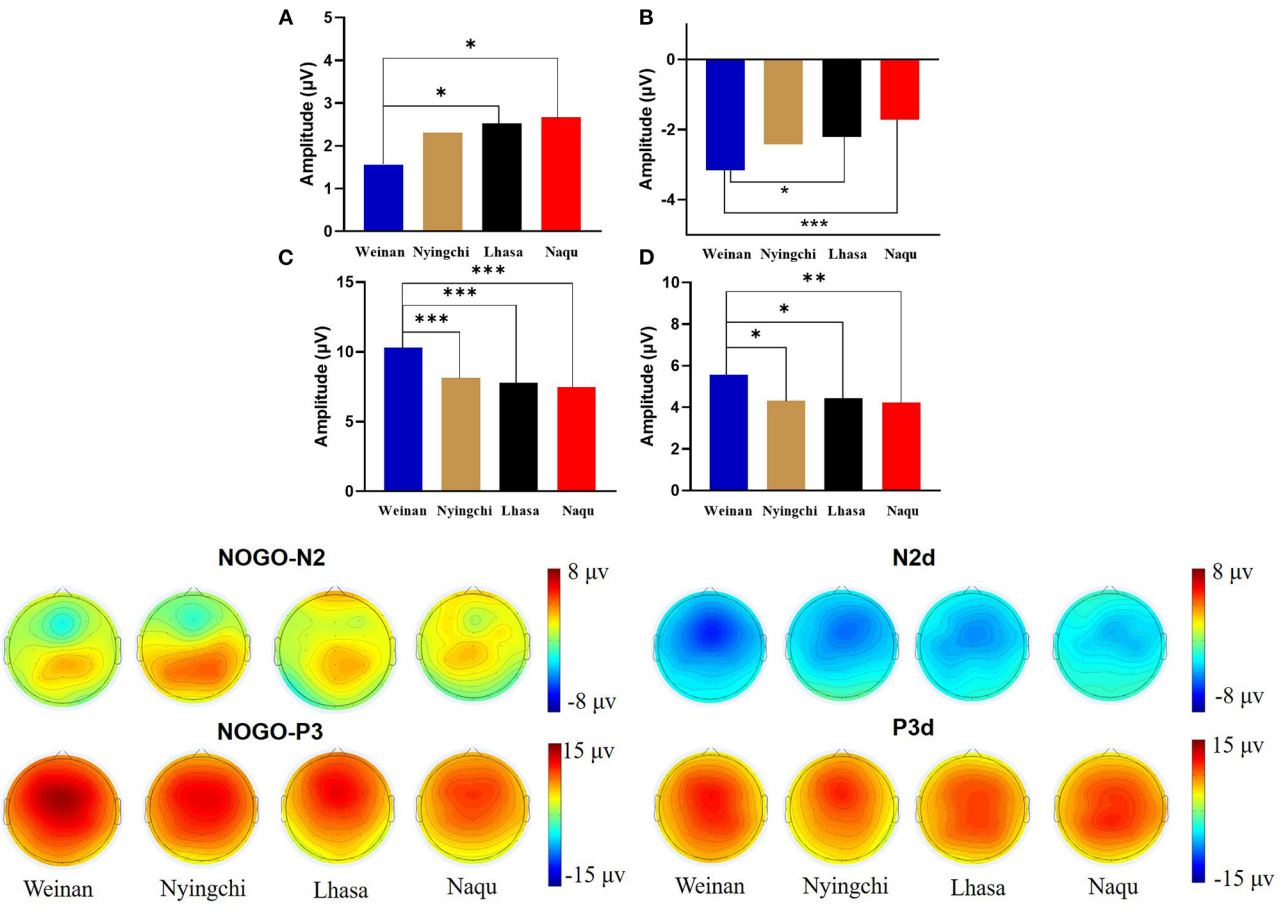

FIGURE 4 | Topographic map of ERP composition at different altitudes.

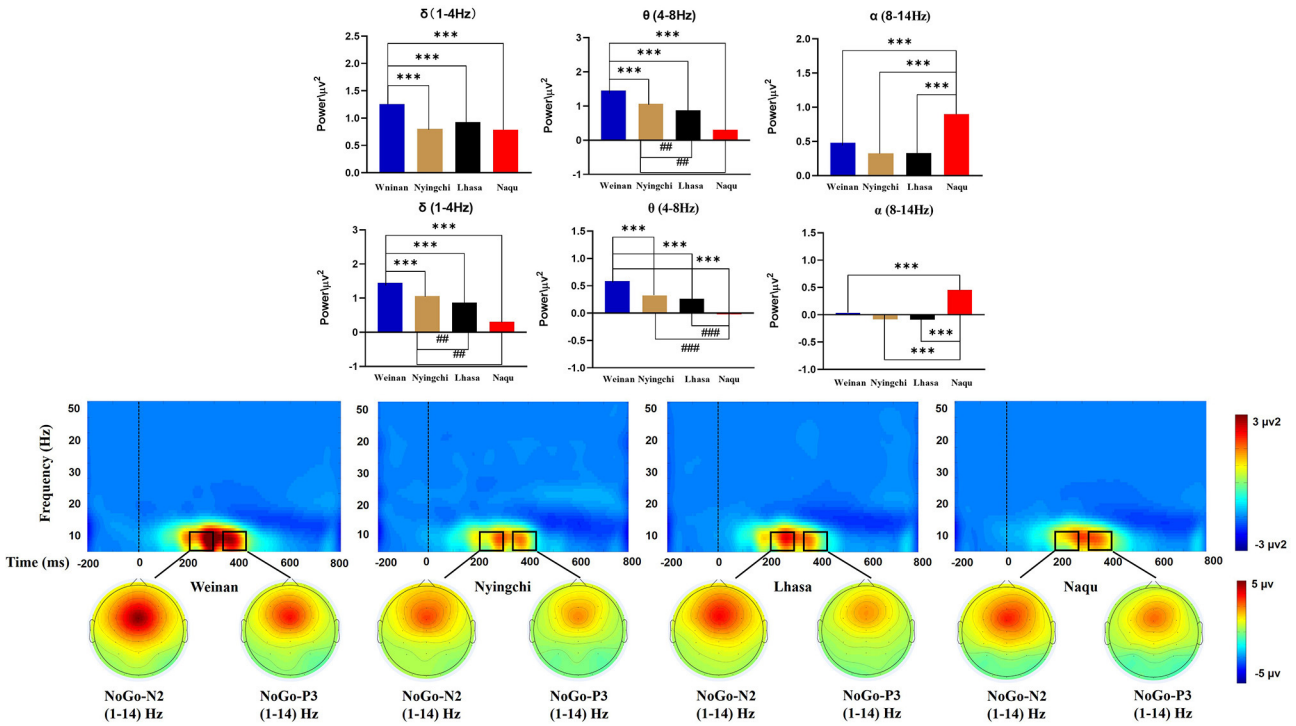

FIGURE 5 | Time-frequency diagram of Nogo stimulation at different altitudes.

The results revealed interaction effects between altitude and trial type [$F_{(6,\ 1{,}455)} = 15.50$, $p < 0.000$, $MSE = 143.38$, $\eta^2 = 0.05$]. Simple main effect analysis found that there was no significant difference under Go condition ($p = 0.527$), but there was difference under NoGo trial [$F_{(24,\ 1{,}455)} = 2.923$, $p = 0.033$, $MSE = 27.036$, $\eta^2 = 0.006$] and a significant difference in difference wave N2d [$F_{(24,\ 1{,}455)} = 42.73$, $p < 0.000$, $MSE = 394.95$, $\eta^2 = 0.081$]. Further analyses revealed that at NoGo trial, the amplitudes of N2 was positive for Naqu [$M = 2.671\ \mu V$, 95% CL (1.997, 3.064), $p = 0.019$] and Lhasa [$M = 2.528\ \mu V$, 95% CL (2.033, 4.720), $p = 0.022$] regions than that for the plain [$M = 1.557\ \mu V$, 95% CL (1.023, 2.09)]. The amplitudes of N2d for the plain group [$M = -3.617\ \mu V$, 95% CL (−4.239, −3.103)] were negative than that for Lhasa [$M = -2.235\ \mu V$, 95% CL (−2.730, −1.704), $p = 0.017$] and Naqu [$M = -1.906\ \mu V$, 95% CL (−2.44, −1.373), $p < 0.000$].

There was no interaction effect between altitude and electrodes [$F_{(12,\ 1{,}455)} = 0.618$, $p = 0.829$, $MSE = 5.714$, $\eta^2 = 0.005$], no interaction effect between electrodes and trial type [$F_{(8,\ 1{,}455)} = 1.588$, $p = 0.124$, $MSE = 14.683$, $\eta^2 = 0.009$], or no triple interaction effects among three variables [$F_{(24,\ 1{,}455)} = 0.276$, $p = 1.00$, $MSE = 2.548$, $\eta^2 = 0.005$] (**Figure 3**).

N2 Latency

A 4(altitude) × 3(trial type) × 5(electrode distribution) mixed analysis of variance analysis revealed a significant main effect of electrode on the latencies of N2 [$F_{(24,\ 1{,}455)} = 3.702$, $p = 0.040$, $\eta^2 = 0.049$]. The *post-hoc* test suggested that the latency of N2 at Cz electrode was significantly earlier than that of Fz and FCz electrode (265.46 ± 3.48 vs. 270.52 ± 3.43, $p = 0.034$; 265.46 ± 3.48 vs. 269.08 ± 3.25, $p < 0.001$).

The analysis also revealed significant interaction effects between the altitude and trial type [$F_{(4,\ 1{,}455)} = 2.910$, $p = 0.040$, $\eta^2 = 0.108$]. Simple effect analysis indicated that there was a significant difference in the NoGo trial [$F_{(12,\ 1{,}455)} = 3.913$, $p = 0.012$, $\eta^2 = 0.140$]. The latency of N2 in the Nyingchi group was significantly earlier than that in the plain and the Lhasa groups.

The Amplitude and Peak Latency for P3

P3 Amplitude

A 4(altitude) × 3(trial type) × 5(electrode distribution) mixed analysis of variance was conducted for the peak amplitudes of P3. The main effect of electrodes was significant [$F_{(4,\ 1{,}037)} = 7.602$, $p < 0.000$, $\eta^2 = 0.028$]. The amplitudes of P3 were positive, with electrode FCz showing the maximum amplitude ($M = 8.813\ \mu V$, $SE = 0.402\ \mu V$) and electrode Pz showing the minimum amplitude ($M = 6.250$, $SE = 0.405\ \mu V$). P3d amplitude were larger, with electrode Fz showing the maximum amplitude ($M = 6.979\ \mu V$, $SE = 0.337\ \mu V$) and electrode Pz showing the minimum amplitude ($M = 3.409$, $SE = 0.334\ \mu V$).

The results also showed that the interaction effects between altitude and stimulation type were significantly different [$F_{(6,\ 1{,}455)} = 17.361$, $p < 0.000$, $MSE = 280.611$, $\eta^2 = 0.067$]. The simple main effect analysis found that there was a significant difference for three conditions at different altitudes [$F_{(3,\ 1{,}455)} = 4.583/74.857/13.88$, $p < 0.01$]. A further analysis revealed that

at Go trial, the amplitude of P3 in the plain group [$M = 4.716$ μV, 95% CL (4.01, 5.421)] was significantly positive than that in Nyingchi [$M = 3.202$ μV, 95% CL (2.45, 3.954)] and Naqu [$M = 3.225$ μV, 95% CL (2.54, 3.93), $p = 0.024$] groups. In NoGo trial, the amplitude of P3 in the plain group [$M = 10.295$ μV, 95% CL (9.59, 11.00)] was significantly positive than that in Nyingchi [$M = 7.720$ μV, 95% CL (6.968, 8.472)], Lhasa [$M = 7.799$ μV, 95% CL (6.652, 8.37)], and Naqu groups [$M = 7.464$ μV, 95% CL (6.759, 8.17)], $p < 0.000$. The amplitude of P3d were significant positive for the plain [$M = 6.114$ μV, 95% CL (5.408, 6.819)] than for the Nyingchi [$M = 7.720$ μV, 95% CL (6.968, 8.472), $p = 0.013$], Lhasa [$M = 7.799$ μV, 95% CL (6.652, 8.37), $p = 0.013$], and Naqu [$M = 4.574$ μV, 95% CL (3.868, 5.279), $p = 0.009$] (**Figure 4**).

There was no interaction effect between altitude and electrodes [$F_{(12, 1,455)} = 0.963$, $p = 0.403$, $MSE = 6.51$, $\eta^2 = 0.003$), no interaction effect between trial type and electrodes [$F_{(8, 1,455)} = 1.435$, $p = 0.177$, $MSE = 23.191$, $\eta^2 = 0.008$], or no triple interaction effects among three variables [$F_{(24, 1,455)} = 0.059$, $p = 1.00$, $MSE = 0.956$, $\eta^2 = 0.005$].

P3 Peak Latency

A 4(altitude) × 3(trial type) × 5 (electrode distribution) mixed analysis of variance analysis revealed a significant main effect of altitude for the latencies of P3 [$F_{(6, 14,455)} = 3.399$, $p = 0.038$, $\eta^2 = 0.124$]. The *post-hoc* analysis test suggested that the latency of P3 in the Nyingchi group was significantly earlier than that of the plain, Lhasa, and Naqu groups (355.51 ± 8.15 vs. 386.13 ± 7.52, 378.53 ± 7.52, 387.23 ± 7.71, $p = 0.007$, $p = 0.042$, $p = 0.006$). The analysis also revealed a significant interaction effects between altitude and trial type [$F_{(24, 14,455)} = 4.210$, $p = 0.008$, $\eta^2 = 0.149$]. Further analysis indicated that at the Go task, the latency of P3 in the Nyingzhi group was significantly earlier than that in the other three groups ($p < 0.001$, $p = 0.011$, $p = 0.026$). At the NoGo task, the latency of P3 in the Nyingzhi group was significantly earlier than that in the other three groups ($p < 0.001$). Other main effects and interaction effects were not significant.

Correlation Analyses Between Behavioral Data and ERP Components

Pearson correlation analysis revealed that NoGo-P3 and P3d were negatively related to altitudes ($r = -0.358$, $p < 0.000$) and ($r = -0.276$, $p = 0.004$), respectively, and the NoGo-P3 was positively related to SpO_2 ($r = 0.197$, $p = 0.041$). Correlation analysis also revealed that Go-P3, NoGo-P3, N2d, and P3d were positively related to ITR ($r = 0.203$, $p = 0.036$), ($r = 0.202$, $p = 0.036$), ($r = 0.204$, $p = 0.034$), ($r = 0.228$, $p = 0.018$). The results are shown in **Table 2**.

The Results of Frequency Domain (ERO)

N2

A 4(altitude) × 5(frequency band) × 5(electrodes) mixed ANOVA yielded no triple interaction effects [$F_{(48, 2,275)} = 1.252$, $p = 0.116$, $MSE = 0.449$, $\eta^2 = 0.026$]. The results of analysis revealed a significant interaction effect between the altitude and frequency band [$F_{(12, 2,275)} = 24.599$, $p < 0.000$, $MSE = 8.821$, $\eta^2 = 0.559$]. Simple effect analysis found that in the δ, θ, and α bands, there was a significant difference for N2 at different altitudes [$F_{(3, 2,275)} = 16.359/78.98/24.672$, $p < 0.000$]. A further analysis indicated that the power of δ band in the plain group was the highest [$M = 1.258$, 95% CL (1.153, 1.363)], $p < 0.000$], which was significantly larger than that in the Nyingchi [$M = 0.805$, 95% CL (0.693, 0.917)], Lhasa [$M = 0.926$, 95% CL (0.817, 1.036)], and Naqu [$M = 0.786$, 95% CL (0.681, 0.891)] groups. The power of θ band in plain group was the highest [$M = 1.453$, 95% CL (1.347, 1.558), $p < 0.000$], which was significantly larger than that in Nyingchi [$M = 1.062$, 95% CL (0.952, 1.171)], Lhasa [$M = 0.871$, 95% CL (0.759, 0.983)], and Naqu [$M = 0.307$, 95% CL (0.202, 0.412)]. However, the power of α band in the Naqu group was the highest [$M = 0.9$, 95% CL (0.795, 1.01), $p < 0.000$], which was significantly higher than that in the plain group [$M = 0.479$, 95% CL (0.374, 0.584)], Nyingzhi [$M = 0.329$, 95% CL (0.217, 0.441)], and Lhasa [$M = 0.324$, 95% CL (0.215, 0.434)]. The results of analysis also revealed a significant interaction effect between the electrodeposition and frequency band [$F_{(16, 2,275)} = 14.453$, $p < 0.000$, $MSE = 5.183$, $\eta^2 = 0.092$]. Simple effect analysis found that the power of δ, θ, and α bands was the highest at the FCz electrode, decreased gradually at the Fz, Cz, and CPz electrode, and dropped up to the minimum Pz electrode (**Figure 5**).

P3

A 4(altitude) × 5(frequency band) × 5(electrodes) mixed ANOVA yielded no triple interaction effect [$F_{(48, 2,275)} = 1.073$, $p = 0.341$, $MSE = 0.143$, $\eta^2 = 0.022$]. The results of the analysis yielded significant interaction effects between the altitude and frequency band [$F_{(12, 2,275)} = 29.044$, $p < 0.000$, $MSE = 3.874$, $\eta^2 = 0.133$]. Simple main effect analysis found that in the δ, θ, and α bands, there was a significant difference for P3 at different altitudes [$F_{(3, 2,275)} = 14.741/58.743/59.699$, $p < 0.000$]. The power of δ band in the plain group [$M = 0.657$, 95% CL (0.592, 0.721)] was significantly larger than that in the Nyingchi [$M = 0.38$, 95% CL (0.311, 0.448)], and Lhasa [$M = 0.419$, 95% CL (0.353, 0.486)], $p < 0.000$. Naqu [$M = 0.38$, 95% CL (0.311, 0.448)] was significantly larger than Nyingchi and Lhasa, $p < 0.05$. The power of θ band in the plain group was the highest [$M = 0.589$, 95% CL (0.525, 0.653), $p < 0.000$], which was significantly larger than that in Nyingzhi [$M = 0.261$, 95% CL (0.193, 0.33)], Lhasa [$M = 0.323$, 95% CL (0.256, 0.389)], and Naqu [$M = -0.021$, 95% CL (−0.085, 0.043)]. The power of θ band in Naqu was the lowest, which is significantly lower than that in Nyingchi and Lhasa. However, the power of α in Naqu [$M = 0.453$, 95% CL (0.389, 0.517), $p < 0.000$], which was significantly higher than that in Weinan [$M = 0.035$, 95% CL (−0.029, 0.099)], Nyingzhi [$M = -0.091$, 95% CL (−0.159, −0.023)], and Lhasa [$M = -0.083$, 95% CL (−0.149, −0.016)].

DISCUSSION

Changes in Response Inhibition Function in Soldiers From Different Areas

In this study, physiological indexes and ERPs were used to explore the dynamic brain changes and neurons' oscillating hypoxia hypobaric environment exposure characteristics to varying altitudes on soldiers' inhibition control ability.

TABLE 2 | Results of correlation analysis.

	NoGo-N2		NoGo-P3		N2d		P3d	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Altitudes	−0.021	0.829	−0.358	**0.000**	−0.062	0.52	−0.276	**0.004**
ITR	−0.022	0.819	0.202	**0.036**	0.204	**0.034**	0.228	**0.018**
SpO_2	0.020	0.837	0.197	**0.041**	0.015	0.874	0.157	0.104

Altitudes: Weinan (347 m), Nyingchi (2,895 m), Lhasa (3,890 m), and Naqu (4,860 m).
ITR, information translation rate.
The bold indicates a significant correlation.

The results showed that the SpO_2 of soldiers in all three plateau areas was significantly lower than the plain group. The results of HR analysis showed an opposite trend to SpO_2. Studies have shown that SpO_2 was negatively correlated with stationing altitude, and HR was positively correlated with stationing altitude (Rivera-Ch et al., 2008; Hilty et al., 2016; Ottestad et al., 2017). These study results are consistent with previous studies' results, and the overall SpO_2 level and HR of the participants were in the normal range.

The classical Go/NoGo task used in this study presented button stimuli as simple visual stimuli. The RT results showed that both for Go and NoGo stimulation, there was a highly significant increase in the Naqu group compared to the plain group. However, there was no significant difference between the Nyingchi and Lhasa and the plain. The FARs of soldiers in the plateau groups were all higher than that of the plain group, but only the Nagqu and the plain group had significant differences. The analysis of ITR shows that there is no substantial change in the transmission rate from 347 to 3,860 m (Lhasa), but it decreases significantly at the altitude of 4,890 m (Naqu). Previous long-term exposure studies using different cognitive tasks have found that reaction times appear shortened at high altitudes (Richardson et al., 2011). The disappearance of the effects of prolonged exposure on behavior may be due to adaptation supported by compensatory mechanisms. However, the significant increase in the Nagqu area may be due to the decline in executive function caused by prolonged exposure. One study found that the FAR and RT increased in the highland region, indicating that hypoxia exposure led to the decline in executive function (Trompetero et al., 2015). The findings are consistent with the earlier results that the plateau's hypoxic environment has a relatively low effect on simple cognitive tasks. In contrast, the hypoxic environment may impact complex cognitive functions dominated by coordination and accuracy.

The ERPs can reflect information on the neural mechanisms of response inhibition in warriors exposed to high-altitude environments for long periods. In this study, significant response inhibition effects were successfully evoked at four groups, that is, NoGo-N2 and NoGo-P3 components induced by NoGo stimulation were successfully inhibited. Comparing the differences between the three plateau groups and one plain group revealed that the three plateau groups' soldiers showed a significant decrease in amplitude both in the N2 component of conflict monitoring and the P3 component of late processing of information. Studies have indicated that N2 and P3 are considered to function as separable processes in Go/NoGo tasks (Randall and Smith, 2011; Burkhard et al., 2018). The NoGo-N2 component response conflict detection and response inhibition can effectively represent the soldiers' cognitive control degree (Enriquez-Geppert et al., 2010; Burkhard et al., 2018). The NoGo-P3 component amplitude is associated with late processing of response inhibition (Groom and Cragg, 2015). It shows that with the increase in altitude, functional declines in soldiers' response inhibition control become more and more serious, manifested by the decrease in conflict monitoring ability and inhibition control ability in the inhibition processing stage. The amplitude of P3d also decreased significantly in the three plateau groups, especially at the Naqu group. The study showed that the amplitude of P3d was weakened considerably or even missing, suggesting that the function of inhibition control (i.e., response inhibition or movement inhibition) was declined at high altitude. In addition to the response inhibition domain, the P3 component is usually considered the late stage of information processing: the allocated attentional resources (Meinhardt and Pekrun, 2003). It has been shown that high-altitude populations have higher cognitive demands that limit attentional resources for inhibitory control (Hill et al., 2014; Hu et al., 2016). A high-altitude study using an attention task also found lower P3 amplitudes in the high-altitude group than in the low-altitude group (Wang et al., 2014). Chronic high-altitude exposure leads to reduced attentional resource availability (Nieuwenhuis et al., 2005). This study proved that the attention resources of the plateau group decreased compared with the plain control group.

Notably, among the three groups of high-altitude regions, the hypoxic environment in the Nagqu region had the most pronounced effect on the soldiers' inhibitory control function. The primary manifestation was the smallest amplitude of the P3 component of the difference wave, which is considered the neural activity induced by afferent stimuli and reflects the degree of perceptual information processing and an alteration (Godde et al., 2010; Martin et al., 2012; Walsh et al., 2020). This result suggests that a higher altitude leads to a lower degree of inhibitory control activity on information processing due to hypoxia. Studies also show that people's mental and physical working ability in the highland environment is significantly reduced along with the increase in altitude (Tong et al., 2007; Zhu and Fan, 2017). Although the P3 latency is thought to reflect a slowdown in signal processing caused by hypoxia (Ramautar

et al., 2006), there was no difference in P3 latency among groups. It may be that the relatively long exposure to high altitude in the subjects of this study may have acclimated to hypoxia. Therefore, the changes in P3 latency at high altitudes found in previous studies may depend on how long individuals live at high altitudes (Mazzeo, 2008). The amplitude of the N2 component, which is a conflict monitor, also tends to weaken, but significant differences are observed in Lhasa at the height of 3,160 m. The results are similar to previous studies' findings that under mild hypoxic conditions, the organism showed increased neural activity, but reduced availability of attentional resources produced neuroexcitatory inhibition, increased impulsivity, and reduced conflict monitoring sensitivity post-conflict processing (Pontifex et al., 2009; Turner et al., 2015; Lefferts et al., 2016; McMorris et al., 2017). The results suggest that cognitive function effects are not easily perceived under mild hypoxic conditions and when complex tasks are not performed. Mental health maintenance should be done in advance, with particular attention to strengthening emotional stability regulation.

The Variation in δ-, θ-, and α-Band Power in Response Inhibition Function

This study also explored the effects of different altitude plateau on the soldier's response inhibition function near convulsions, focusing on the conflict monitoring and response inhibition phases. Hypoxic exposure can change the neural network's spatial representation and be reflected by EEG's different nervous oscillations (Cvetkovic et al., 2004). The results showed decreased the δ- and θ-band powers of soldiers in three high-altitude areas than those in the plain area. One study showed reduced brain wave activity in the δ and θ bands in a hypoxic environment (more than 30 days) (Zhao et al., 2016). However, the opposite result was found in the simulation module simulating the anoxic environment at high altitude (23 min): the δ and θ bands' activity increased (Papadelis et al., 2007). The reason may be related to the time of hypoxic exposure. This study's subjects are soldiers stationed in various areas for 2 years, indicating that prolonged exposure to a plateau environment can affect brain activity changes. Some studies have explored brainwave activity characteristics in adolescents living at high altitudes and found that δ- and β-frequency activity decreased (Richardson et al., 2011). In this study, there is no difference in the β-frequency activity of soldiers in different regions.

Studies have shown that EEG activity decreases at the beginning of an acute hypoxic exposure and increases with the emergence of α-band activity (Schellart and Reits, 2001; Papadelis et al., 2007). This study shows that the α-band power activity in the conflict monitoring phase of response inhibition function decreases at Nyingchi and Lhasa. There was no significant difference compared with the plain area. However, the α-band power at Naqu was significantly higher than that at the other three groups. This change of power in the specific frequency band of EEG is caused by a decreased synchronization of related nerve oscillations in the cortex (Pfurtscheller and Aranibar, 1979). α-Rhythm synchronization means that this region of the brain is resting or dormant (Pfurtscheller and Aranibar, 1979; Pfurtscheller et al., 1997). α-Rhythmic ERD phenomenon can reveal some pathological states to some extent: some studies have found that Parkinson's patients have α- and β-band ERD phenomenon, showing a hyperactive motor cortex (Heida et al., 2014). Combined with the behavioral results, the above studies further show that soldiers' low information transmission speed in the Naqu was related to weakening of the α-band ERD phenomenon. The study also found that the α-band power in the conflict detection stage of response inhibition was more active than that in the response inhibition stage, indicating that long-term hypobaric hypoxia (HH) exposure caused long-term excitation of the central nervous system, resulting in increased brain loss and gradual decline inactivity. This further reveals that soldiers need to consume more cognitive resources to complete tasks in an anoxic environment.

Several limitations of the present study should be noted. In this study, all the subjects lived at the site for more than 24 months and completed the process of acclimatization at a high altitude. As the experiment was carried out in spring, Nyingchi and Lhasa's oxygen contents are relatively abundant. The sample size involved in the study is relatively small, so the research results may be affected and have some limitations. Second, we did not measure any cerebral oxygen delivery parameters, such as cerebral oxygenation and cerebral blood flow. Future studies should directly measure cerebral oxygenation, cerebral blood flow, and cerebral oxygen delivery and clarify the mechanism of decreased executive function under hypoxia (Ochi et al., 2018).

CONCLUSION

In summary, this study found that chronic hypoxia exposure at different altitudes of plateau affects response inhibition, mainly in the conflict monitoring phase and post-processing phase. Further analysis revealed that 3,860 m was the height of decline in the brain's response inhibition function during the conflict monitoring phase, and 2,950 m was the height of decrease during the response inhibition phase. In addition, the soldier's brain executive function was closely related to SpO_2, and a reduction in SpO_2 may lead to a decline in response inhibition.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The study was conducted following the Declaration of Helsinki, and all procedures were carried out with the adequate understanding and written informed consent of the subjects. The study protocol was approved by the Ethics Committee of the Fourth Military of Medical University.

AUTHOR CONTRIBUTIONS

XW and SZ were involved in the field for the data collection, troubleshooting, manuscript finalization, organized the data, carried out the analyses, drafted the manuscript, and took the lead in the manuscript finalization and submission. XN and AC were involved in the conceptualization, design, and planning of the study. All authors went through all the versions of the manuscript and approved them.

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REVIEW
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Hypoxia and Inflammation: Insights From High-Altitude Physiology

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The key regulators of the transcriptional response to hypoxia and inflammation (hypoxia inducible factor, HIF, and nuclear factor-kappa B, NF-κB, respectively) are evolutionarily conserved and share significant crosstalk. Tissues often experience hypoxia and inflammation concurrently at the site of infection or injury due to fluid retention and immune cell recruitment that ultimately reduces the rate of oxygen delivery to tissues. Inflammation can induce activity of HIF-pathway genes, and hypoxia may modulate inflammatory signaling. While it is clear that these molecular pathways function in concert, the physiological consequences of hypoxia-induced inflammation and how hypoxia modulates inflammatory signaling and immune function are not well established. In this review, we summarize known mechanisms of HIF and NF-κB crosstalk and highlight the physiological consequences that can arise from maladaptive hypoxia-induced inflammation. Finally, we discuss what can be learned about adaptive regulation of inflammation under chronic hypoxia by examining adaptive and maladaptive inflammatory phenotypes observed in human populations at high altitude. We aim to provide insight into the time domains of hypoxia-induced inflammation and highlight the importance of hypoxia-induced inflammatory sensitization in immune function, pathologies, and environmental adaptation.

Keywords: hypoxia, inflammation, high altitude, hypoxia inducible factor, nuclear factor-κB

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INTRODUCTION

Inflammation plays a key role in the physiological response to hypoxic stress. Tissues experience hypoxia during injury, infection, hypoperfusion, ischemia, or hypoxemia secondary to sleep apnea, pulmonary disease, anemia, high-altitude exposure, or other causes (Celli et al., 2004; McNicholas, 2009; Brill and Wedzicha, 2014; Hirota, 2015; Couzin-Frankel, 2020; Tobin et al., 2020; Xie et al., 2020). Cellular hypoxia can trigger the expression of several inflammatory mediators which signal tissue damage and initiate survival responses. However, while hypoxia-induced inflammation may serve a protective role by initiating an immune response and promoting tissue healing, it can also contribute to several pathologies, particularly in the context of chronic hypoxia. In this review, we summarize the known crosstalk between the transcriptional responses to hypoxia and inflammation and highlight the physiological consequences that occur as a result of maladaptive hypoxia-induced inflammation. Finally, we review what we know about adaptive regulation of inflammation under chronic hypoxia by investigating inflammatory phenotypes in human populations adapted to high altitude.

MOLECULAR MECHANISMS

Hypoxia-Inducible Factor Pathway

The transcriptional response to hypoxia is controlled by the hypoxia-inducible factor (HIF) signaling cascade (Semenza, 2009). HIF is a heterodimer protein composed of an oxygen-sensitive alpha subunit and constitutively expressed beta subunit (Biddlestone et al., 2015). The three HIF isoforms (HIF-1, HIF-2, and HIF-3) have some overlapping roles but also demonstrate distinct functions in different cell types (Carroll and Ashcroft, 2006; Dengler et al., 2014; Watts and Walmsley, 2019). Under normoxic conditions, HIF-α is hydroxylated by oxygen-dependent prolyl hydroxylases (PHDs 1–3 in humans; **Figure 1**). Upon hydroxylation, HIF-α is ubiquitinated by the von Hippel-Lindau tumor suppressor protein (pVHL) and degraded by proteasomes. Since PHD requires oxygen as a co-substrate, its activity decreases under hypoxic conditions, allowing HIF-α to dimerize with HIF-β and translocate to the nucleus. The HIF complex can then bind to hypoxia response elements (5'-RCGTG-3') in gene promoters to regulate expression of at least 100 genes to coordinate increased oxygen supply to hypoxic tissue (Kaelin and Ratcliffe, 2008; Semenza, 2009). HIF pathway activity is associated with activation of genes involved in metabolic adaptation, such as phosphoglycerate kinase (*PGK*) and lactate dehydrogenase A (*LDHA*), vascularization *via* vascular endothelial growth factor (*VEGF*), as well as red blood cell production *via* erythropoietin (*EPO*), and several other genes involved in improving oxygen delivery and use efficiency (Dengler et al., 2014; Villafuerte et al., 2014).

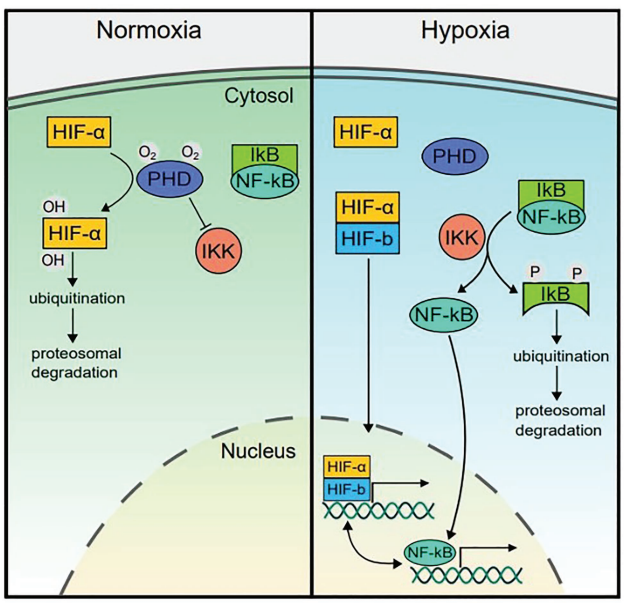

FIGURE 1 | Hypoxia inducible factor (HIF)-nuclear factor (NF)-kappa B (NF-κB) crosstalk. In normoxic conditions, prolyl hydroxylases (PHDs) hydroxylate HIF-α and the IKKβ subunit of the IκB kinase (IKK) complex, marking them for degradation and thereby reducing transcriptional activity of HIF and repressing (but not completely blocking) NF-kB activity. In hypoxia, PHD activity decreases since it utilizes oxygen as a cofactor. Therefore, HIF-α is stabilized and can dimerize with the constitutively active HIF-β subunit. The complex translocates to the nucleus to upregulate expression of genes involved in the hypoxia response. In hypoxia, with reduced PHD activity, the rate of IKK degradation of IκB increases, releasing repression of NF-κB and allowing it to translocate to the nucleus at higher rates and upregulate inflammatory gene expression. PHD, prolyl hydroxylase; HIF, hypoxia-inducible factor; NF-κB, Nuclear factor kappa B; IKK, IκB kinase complex (composed of IKK-α, IKK-β, and IKK-γ subunits); and IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor.

HIF and NF-κB Crosstalk

While the HIF pathway primarily responds to hypoxia, HIF expression is also increased in response to non-hypoxic stimuli, including bacterial lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), reactive oxygen species, hepatocyte growth factor, and interleukin (IL)-18 *via* crosstalk with the nuclear factor-κB (NF-κB) pathway (Figueroa et al., 2002; Zhou et al., 2003; Frede et al., 2006; Taylor, 2008). The NF-κB transcription factor is a master regulator of inflammation. NF-κB is kept localized in the cytoplasm by inhibitory IκB proteins (Lawrence, 2009; Oeckinghaus and Ghosh, 2009), which thereby inhibit DNA binding by NF-κB (Beg and Jr, 1993; Mitchell et al., 2016). In response to inflammatory stimuli and microbial products, the IκB kinase (IKK) complex phosphorylates IκB, leading to IκB ubiquitination and proteasomal degradation (Israël, 2010). With the degradation of IκB, NF-κB can translocate to the nucleus and upregulate key downstream inflammatory pathways (Hayden and Ghosh, 2004; Perkins, 2006; Mitchell et al., 2016; **Figure 1**).

The NF-κB pathway can also upregulate HIF-1α (BelAiba et al., 2007). NF-κB subunits bind to the NF-κB binding element within the HIF-1α gene promoter region and induce HIF-1α mRNA expression (Van Uden et al., 2008). Several studies support this NF-κB-dependent HIF-1α expression. For example, in cell culture models (HEK293 cells and pulmonary artery smooth muscle cells); NF-κB transfection resulted in increased HIF-1α mRNA and protein expression. Additionally, when these cells were co-transfected with a mutated dominant negative IκB (which cannot be phosphorylated by IKK) to reduce NF-κB translocation to the nucleus, HIF-1α mRNA and protein expression decreased (BelAiba et al., 2007; Bonello et al., 2007; Görlach and Bonello, 2008).

In addition to this direct link, the HIF and NF-κB pathways also share common regulators. Like HIF-α, the IKK complex responsible for regulating NF-κB activity is also a target of PHD and therefore its NF-κB regulatory activity is oxygen dependent. In normoxic conditions, PHD hydroxylates IKKβ, therefore repressing NF-κB nuclear translocation and transcriptional activity. When PHD is rendered inactive in hypoxic conditions, the IKK complex can proceed to remove IκB from NF-κB, increasing its rate of translocation to the nucleus and upregulating inflammatory gene expression (**Figure 1**; Cummins et al., 2006; Taylor, 2008).

PATHOPHYSIOLOGICAL CONSEQUENCES

Hypoxia-Induced Inflammation: Adaptive or Maladaptive?

At short timescales, and at the tissue level, inflammatory signaling in response to hypoxia is an adaptive mechanism which evolved to promote cell survival during infection, injury, or oxygen limitation (Walmsley et al., 2014). However, chronic and/or systemic hypoxia can produce maladaptive inflammation which can contribute to disease development. For example, in a clinical context, the crosstalk between hypoxia and inflammation may contribute to several inflammation-mediated metabolic and cardiovascular comorbidities that accompany hypoxia-promoted diseases such as chronic obstructive pulmonary disease or obstructive sleep apnea (Tasali and Ip, 2008; Quercioli et al., 2010; Cavaillès et al., 2013). This can also be investigated in the context of high-altitude exposure, where inflammatory signaling pathways and immune function must respond and adapt to acute, chronic, or lifelong hypoxemia. Here, we will examine our current understanding of how the interaction between hypoxia and inflammation contributes to high-altitude illness and what we might learn about the adaptive regulation of hypoxia-induced inflammation from high-altitude native populations (**Figure 2**).

The Role of Inflammation in High-Altitude Illness

High-altitude illnesses are a common risk for sojourners to high altitude (>2,500 m elevation). Within the first week of exposure, sojourners often present with Acute Mountain Sickness (AMS), which is characterized by headache, nausea, fatigue, and gastrointestinal issues (Roach et al., 2018). AMS typically resolves with acclimatization (Gallagher and Hackett, 2004). However, in serious cases, sojourners may develop severe and potentially fatal illnesses, such as high-altitude pulmonary edema (HAPE), high-altitude pulmonary hypertension (HAPH), or high-altitude cerebral edema (HACE; Gallagher and Hackett, 2004; Mehta et al., 2008; Luks et al., 2017). The incidence and severity of HAPH, HAPE and HACE vary depending on multiple factors, such as ascent time, altitude, and timely recognition and treatment (Gallagher and Hackett, 2004). Despite several decades of examining the physiology of AMS, HAPH, HAPE, and HACE, some questions remain regarding the pathophysiology of these conditions and the extent to which inflammation contributes to their onset and progression.

Acute Mountain Sickness

AMS development results from a complex network of physiological responses to hypoxia (i.e., inflammation, vasogenic edema, and acidosis) as well as anatomical factors (i.e., insufficient cerebrospinal fluid production, varied cerebral venous blood flow; West, 2004; Luks et al., 2017). While there is substantial research on potential contributors to AMS (reviewed in Imray et al., 2010; Luks et al., 2017), the exact biological pathways and molecular mechanisms behind AMS development remain unclear.

Recent studies support a potential role of inflammation in AMS. There is a general consensus that pro-inflammatory cytokines and other inflammatory markers (most notably C-Reactive protein (CRP), IL-1β, and IL-6) are increased in individuals acutely exposed to hypoxia or high altitude (Hartmann et al., 2000; Song et al., 2016; Lundeberg et al., 2018;

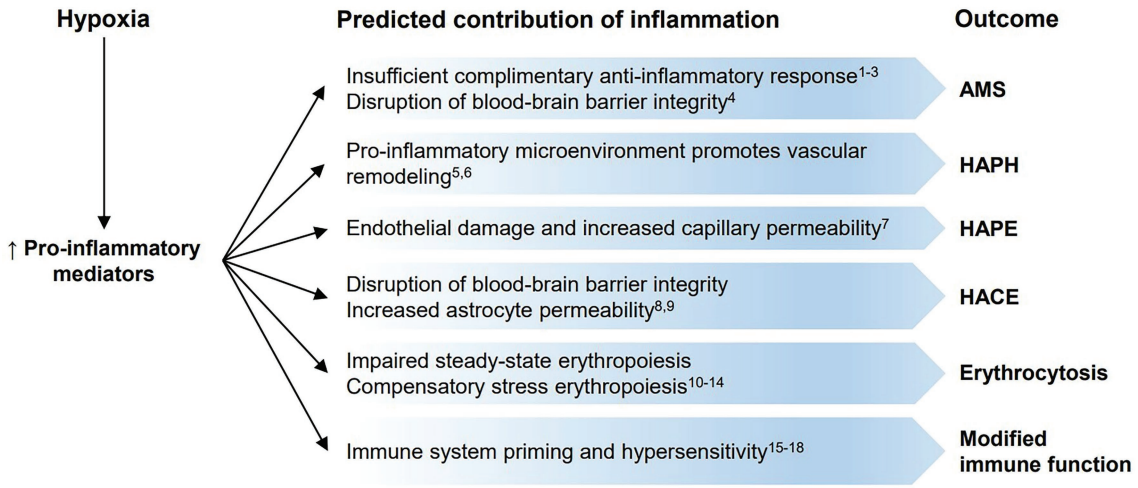

FIGURE 2 | Predicted contributions of inflammation to high-altitude illnesses, erythrocytosis, and immune function. Hypoxic conditions lead to increases in pro-inflammatory mediators, which may play a role in the development of high-altitude illnesses [(Acute Mountain Sickness (AMS), high-altitude pulmonary hypertension (HAPH), high-altitude pulmonary edema (HAPE), and high-altitude cerebral edema (HACE)] and erythrocytosis, or modify immune function. (1) Julian et al. (2011); (2) Liu et al. (2017); (3) Wang et al. (2018); (4) Varatharaj and Galea (2017); (5) Wilkins et al. (2015); (6) Brito et al. (2020); (7) Mishra et al. (2016); (8) Song et al. (2016); (9) Zhou et al. (2017); (10) Jackson et al. (2010); (11) Liao et al. (2018); (12) Bennett et al. (2019); (13) Imagawa et al. (1990); (14) Haase (2013); (15) Kvamme et al. (2013); (16) Baze et al. (2011); (17) Facco et al. (2005); and (18) Feuerecker et al. (2019).

Wang et al., 2018; Malacrida et al., 2019; Kammerer et al., 2020). In some cases, inflammatory mediator expression appears to differ across individuals who develop AMS and those who do not. For example, individuals who develop AMS have been reported to show decreased plasma levels of IL-10 and increased IL-1β, IL-6, and TNF-α compared to non-AMS controls (Liu et al., 2017; Wang et al., 2018). Furthermore, the association between plasma IL-6 and AMS score has been identified by multiple independent groups (Boos et al., 2016; Wang et al., 2018).

While these studies indicate that some circulating pro-inflammatory markers may be associated with AMS development, it is not yet clear what specific role they may play. It is possible that hypoxia initiates the release of inflammatory and angiogenic mediators which disrupt the blood-brain barrier and promote vasogenic edema. A complementary hypothesis is that protection from AMS may be driven by a robust anti-inflammatory response which protects against potential increased blood-brain barrier permeability caused by acute systemic inflammation (Varatharaj and Galea, 2017). This is supported by data from Julian et al. (2011), who found higher levels of anti-inflammatory marker (IL-1RA, HSP70, and adrenomedullin) expression in AMS-resistant compared to AMS-susceptible individuals (Julian et al., 2011).

Finally, both steroids and nonsteroidal anti-inflammatory drugs (NSAIDS) equally reduce AMS incidence despite their different modes of action (Dumont et al., 2000; Gertsch et al., 2012; Zheng et al., 2014). This suggests that both COX-2 mediated inflammation as well as analgesic mechanisms that mediate nociception contribute to AMS symptomology (Hartmann et al., 2000; Song et al., 2016; Kanaan et al., 2017). Indeed, inflammatory mediators released during tissue injury can activate nociceptors and can contribute to pain hypersensitivity (Kidd and Urban, 2001). However, Lundeberg et al. (2018) note no change in AMS symptoms in individuals receiving Ibuprofen at normal recommended doses of 400 mg three times a day (Lundeberg et al., 2018), perhaps, due to the lower dosage compared to other studies which did observe a reduction in AMS symptoms with a higher Ibuprofen dose (600 mg three times a day; Gertsch et al., 2010, 2012; Lipman et al., 2012).

High Altitude Cerebral Edema

HACE is a severe and potentially fatal complication that can occur in individuals who travel above 2000 m. HACE is accompanied by symptoms including headache, ataxia, declines in cognitive function, and can lead to seizures (Hackett, 1999). As with AMS, HACE is most common with rapid ascent. HACE is frequently preceded by some AMS symptoms, and both illnesses are influenced by cerebral hemodynamics. As a result, HACE is sometimes considered a more severe form of AMS, however, additional distinct pathophysiological mechanisms contribute to HACE development (Hackett and Roach, 2004; Brugniaux et al., 2007; Li et al., 2018). HACE can develop spontaneously at very high altitudes in acclimatized individuals (Clarke, 1988). HACE appears to occur as a result of hypoxia-mediated cerebral vasodilation and a subsequent impairment of the autoregulation of cerebral blood flow, loss of blood-brain barrier integrity, and a rise in intracranial pressure (Hackett, 2000). Ultimately, cytotoxic and vasogenic edema leads to microvascular disruption and microbleeds (Hackett et al., 2019).

While some aspects of HACE pathophysiology remain uncertain, the underlying mechanisms producing HACE are likely similar to those of AMS and inflammation may play a role in its development (Bailey et al., 2009; Song et al., 2016; Zhou et al., 2017). In both mouse and rat models, pro-inflammatory cytokines are significantly increased in the brain cortex after hypoxia exposure. Furthermore, when pre-treated with bacterial LPS to produce a systemic inflammatory response, subsequent hypoxia exposure results in cerebral edema (Song et al., 2016; Zhou et al., 2017). It is proposed that pre-existing inflammation increases aquaporin 4 (AQP4) activity in astrocytes *via* toll-like receptor 4 (TLR4), mitogen-activated protein kinase (MAPK), and NF-κB signaling, thereby increasing astrocyte permeability. Together, these data indicate that when challenged with a combination of hypoxia and inflammation, the combination of increased pro-inflammatory cytokines and increased blood vessel permeability produces vasogenic edema (Song et al., 2016; Zhou et al., 2017).

Although this data provide intriguing support for a role of hypoxia-induced inflammation and HIF-NF-kB crosstalk in HACE onset, it remains unclear if inflammation is a proximal cause of AMS or HACE. Nonetheless, inflammation likely plays a contributing role in susceptibility and progression of these illnesses. Further work is required to reach a unified explanation for HACE development.

High-Altitude Pulmonary Hypertension and High-Altitude Pulmonary Edema

HAPH occurs due to general hypoxic pulmonary vasoconstriction (HPV). Under typical conditions, local HPV aids in redistributing blood flow away from lung regions with poor ventilation and improves pulmonary gas exchange. However, at high altitude, global reductions in alveolar oxygen pressure can lead to HPV throughout the lung and increase mean pulmonary artery pressures (Swenson, 2013). HAPH is estimated to impact up to 10% of high-altitude residents (León-Velarde et al., 2005). The clinical presentation of HAPH includes dyspnea, general fatigue, exercise intolerance, and in severe cases, chest pain and mental alterations, and ultimately cor pulmonale.

While acute HAPH is primarily driven by increased vasomotor tone, chronic hypoxic stress and persistent pulmonary hypertension can produce vascular remodeling and exacerbate pulmonary artery pressures (Groves et al., 1987; Wilkins et al., 2015). The hallmark of vascular remodeling in chronic hypoxia is increased vessel muscularization (Arias-Stella and Saldana, 1963; Hislop and Reid, 1976). It is predicted that inflammation plays an important role in this remodeling process. Chronic hypoxia produces a proinflammatory microenvironment in pulmonary artery walls (Burke et al., 2009). Resident fibroblasts, immune cells, and progenitor cells in the vascular adventitia respond

to this local cellular stress by releasing additional inflammatory mediators and growth factors which impact vascular wall cell phenotypes and contribute to increased muscularization (Stenmark et al., 2013). This inflammatory microenvironment also promotes recruitment, retention, and differentiation of additional inflammatory cells (Burke et al., 2009).

HAPE occurs most commonly during rapid ascent. This is due to exaggerated HPV which causes acute pulmonary hypertension, increased capillary permeability, and alveolar fluid buildup (Talbot et al., 2005; Dunham-Snary et al., 2017; Brito et al., 2020; Swenson, 2020; Sydykov et al., 2021). HAPE onset is primarily due to this non-inflammatory hydrostatic pulmonary edema (Swenson et al., 2002). However, inflammation may play a downstream role in HAPE pathology. Several studies report increased inflammatory mediators and chemokines in bronchoalveolar fluid in later stages of the illness including leukotriene B_4 and complement fragments (C5a; Schoene et al., 1986, 1988), plasma endothelin-1 (ET-1; Droma et al., 1996), lactate dehydrogenase, IL-1β, IL-6, IL-8, TNF-α, and IL-1RA (Kubo et al., 1996, 1998). Several of these studies also report increased leukocyte counts in bronchoalveolar lavage fluid. This late-stage inflammation could exacerbate pulmonary vascular leakage by causing endothelial damage and increasing capillary permeability (Mishra et al., 2016).

Some studies have also suggested that inflammation contributes to HAPE susceptibility. Individuals with a history of HAPE are more susceptible to developing HAPE again upon re-entry to high altitude (Lakshminarayan and Pierson, 1975; Bärtsch, 1999; Gallagher and Hackett, 2004). This increased susceptibility is attributed to higher baseline inflammation (Mishra et al., 2016). Furthermore, one report suggests that HAPE-susceptible individuals also demonstrate reduced lung function compared to HAPE-resistant individuals (Gupta et al., 2017), which would limit the adaptive compensatory ventilatory response to hypoxia. The reduced lung function in these individuals is further correlated with increased plasma CRP (Shaaban et al., 2006; Hancox et al., 2007). This association between chronic inflammation and poor lung function is hypothesized to contribute to HAPE-susceptibility, although it does not explain the direct cause of onset. Additionally, pre-existing pulmonary vascular remodeling, driven partially by inflammation, may exacerbate pulmonary pressures and blood flow distribution and thereby increase HAPE susceptibility (Wilkins et al., 2015).

Inflammation and Erythropoiesis

Erythropoiesis is tightly regulated to maintain homeostatic balance between red blood cell production and degradation. This balance is crucial for optimal oxygen delivery to tissues. An inadequate number of erythrocytes lead to tissue hypoxia, while high erythrocyte concentrations can increase blood viscosity, impair blood flow, and increase risk of thrombosis (Keohane et al., 2013). This is particularly relevant in Chronic Mountain Sickness (CMS), a clinical syndrome commonly presented in high-altitude natives and life-long residents, which frequently coincides with excessive erythrocytosis (Hb ≥ 19 g/dL for women, Hb ≥ 21 g/dL for men) in some high altitude groups (León-Velarde et al., 2005; Oberholzer et al., 2020). The HIF pathway plays a key role in regulating erythropoiesis. HIF activation results in increased transcription of the *EPO* gene in the kidney and liver, and EPO serum concentration can increase up to several 100-fold in response (Imagawa et al., 1990; Haase, 2013). Additionally, HIF activity plays a role in regulating iron uptake from the gut and mobilizing erythroid progenitor cells in the bone marrow (Nemeth, 2008).

Chronic inflammation typically leads to anemia through several mechanisms: prioritizing myeloid cell production, sequestering iron, and increasing erythroid turnover rate. Inflammation impacts crucial sites of erythrocyte production to redistribute resources toward myeloid cell production and therefore reduces lymphoid and erythroid output. For example, expression of pro-inflammatory IL-1β, interferon gamma (IFNγ), and IL-6 skew multipotential hematopoietic progenitors toward myeloid cell development (Tie et al., 2019). This is an adaptive response since myeloid cells are needed to fight infection; however, this comes at the cost of bone marrow erythropoiesis. Additionally, while iron is an essential component of hemoglobin synthesis, it is also an essential micronutrient to pathogens. During inflammation, increased IL-6 induces hepcidin production, interrupting iron absorption from the gut and blocking iron release from macrophages, leading to hypoferremia (Nemeth et al., 2004; Nemeth, 2008; Zaritsky et al., 2009; Weiss et al., 2019). By decreasing iron availability, the immune system limits pathogen proliferation; however, a consequence of iron sequestration is that this also limits iron availability for red blood cell production.

When steady state erythropoiesis becomes insufficient, or red blood cells are broken down at high rates, a compensatory extramedullary mechanism called stress erythropoiesis is initiated to prevent lethal anemia (Paulson et al., 2011). Stress erythrocytosis produces a burst of new erythrocytes to maintain red cell concentrations until bone marrow erythropoiesis recovers. The mechanism of stress erythropoiesis has been studied extensively in mice, where it occurs in the spleen and liver in response to anemia, hypoxia, or sterile inflammation. Studies in mice revealed that sterile inflammation produced by phenylhydrazine injections lead to decreased erythroid progenitor cells in the bone marrow and increased erythroid progenitor cells in the spleen. It was discovered that bone marrow erythroid progenitor cells and splenic erythroid progenitor cells respond to different factors, indicating that splenic progenitors are distinct from bone marrow progenitors. This splenic stress erythropoiesis response depends on TLR signaling molecules Myd88 and TRIF (Jackson et al., 2010). Furthermore, pro-inflammatory cytokines TNF-α and IL-1β promote the expansion of splenic erythroid progenitors (Liao et al., 2018; Bennett et al., 2019). It is currently unknown if similar mechanisms are responsible for stress erythropoiesis in humans (Kim et al., 2015; Mairbäurl, 2018). Future studies may investigate biomarkers of stress erythropoiesis signaling

to determine if this protective mechanism is active during high-altitude exposure or other forms of hypoxic stress.

Impacts of Chronic Hypoxia on Immune Function and Inflammatory Signaling

Immune cells are exposed to hypoxia when they are recruited to sites of inflammation. In physiological immunological niches (bone marrow, placenta, gastrointestinal tract mucosal surfaces, and lymph nodes), the maintenance of sustained and moderate physiological hypoxia is an adaptive mechanism to regulate metabolic pathways and immune homeostasis. However, in pathological immunological niches (tumors and chronically inflamed and ischemic tissue), severe and unregulated hypoxia can lead to maladaptive inflammation and disease development (Taylor and Colgan, 2017). In either case, the immune cell response to hypoxia hinges upon the ability to mount a transcriptional response. Therefore, HIF activity is essential to immune cell survival and function.

Several studies demonstrate the significance of HIF signaling in immune function. HIF-1α deletion in myeloid cells (granulocytes and monocytes/macrophages) impairs their mobility, aggregation, antibacterial activity, and survival (Cramer et al., 2003; Walmsley et al., 2005). On an organismal level, HIF-1α deletion in macrophages can reduce mortality in LPS-induced sepsis in mice (Peyssonnaux et al., 2007). HIF-2α can also directly regulate pro-inflammatory cytokine expression in myeloid cells (Imtiyaz et al., 2010). In addition to HIF's essential role in immune cell function, NF-kB is also critical to cell survival in hypoxia. Walmsley et al. (2005) demonstrated that the transcription of the NF-κB p65 subunit is regulated by hypoxia. Neutrophils cultured in hypoxia and treated with NF-κB inhibitors had reduced survival rates, suggesting that activation of the NF-κB pathway promotes neutrophil survival in hypoxia (Walmsley et al., 2005).

Despite this important interplay between hypoxia and inflammation in immune function, we know little about how inflammatory signaling and immune function adapt to chronic sustained or chronic intermittent hypoxia. Studies in animal models demonstrate potential blunting or sensitization of inflammatory responses to infection as a result of chronic hypoxia exposure. A study in salmon exposed to chronic hypoxia for 58 days found blunted expression of pro-inflammatory genes in macrophages and the head kidney in response to a viral inflammatory stimulus (Kvamme et al., 2013). Alternatively, in a mouse model, 36 days of hypoxia exposure lead to an enhanced immunological response to LPS illustrated by higher antibody titers and higher TNF-α expression, indicating that hypoxia may stimulate innate and adaptive immune responses (Baze et al., 2011).

Among studies in humans, chronic hypoxia also appears to modulate immune function and inflammatory signaling. A study of women exposed to high altitude (5,050 m) for 21 days showed increased white blood cells, reductions in CD3+ and CD4+ T-cells, an increase in natural killer cells, and a decrease in IFNγ expression by circulating T-cells (Facco et al., 2005). Another recent study in humans exposed to high altitude (3,232 m) for up to 11 months shows that cytokine expression in response to inflammatory stimuli were also higher than sea-level values (Feuerecker et al., 2019). The possibility that chronic hypoxia exposure in humans leads to immune sensitization and hypersensitivity to inflammatory stimuli warrants future study. Since several critical and chronic illnesses are associated with hypoxemia (e.g., sepsis, acute respiratory distress syndrome, chronic obstructive pulmonary disease, and sleep apnea), it is possible that inflammatory dysregulation in these conditions may be caused in part by this concurrent hypoxic stress.

LESSONS FROM HIGH-ALTITUDE ACCLIMATIZED AND ADAPTED GROUPS

High altitude environments are physiologically stressful due to low atmospheric pressure and oxygen limitation. Despite this, humans have survived in these environments for thousands of years and different high-altitude native populations exhibit distinct physiological adaptations that may be associated with genetic variants. In this section, we discuss what can be learned from high-altitude acclimatized and adapted populations about how inflammatory pathways respond to chronic hypoxia.

Inflammatory Pathway Genes Under Selection in High-Altitude Groups

Several studies have identified genes under evolutionary selection in high-altitude native populations. HIF pathway genes, particularly *EGLN1* and *EPAS1*, are consistently highlighted across studies. Additionally, several genes associated with inflammation also show signals of selection but have received less attention (Foll et al., 2014). The inflammatory pathway genes *IL6*, *IL1A*, *IL1B*, *NOS1*, *NOS2*, and *TNF* show signals of evolutionary selection in both Andean and Tibetan high-altitude native populations (Beall, 2007; Bigham and Lee, 2014; Moore, 2017). *NOS1* and *NOS3* have also been shown to be under positive selection in the Sherpa population (Droma et al., 2006; Zhang et al., 2017). Additional inflammation-related genes have been reported to be under selection in individual groups, including *HLA-DQB1*, *PPARA*, and *TGFBR3* in Tibetans (Yang et al., 2017), *PPARA* in Sherpas (Horscroft et al., 2017), *BRINP3*, *DUOX2*, and *CLC* in Andeans (Crawford et al., 2017; Jacovas et al., 2018), and *AIMP1* in Ethiopians (Scheinfeldt et al., 2012). **Table 1** summarizes inflammation-related genes found to be under evolutionary selection in high altitude human populations.

IL6 is particularly interesting given that its expression has been associated with AMS development. IL-6 is also noted to increase hematopoietic stem cell proliferation under hypoxic conditions, and its effects on red cell production are synergistic with other pro-inflammatory cytokines, including IL-1α and TNF-α (Faquin et al., 1992). Together, this data suggest that IL-6 plays a role in adaptation to chronic hypoxia. It is currently unknown what the precise adaptive *IL6* variants are, but it is possible that blunting IL-6 expression in response to chronic hypoxia or chronic intermittent hypoxia may provide an advantage by preventing hypoxia-induced chronic low-grade systemic inflammation. Another important inflammatory gene under

TABLE 1 | Inflammation-related genes identified as top candidates showing signals of evolutionary selection in high-altitude human populations.

Gene name	Protein encoded	Population	Function	Reference
IL6	Interleukin-6	Andean, Tibetan	Pro-inflammatory cytokine	Bigham and Lee, 2014
			Anti-inflammatory myokine	Foll et al., 2014
TNF	Tumor necrosis factor, TNF-a	Andean, Tibetan	Pro-inflammatory cytokine	Bigham and Lee, 2014
IL1A	Interleukin 1 alpha	Andean, Tibetan	Pro-inflammatory cytokine	Bigham and Lee, 2014
IL1B	Interleukin 1 beta	Andean, Tibetan	Pro-inflammatory cytokine	Bigham and Lee, 2014
HMOX2	Heme oxygenase 2	Tibetan	Heme protein catabolism	Yang et al., 2016
				Peng et al., 2011
NOS1	Nitric oxide synthase 1 (neuronal)	Andean, Tibetan, Sherpa, Ethiopian	Nitric oxide production	Bigham and Lee, 2014
				Horscroft et al., 2017
NOS2	Nitric oxide synthase 2	Andean, Tibetan	Nitric oxide production	Crawford et al., 2017
				Bigham and Lee, 2014
				Bigham et al., 2010
NOS3	Nitric oxide synthase 3 (endothelial)	Sherpa	Nitric oxide production	Droma et al., 2006
BRINP3	BMP/Retinoic acid inducible neural specific 3	Andean	Associated with vascular inflammation	Crawford et al., 2017
CLC	Galectin-10	Andean	Immune response regulation	Jacovas et al., 2018
HLA-DQB1	HLA class II histocompatibility antigen, DQ beta 1 chain	Tibetan	Immune response regulation	Yang et al., 2017
			Detection of foreign proteins	
AIMP1	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	Ethiopian	Inflammatory cytokine activity	Scheinfeldt et al., 2012
			Involved in angiogenesis, inflammation, wound healing, and glucose homeostasis	
PPARA	Peroxisome proliferator-activated receptor alpha	Tibetan, Sherpa	Regulation of inflammation and immune response	Simonson et al., 2010
				Horscroft et al., 2017
TGFBR3	Betaglycan, Transforming growth factor beta receptor type 3	Tibetan	Involved in inflammatory cytokine (TGF-beta) signaling	Peng et al., 2011

selection in both Tibetan and Andean groups is *TNF* (Moore, 2017), a multifunctional pro-inflammatory cytokine. TNF-α levels have also been found to be elevated in individuals traveling acutely to high altitude (Lundeberg et al., 2018). Like *IL6*, the adaptive *TNF* variant is unknown but modulation of *TNF* expression in response to chronic hypoxia may be important for preventing chronic inflammation at high altitude.

In addition to these genetic changes, epigenetic mechanisms also play a critical role in regulating expression of inflammatory genes and likely influence adaptation of inflammatory pathways to chronic hypoxia (Bayarsaihan, 2011). Epigenetic changes alter gene expression without altering the underlying genetic code. These changes include DNA methylation, histone modifications, and microRNA expression. These epigenetic changes play key roles in environmental adaptation during development and exposure in adulthood (Fernández et al., 2014). There are several HIF-dependent and independent mechanisms by which hypoxia significantly impacts epigenetic modifications (Kim and Park, 2020). For example, HIF induces expression of several histone methyltransferases and demethylases (Batie et al., 2019). Additionally, hydroxylation of histone methyl transferases as well as activity of histone demethylases, are oxygen dependent. DNA methylation patterns are also altered by hypoxia, in part due to the impact on ten-eleven translocation (TET) enzyme expression and activity (Thienpont et al., 2016). The HIF consensus binding site also contains a CpG dinucleotide, indicating that expression of HIF-pathway genes may be dependent on DNA methylation (Wenger et al., 2005; Chen et al., 2020; D'Anna et al., 2020). Given that immune cell phenotypes, including inflammatory macrophage phenotypes, can be regulated by epigenetic modifications, hypoxia has the potential to significantly alter immune cell function *via* its impact on epigenetics (Davis and Gallagher, 2019). These changes may occur within or across generations of exposure. Future work should investigate if high-altitude acclimatized or adapted groups develop particular patterns of DNA methylation or histone modification, which protect against chronic hypoxia-induced inflammation. Over generations, mutations at loci containing CpG sites could also assist in blunting inflammatory gene expression in the face of chronic hypoxic stress.

Nitric Oxide in Native and Acclimatized High-Altitude Groups

Nitric oxide (NO) is a natural vasodilator that plays a crucial role in regulating vasodilation in vascular smooth muscle. Exhaled NO is also used as marker of airway inflammation (Kharitonov and Barnes, 2001; Birrell et al., 2006). The vasodilatory function of NO protects against pulmonary hypertension at high altitude (Feelisch, 2018). Individuals exposed acutely to high altitude tend to show reduced gas phase NO, which typically returns to baseline levels after a couple days then exceeds baseline levels by 5 days. However, gas phase NO has been demonstrated to remain low in HAPE-sensitive individuals, perhaps, indicating that deficits in pulmonary epithelial NO synthesis contribute to exaggerated pulmonary vasoconstriction and edema (Duplain et al., 2000). Tibetan and Bolivian high-altitude adapted populations also show elevated exhaled NO compared to sea-level residents (Beall et al., 2001, 2012;

Erzurum et al., 2007). This phenotype correlates with higher pulmonary blood flow and protection from HAPH. However, He et al. (2017) demonstrate that enhanced NO production may not be unique to high-altitude native populations since individuals of Han Chinese ancestry living at high altitude (3,660–3,700 m) demonstrated an even higher average NO metabolite production than Tibetans at the same altitude (He et al., 2017). Another study reports that the native Sherpa population has a lower level of circulating NO in serum and no differences in circulating NO metabolites in comparison to low-altitude samples (Droma et al., 2006; Horscroft et al., 2017). Interestingly, this finding contrasts the high exhaled NO among Tibetans. This discrepancy in NO substrate bioavailability in Tibetans vs. Sherpas also suggests that these two high-altitude populations may have some distinct adaptation in the nitric oxide pathway (Zhang et al., 2017).

Elevated NO may also be an important adaptation of the immune system to chronic hypoxia-induced inflammation. Under normal physiological conditions, NO plays an anti-inflammatory role and inhibits platelet aggregation and rolling, adherence, and transmigration of leukocytes (Grisham et al., 1998; Coleman, 2001). During inflammatory reactions, the production of the inducible form of nitric oxide synthase in many immune cells, including monocytes, macrophages, and neutrophils leads to very large increases in NO by up to 1,000-fold (Cook and Cattell, 1996; Sharma et al., 2007). NO can then become oxidized to reactive nitrogen oxide species which nitrosate thiol groups in glutathione. This inhibits the activity of many proteins including mitochondrial enzymes and transcription factors. As a result, elevated NO production can impair pathogen function (Sethi and Dikshit, 2000; Bogdan, 2001; Coleman, 2001). At high concentrations, NO also stabilizes HIF by inhibiting HIF PHDs (Taylor and Moncada, 2010). Therefore, NO production during periods of inflammation permit increased expression of HIF-pathway genes as a protective mechanism since tissue inflammation often leads to cellular hypoxia. However, Lüneburg et al. (2016) demonstrated in a rat model that while there is an increase in endothelial nitric oxide synthase (eNOS; NOS3) in chronic hypoxia and chronic intermittent hypoxia conditions, NO bioavailability may be impaired due to increases in asymmetric dimethylarginine (ADMA), a competitive nitric oxide synthase inhibitor that regulates NO production, and hypoxia-induced increase in oxidative stress (Lüneburg et al., 2016). ADMA displaces L-arginine, a NOS substrate, and therefore an increase in ADMA concentrations may result in lower NO substrate bioavailability (Brito et al., 2007). Oxidative stress may lead to superoxide radical production, which may mediate NO degradation and attenuate NO bioavailability (Siques et al., 2014).

Since both *NOS1* (nitric oxide synthase 1, neuronal) and *NOS2* (nitric oxide synthase 2, inducible) are under selection in multiple high-altitude groups, it is clear that adjusting NO production is a key adaptive phenotype for life at high altitude. While the precise adaptive mechanism is unknown, it likely involves protection against pulmonary hypertension and/or modulation of hypoxia-induced inflammation.

Carbon Monoxide in Adapted High-Altitude Groups

Carbon monoxide (CO) is emerging as a potential therapeutic target for inflammatory modulation due to its anti-inflammatory, anti-apoptotic, and anti-proliferative effects (Knauert et al., 2013; Ryter and Choi, 2016). CO is also used as a clinical marker for inflammation and red blood cell turnover since endogenous CO is only produced through the heme oxygenase (HO) pathway (Maines, 1997). HO is responsible for catabolizing free heme proteins into Fe^{2+}, CO, and biliverdin. These end products provide cytoprotective benefits and protect cells from programmed cell death in response to pro-inflammatory agonists (Gozzelino et al., 2010). The HO-CO pathway may be a key regulator of hypoxia-induced inflammation since the gene encoding heme oxygenase-1 (*HMOX1*) is upregulated in response to HIF-pathway activation and HO-1 can inhibit NF-κB (Seldon et al., 2007).

High altitude exposure has been shown to increased exhaled CO levels, and carboxyhemoglobin levels are positively associated with hematocrit in Andean high-altitude residents (Tift et al., 2020). This is suspected to be caused by increased red blood cell turnover, a persistent stress erythropoiesis response, or a unique HO/CO pathway mechanism. Interestingly, the gene encoding the constitutively expressed heme oxygenase-2 (*HMOX2*) is under evolutionary selection in Tibetan high-altitude native groups (Yang et al., 2016). The adaptive *HMOX2* variants in Tibetans are predicted to play a critical role in regulating red blood cell turnover and potentially contribute to the low hemoglobin concentrations in this group by increasing heme oxygenase activity. Due to the key role that the HO-CO pathway plays in linking the molecular responses to hypoxia and inflammation, as well as evidence that HO is a top candidate for evolutionary selection in high-altitude populations, future research should continue to investigate potential therapeutic uses of exogenous CO and HO pathway activation for modulating inflammatory responses, especially in hypoxia-promoted pathologies.

CONCLUSION

HIF-NF-κB crosstalk plays an essential role in the transcriptional response to both hypoxia and inflammation. However, additional research is necessary to understand the physiological implications of these pathway interactions at an organismal level. Several studies have demonstrated that select inflammatory mediators are upregulated at high altitude, or in the presence of acute hypoxia, even in the absence of infection. Since these studies have focused on candidate inflammatory markers, large scale transcriptomic or proteomic studies would provide a better understanding of how inflammatory networks are shifted during chronic hypoxia. Further, our understanding of the time domains of hypoxia-induced inflammation and the impact on immune function may also provide insight into pathology of high-altitude illnesses and highlight the importance of hypoxia-induced inflammatory sensitization in

critical and chronic illnesses including chronic obstructive pulmonary disorder, sepsis, and COVID-19. This work may identify novel therapeutic targets for mitigating excessive inflammation in patients with concomitant hypoxemia and systemic inflammation.

AUTHOR CONTRIBUTIONS

KaP drafted versions of the manuscript with input and revisions from KeP and EH. All authors contributed to the article and approved the submitted version.

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Genome-Wide DNA Methylation Changes Associated With High-Altitude Acclimatization During an Everest Base Camp Trek

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The individual physiological response to high-altitude hypoxia involves both genetic and non-genetic factors, including epigenetic modifications. Epigenetic changes in hypoxia factor pathway (HIF) genes are associated with high-altitude acclimatization. However, genome-wide epigenetic changes that are associated with short-term hypoxia exposure remain largely unknown. We collected a series of DNA samples from 15 participants of European ancestry trekking to Everest Base Camp to identify DNA methylation changes associated with incremental altitude ascent. We determined genome-wide DNA methylation levels using the Illumina MethylationEPIC chip comparing two altitudes: baseline 1,400 m (day 0) and elevation 4,240 m (day 7). The results of our epigenome-wide association study revealed 2,873 significant differentially methylated positions (DMPs) and 361 significant differentially methylated regions (DMRs), including significant positions and regions in hypoxia inducible factor (HIF) and the renin–angiotensin system (RAS) pathways. Our pathway enrichment analysis identified 95 significant pathways including regulation of glycolytic process (GO:0006110), regulation of hematopoietic stem cell differentiation (GO:1902036), and regulation of angiogenesis (GO:0045765). Lastly, we identified an association between the *ACE* gene insertion/deletion (I/D) polymorphism and oxygen saturation, as well as average *ACE* methylation. These findings shed light on the genes and pathways experiencing the most epigenetic change associated with short-term exposure to hypoxia.

Keywords: epigenetics (DNA methylation), genome-wide DNA methylation analysis, high altitude acclimatization, HIF pathway, hypoxia

INTRODUCTION

Altitude acclimatization in humans is characterized by complex physiological responses, which include the cardiovascular, hemopoietic, respiratory, and metabolic systems [for review, see Palmer (2010)]. Each system responds uniquely to low oxygen environments. For example, cardiovascular output increases (i.e., increased heart rate and stroke volume) upon initial altitude exposure and

returns to pre-altitude baseline after several days of acclimatization [for review, see Naeije (2010)]. The respiratory system's response is to initiate hyperventilation. The hypoxic ventilatory response (HVR) is elicited shortly upon exposure to high altitude, with ventilatory acclimatization emerging following 5–7 days of sustained exposure to hypoxia (Powell et al., 1998). Lastly, the hemopoietic response in the form of increased erythrocyte production is evident after several days to weeks of exposure (Rodriguez et al., 2000). Each of these responses facilitates acute acclimatization to the low ambient oxygen tension present at high altitudes, allowing humans to acclimatize to hypoxic conditions.

Epigenetic change is one mechanism through which physiological acclimatization may occur. Epigenetic modifications can affect gene expression and include DNA methylation, histone tail modifications, and short RNA regulation. The most well-studied epigenetic mark is DNA methylation, the addition of a methyl group primarily to cytosine bases. DNA methylation patterns can change upon exposure to various environmental conditions, including exposure to different diets, stress, and toxicants (Dolinoy et al., 2007; Baccarelli et al., 2009; Colacino et al., 2012; Childebayeva et al., 2019b). Previous studies have demonstrated that changes in DNA methylation are associated with exposure to the low oxygen environment of high altitude (Alkorta-Aranburu et al., 2012; Childebayeva et al., 2019a,b, 2020). These studies show that genes in the hypoxia inducible factor (HIF) pathway exhibit changes in DNA methylation associated with high-altitude exposure.

The HIF pathway is the main oxygen sensing pathway that regulates cellular homeostasis in metazoans (Bigham and Lee, 2014). The pathway takes its name after the master transcriptional regulator HIF, a heterodimeric transcription factor that is formed by one of three α-subunits (HIF-1α, HIF-2α, or HIF-3α) and a β-subunit (also known as ARNT). In normoxia, HIF1A is hydroxylated and subsequently degraded by the ubiquitin–proteosome pathway. Under hypoxia, this hydroxylation is inhibited by the lack of oxygen availability, leading to the dimerization of HIF and activation of target genes. HIF is responsible for transducing changes in oxygen tension to changes in gene expression through hypoxia response elements (HREs) (Wang and Semenza, 1995; Kaelin and Ratcliffe, 2008; Semenza, 2012). The renin–angiotensin system or RAS is a second pathway that is involved in the response to hypoxia. It is one of the body's most important regulators of blood pressure and inflammation (Muller et al., 1997; Rupert et al., 2003). The RAS protein, angiotensin converting enzyme (ACE), is a central peptide in blood-pressure regulation responsible for converting angiotensin-I to the vasoconstrictor, angiotensin-II. An insertion/deletion (I/D) polymorphism in ACE is associated with physical performance at high altitude (Woods et al., 2002; Tsianos et al., 2005). The I-allele has been associated with higher levels of submaximal oxygen saturation (SaO_2) among Andean Quechua (Bigham et al., 2008), and in trekkers of European ancestry (Woods et al., 2002).

Previous research by our group has shown that acclimatization to hypoxia is associated with DNA methylation changes in HIF pathway genes including *EPAS1*, *EPO*, *PPARa*, and *RXRA* (Childebayeva et al., 2019a). However, it is not well understood what other genes and pathways display DNA methylation changes upon exposure to hypoxia. To understand how acclimatization to hypoxia affects genome-wide DNA methylation patterns, we performed an epigenome-wide association study in individuals trekking to Everest Base Camp. Our analysis compared baseline methylation measured in Kathmandu, Nepal at 1,400 m (day 0) with methylation measured at a high-altitude location, Pheriche, Nepal at 4,240 m (day 7 of the trek).

MATERIALS AND METHODS

Ethics Statement

Ethical approval was received from the Syracuse University Institutional Review Board (Protocol 18-006) and the University of Michigan Institutional Review Board (HUM00141118). The study abided by the Canadian Government Tri-Council policy on research ethics with human participants (T2) and the Declaration of Helsinki, except for registration in a database. Ethical approval was received also from the Mount Royal University Human Research Ethics Board (Protocol 100012 and 101361) and harmonized with the Nepal Health Research Council (Protocol 109-2017).

Study Design and Sample Collection

Thirty-two samples (16 samples at 1,400 m and 16 samples at 4,240 m) corresponding to 16 unique individuals were selected from a larger participant cohort from the research expedition to Everest Base Camp in the Nepal Himalaya (Childebayeva et al., 2019a). Briefly, study participants and researchers flew from Kathmandu (baseline) to Lukla from where the research group trekked for 10 days from 2,800 to 5,160 m (**Figure 1**). In the morning between 06:00 and 08:00 local time at 1,400 m (Kathmandu; day 0) and 4,240 m (Pheriche; day 7), saliva samples for DNA and physiological measures were taken following one night of sleep at each altitude. Physiological measurements included hemoglobin concentration [Hb], patient end-tidal carbon dioxide ($P_{ET}CO_2$), a measure of CO_2 partial pressure in expired air, which reflects the CO_2 level in the arterial blood, and peripheral oxygen saturation (SaO_2). Detailed information on phenotype collection and sampling is provided in Childebayeva et al. (2019a). All participants were healthy, non-pregnant, non-lactating, non-smokers between 19 and 41 years of age. All participants were of self-reported European ancestry and had at least 1 year since their last altitude experience. Participant characteristics can be found in **Table 1**.

Phenotype Testing

We performed linear mixed models using the R package lmerTest to test for significant differences in phenotypes between Kathmandu and Pheriche (**Table 1**). The following model was tested: Phenotype $\sim$ Altitude + Sex + Age + (1 | ID).

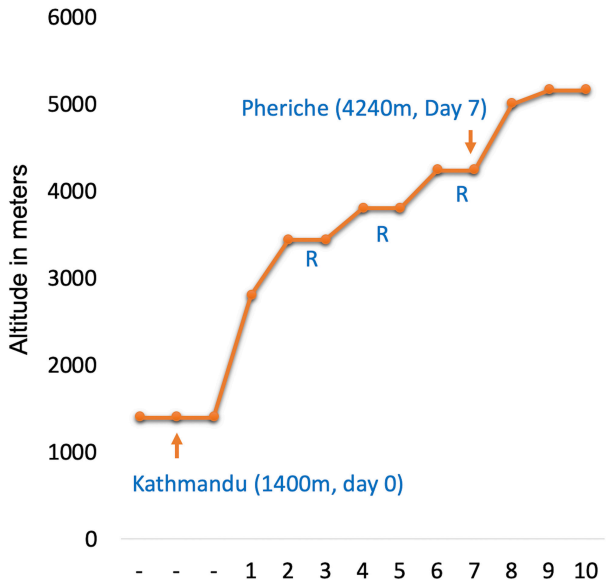

FIGURE 1 | Ascent profile with sample collection altitudes indicated with arrows and labels. Study participants flew from baseline (day 0) Kathmandu to 2,800 m to begin the trek. Three non-trekking rest days are indicated by "R." Epigenome wide association study was performed on matched samples collected at 1,400 m (day 0) and 4,240 m (day 7).

TABLE 1 | Participant characteristics.

	1,400 m (day 0)	4,240 m (day 7)
Hemoglobin (mg/L)*	13.1 (1.8)	14.8 (1.4)
BMI (kg/m^2)#	22.6 (2.4)	22.4 (2.2)
$P_{ET}CO_2$ (Torr)**	30.7 (3.2)	22.1 (2.9)
SaO_2 (%)**	97.1 (1.1)	89.8 (2.4)
% Female	53%	
Age, year	23.6 (6.0)	

Data are means (SD).
Significance symbols denote the difference between Kathmandu baseline and each altitude.
*#p-value < 0.10; *p-value < 0.01; and **p-value < 0.001.*

DNA Methylation

We generated DNA methylation data for ∼850,000 CpG sites using the Illumina Infinium® MethylationEPIC BeadChip assay for 32 samples in our study. We used the EZ-96 DNA Methylation™ Kit (Zymo Research, Irvine, CA, United States) to bisulfite convert each DNA sample following the standard protocol with alternative incubation conditions optimized for the Illumina Infinium® MethylationEPIC BeadChip assay. We used R for data processing and analysis implementing the packages minfi, ChAMP, and SmartSVA (Aryee et al., 2014; Morris et al., 2014; Chen et al., 2017). Based on QC metrics, two samples from the same participant failed and were excluded from all analyses; thus, the final sample size was n = 30 (15 at 1,400 m and 15 at 4,240 m).

Data normalization was performed using the funnorm normalization function in minfi (Aryee et al., 2014). We removed all probes that were above the 10e5 detection p-value threshold (N = 8,126) in more than 5% of the samples, all cross-reactive probes, probes associated with sex chromosomes, probes containing SNPs with MAF > 5% at target CpG sites, single base extension sites of type I probes, and in the body of the probe (Chen et al., 2013). All analyses were performed with N = 657,569 sites after normalization and probe removal. Samples were tested for batch effects using singular value decomposition (SVD) analysis in champ. SmartSVA (Chen et al., 2017) was used to perform a surrogate variable test, and the surrogate variable 1 was used for correcting for any saliva cell type differences associated with altitude. SmartSVA is a surrogate variable analysis method that can be used for reference-free adjustment for cell mixtures (Chen et al., 2017).

Differential Methylation Testing

Fully processed M-values were tested for differential methylation using the package limma (Ritchie et al., 2015). The following model was used to test for the differentially methylated positions (DMPs): DNA methylation ∼ Sample ID + Altitude + Surrogate Variable 1 (from smartSVA). P-values were adjusted for multiple testing using the false-discovery rate (FDR) following the Benjamini–Hochberg procedure (Hochberg and Benjamini, 1990) to produce FDR-corrected q-values. Differentially methylated regions (DMRs) were determined using DMRcate with default parameters (lambda = 1,000, C = 2, min.CpG sites = 2) (Peters et al., 2015). Pathway enrichment was performed using the package methylGSA (Ren and Kuan, 2019).

Angiotensin converting enzyme genotyping was performed using the same protocol as in Bigham et al. (2008). We extracted *ACE* CpG sites from the MethylationEPIC array to assess its methylation status independent from the epigenome-wide association analysis. We tested the relationship between ACE I/D status and SaO_2 separately for Kathmandu and Pheriche using linear modeling and adjusting for age and sex. The relationship between *ACE* genotype and phenotypes, as well as *ACE* genotype and *ACE* DNA methylation, was tested using linear mixed modeling in R using the lmerTest package (Kuznetsova et al., 2017). The linear mixed model was adjusted for altitude, age, sex, and individual IDs. Plotting was performed using the ggplot2 package (Wickham, 2009).

RESULTS

Participant Demographics

Our study group included n = 15 participants of self-reported European ancestry, with 53% females and the average BMI of 22.60 (SD 2.36) at baseline. Participant characteristics can be found in **Table 1**.

Physiological Changes With Altitude Exposure

We detected significant physiological changes between altitude 1,400 m (day 0) and 4,240 m (day 7) (henceforth physiological variables are referred to as phenotypes in this manuscript) in

arterial oxygen saturation (SaO_2), hemoglobin concentration [(Hb)], and end-tidal carbon dioxide partial pressure ($P_{ET}CO_2$) (**Table 1**). Briefly, we observed a significant increase in [Hb] and a significant decrease in SaO_2 and $P_{ET}CO_2$ with increasing altitude. The physiological responses we have reported are expected at high altitude, i.e., lower arterial oxygen saturation due to decreased ambient PO_2, a decrease in $P_{ET}CO_2$ indicating an increase in alveolar ventilation, and higher [Hb], reflecting the body's physiological response to low-oxygen conditions by increasing hemoglobin production.

Differential Methylation Analysis

We generated DNA methylation data for ∼850,000 CpG sites using the Illumina Infinium® MethylationEPIC BeadChip. After QC, we performed differential methylation analysis on 755,636 probes. We identified 2,873 DMPs at q-value < 0.10 (**Supplementary Table 1**) that differed between baseline 1,400 and 4,240 m genome-wide inflation factor λ = 1.2. Among these, we identified HIF pathway genes: *ANGPT1, CREBBP, CUL2*, *HIF1A*, *HK1*, *HMOX1*, *PDK1* (two significant CpG sites), *PIK3R3*, *PLCG1*, *PRKCG*, *RELA*, and *STAT3,* and RAS pathway genes: *ABL1*, *ANGPT1*, *EFNA3*, *FGFR1*, *GAB1*, *GNB1*, *GNB3*, *GNB4*, *GRB2*, *KITLG*, *KRAS*, *MAPK10*, *PAK1*, *PAK2*, *PDGFA*, *PIK3R3*, *PLCG1*, *PRKCG*, *PTPN11*, *RALA*, *RAP1A*, *RAP1B*, *RASA3* (five significant CpG sites), *RASSF1*, *RELA*, *RGL2*, and *RIN1* (**Table 2**). We also identified genes associated with inflammation: *IL12B*, *TRIM31, NLRP3, IL1RAP,* among others, and genes associated with cognitive function: *ASH1L* and *TNIK*.

In order to detect biological pathways overrepresented among the significant CpG sites from the analysis of differential methylation, we performed a pathway enrichment analysis using the methylgometh function in the R package methylGSA (Ren and Kuan, 2019). Ninety-five significant pathways were identified by methylgometh including the GO pathways regulation of glycolytic process (GO:0006110), regulation of hematopoietic stem cell differentiation (GO:1902036), and regulation of angiogenesis (GO:0045765) (**Supplementary Table 2**). Other pathways of interest included brain development (GO:0007420), negative regulation of neuron differentiation (GO:0045665), and interleukin-1-mediated signaling pathway (GO:0070498).

We then tested for DMRs, i.e., contiguous regions in the genome that show differential methylation between phenotypes or groups. We used DMRcate (Peters et al., 2015) to find DMRs between low- (1,400 m) and high-altitude (4,240 m) samples. Using this approach, we identified 361 significant DMRs out of 657,408 possible DMRs (**Supplementary Table 3**). These included DMRs near/in genes associated with the HIF pathway: *HIF1A* and *ENO1* (glycolytic enzyme), and the RAS pathway: *ABL1*, *FGFR3*, *KRAS*, *RASA3*, and *RGL2*.

Phenotype Associations

To determine if changes in DNA methylation could be driving acclimatization, we performed association testing between significant genome-wide methylation positions and phenotypes associated with high-altitude acclimatization. To do so, we focused our analysis on significant CpG sites identified in the DMP analysis (N = 2,873) and phenotypes that were significantly different between the groups (**Table 1**) including SaO_2, [Hb], and $P_{ET}CO_2$. Two CpG sites, cg16546681 (chr1:155244518, q-value = 0.01, β regression coefficient = 6.46) in the gene *CLK2* and cg14548038 (chr9:140178418, q-value = 0.03, β regression coefficient = 4.73) upstream of the gene *TOR4A,* were significantly positively associated with SaO_2 (%). No significant associations were identified for [Hb] or $P_{ET}CO_2$ after correcting for multiple comparisons.

ACE I/D, Oxygen Saturation, and DNA Methylation

We tested the relationship between *ACE*, a gene associated with high-altitude performance, and high-altitude phenotypes [SaO_2, $P_{ET}CO_2$, (Hb)]. Individuals in this study were genotyped for the *ACE* I/D (rs4646994) polymorphism. We performed a genotypic test, wherein I/I and I/D genotypes were compared to D/D genotype, and identified a significant association between *ACE* genotype and SaO_2. Individuals with genotypes I/D (β regression coefficient = 1.69, p-value < 0.01) and I/I (β regression coefficient = 1.85, p-value < 0.05) had significantly higher SaO_2 than individuals with the D/D genotype at 1,400 m (Kathmandu); the relationship was not significant for 4,240 m (Pheriche) (**Figure 2**). In an additive model, the I-allele was associated with increased SaO_2 (β regression coefficient = 1.03, p-value < 0.05) at 1,400 m; the relationship was also not significant for 4,240 m. In a dominant model, individuals who were either heterozygotes or homozygotes for the I-allele (grouped together) displayed higher SaO_2 (β regression coefficient = 1.71, p-value < 0.01) at 1,400 m; the relationship was approaching significance (β regression coefficient = 2.79, p-value = 0.09) for 4,240 m. Our results suggest that the dominant model, wherein individuals carrying either the I/D or I/I alleles have higher oxygen saturation than individuals carrying the D/D allele, is best suited to explain the relationship between SaO_2 ACE I/D in our study.

Average *ACE* methylation was positively associated with the I-allele when we tested the relationship using an additive model (β regression coefficient = 0.31, p-value = 0.03) (**Figure 2B**). We also tested the relationship between individual *ACE* CpG sites and high-altitude phenotypes. *ACE* CpG sites, cg02040921 and cg09920557, were associated with SaO_2 (cg02040921: p-value = 0.04; cg09920557: p-value = 0.06). Increased methylation of *ACE* CpGs was associated with increased SaO_2 (**Figures 2C,D**). No significant associations were identified for [Hb] or $P_{ET}CO_2$.

DISCUSSION

The role of epigenetic change, including DNA methylation, in acclimatization to short-term hypoxia exposure is not well characterized. We aimed to fill this gap using genome-wide DNA methylation data from the same individuals measured at different altitudes during a trek to Everest Base Camp. We identified significant associations between genome-wide DNA methylation and short-term altitude exposure, among which were CpG sites and regions associated with HIF pathway, including *HIF1A*, and RAS pathway genes.

TABLE 2 | Significant CpG sites associated with HIF and RAS pathways.

Pathway	Gene	CpG	*p*-value	*q*-value	Chr	Position (hg19)	Relation to island
HIF	*ANGPT1*	cg09443479	1.96E-04	0.08	8	108,511,174	OpenSea
	CREBBP	cg16560077	7.48E-05	0.05	16	3,781,408	Island
	CUL2	cg09080721	1.76E-04	0.07	10	35,361,575	OpenSea
	HIF1A	cg16788202	2.45E-04	0.08	14	62,162,340	Island
	HK1	cg06506461	3.14E-04	0.09	10	71,112,319	OpenSea
	HMOX1	cg15724965	1.87E-05	0.03	22	35,777,001	Island
	PDK1	cg13462525	7.98E-05	0.05	2	173,420,046	N_Shore
	PDK1	cg11703569	4.63E-05	0.04	2	173,421,320	Island
	PIK3R3	cg12800095	9.33E-05	0.06	1	46,594,087	OpenSea
	PLCG1	cg13312309	5.68E-05	0.05	20	39,799,964	OpenSea
	PRKCG	cg14975881	3.28E-05	0.04	19	54,389,945	N_Shelf
	RELA	cg04962756	2.35E-06	0.01	11	65,425,928	OpenSea
	STAT3	cg09804439	1.59E-04	0.07	17	40,540,457	Island
RAS	*ABL1*	cg13609937	4.40E-05	0.04	9	133,588,314	Island
	ANGPT1	cg09443479	1.96E-04	0.08	8	108,511,174	OpenSea
	EFNA3	cg06058618	6.65E-06	0.02	1	155,057,452	Island
	FGFR1	cg00676030	1.74E-05	0.03	8	38,307,962	OpenSea
	GAB1	cg24244452	3.54E-04	0.09	4	144,284,260	OpenSea
	GNB1	cg14953148	1.56E-04	0.07	1	1,792,846	OpenSea
	GNB3	cg06444189	2.38E-05	0.03	12	6,953,740	OpenSea
	GNB4	cg12872693	4.08E-04	0.10	3	179,168,798	Island
	GRB2	cg11495544	3.76E-04	0.10	17	73,402,155	S_Shore
	KITLG	cg22688836	6.67E-05	0.05	12	88,967,594	OpenSea
	KRAS	cg02850821	8.03E-06	0.02	12	25,403,680	OpenSea
	MAPK10	cg03886687	2.43E-04	0.08	4	87,281,409	OpenSea
	PAK1	cg26996201	2.86E-04	0.09	11	77,122,864	Island
	PAK2	cg02319016	1.34E-06	0.01	3	196,469,777	S_Shelf
	PDGFA	cg22466784	2.41E-04	0.08	7	540,176	OpenSea
	PIK3R3	cg12800095	9.33E-05	0.06	1	46,594,087	OpenSea
	PLCG1	cg13312309	5.68E-05	0.05	20	39,799,964	OpenSea
	PRKCG	cg14975881	3.28E-05	0.04	19	54,389,945	N_Shelf
	PTPN11	cg16207631	2.76E-04	0.09	12	112,856,603	Island
	RALA	cg19104112	2.75E-04	0.09	7	39,663,043	Island
	RAP1A	cg25355888	2.83E-04	0.09	1	112,162,642	Island
	RAP1B	cg00758412	2.23E-04	0.08	12	69,033,023	OpenSea
	RASA3	cg21364828	1.18E-04	0.06	13	114,825,608	OpenSea
	RASA3	cg13818243	2.76E-04	0.09	13	114,789,734	S_Shelf
	RASA3	cg04421280	1.20E-04	0.06	13	114,898,225	Island
	RASA3	cg00427150	1.92E-04	0.07	13	114,770,568	N_Shelf
	RASA3	cg20028528	2.20E-04	0.08	13	114,812,184	N_Shore
	RASSF1	cg25486143	3.20E-04	0.09	3	50,378,527	Island
	RELA	cg04962756	2.35E-06	0.01	11	65,425,928	OpenSea
	RGL2	cg08312215	4.75E-05	0.04	6	33,266,943	Island
	RIN1	cg15082918	2.81E-04	0.09	11	66,104,153	S_Shore

We identified both a significant CpG position (DMP) and a DMR associated with hypoxia inducible factor 1A or *HIF1A*, which is a central gene in the body's hypoxic response (Slemc and Kunej, 2016). In normoxia, HIF1A is degraded *via* ubiquitination but is allowed to accumulate in hypoxic conditions. This allows its protein product to bind to a constitutively expressed HIF1B forming a heterodimer that activates downstream genes (Wenger, 2002). HIF1A activity is under epigenetic control in human cancer cells and hematopoietic cell lines (Walczak-Drzewiecka et al., 2010; Nguyen et al., 2013; Cimmino et al., 2019). Importantly, the *HIF1A*-associated DMR identified here overlaps with the promoter region of the gene, suggesting that methylation at this locus may be associated with changes in gene expression.

We found significant CpG sites associated with the RAS pathway, including ones in the genes *ANGPT1* and *RASA3* (RAS P21 protein activator 3). Angiopoietins 1 and 2 are regulated by HIF1, and *ANGPT1* expression is associated with increased number of vessels without excessive permeability (Kelly et al., 2003). *ANGPT1* can be activated and repressed by HIF1 in a cell-specific manner (Kelly et al., 2003). *RASA3* (RAS P21 protein activator 3) is a Ras-GTPase activating protein that causes anemia and thrombocytopenia in mice when mutated

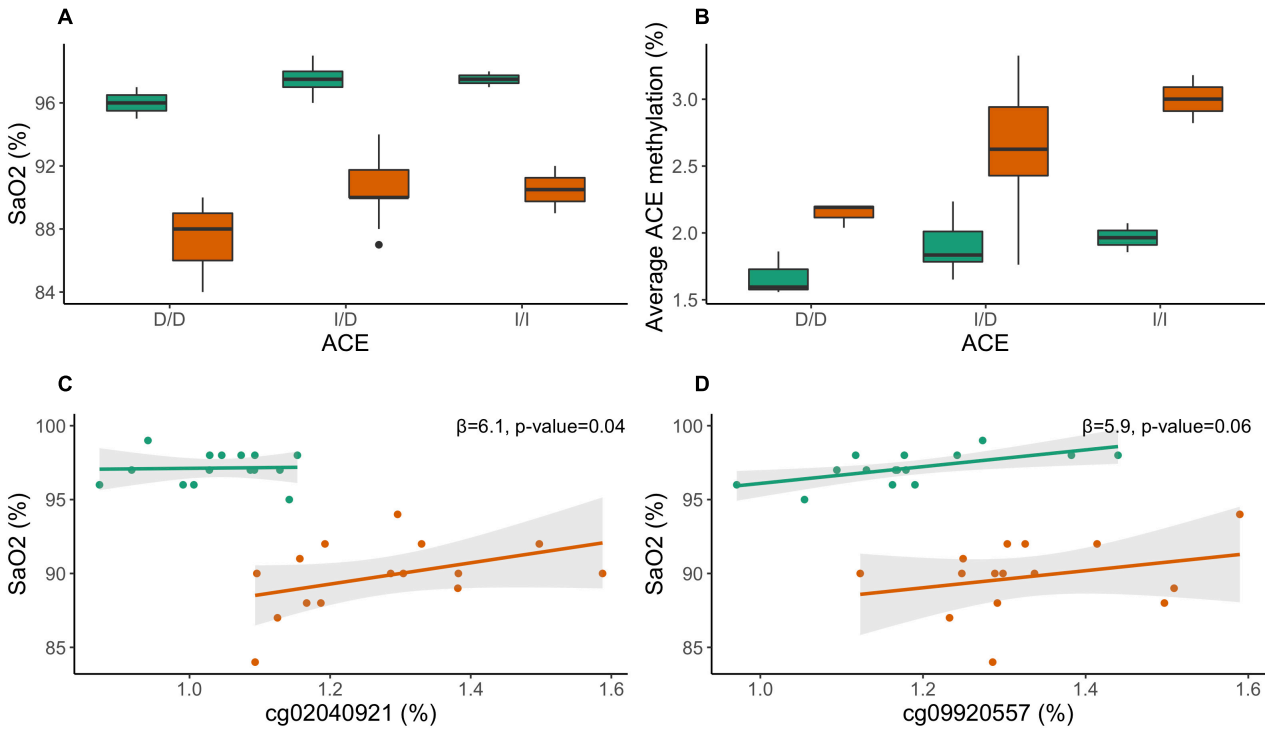

FIGURE 2 | Relationship between ACE I/D, ACE methylation, and SaO_2. **(A)** Boxplot of arterial oxygen saturation by ACE I/D genotype. **(B)** Boxplot of average ACE DNA methylation by ACE I/D genotypes. **(C)** ACE CpG site cg02040921 plotted against SaO_2. **(D)** ACE CpG site cg09920557 plotted against SaO_2. Day 0: 1,400 m (K) is indicated in green and day 7: 4,240 m (P) is noted in orange.

(Blanc et al., 2012). RAS pathway is another canonical hypoxia-induced pathway. RAS has been linked to blood pressure (Fontes et al., 1994), cardiovascular disease (Lee et al., 1993), and primary hypertension (Frossard et al., 1998). The role of RAS in hypoxia has been explored in association with high-altitude pulmonary edema *via* the regulation of the pulmonary vascular tone (Stobdan et al., 2011).

We also found significant DNA methylation changes in genes outside of canonical pathways implicated in high-altitude acclimatization (i.e., HIF and RAS), including significant DNA methylation changes in genes associated with cognitive impairment [*ASH1L* (de Ligt et al., 2012; Crawley et al., 2016; Xi et al., 2020) and *TNIK* (Coba et al., 2012; Anazi et al., 2016)]. Cognitive decline is a common side effect of high-altitude hypoxia (Regard et al., 1989; Yan, 2014; Gao et al., 2015) that becomes apparent 1–2 weeks after initial exposure (Bolmont et al., 2000) and may improve to some degree upon acclimatization (Heinrich et al., 2019). This timing of the cognitive decline is consistent with our study design wherein we identified methylation changes in *ASH1L* and *TNIK* after 1 week of high-altitude exposure. In addition to methylation differences in genes associated with cognitive function, we also identified changes in several genes associated with inflammation. These included CpG sites in the genes *IL12B* (Glas et al., 2012; Liu et al., 2012) and *TRIM31* (Song et al., 2016; Wang et al., 2018).

We specifically focused on the RAS pathway gene *ACE* as it is centrally involved in circulatory homeostasis, and the *ACE* I/D polymorphism has been linked to endurance performance (Myerson et al., 1999), adaptation of highland resident/native populations (Qadar Pasha et al., 2001; Bigham et al., 2008), and performance at altitude (all those other citations). The *ACE* I allele is associated with lower ACE activity (Costerousse et al., 1993) and higher SaO_2 (Woods et al., 2002), potentially as a result of an increased HVR (Patel et al., 2003). We identified an association between *ACE* genotypes I/D and I/I with higher SaO_2, which is consistent with previous research showing a significant relationship between *ACE* and SaO_2 (Woods et al., 2002; Bigham et al., 2008).

We found that the *ACE* I-allele was associated with higher average *ACE* methylation, which has been shown before in a study of birth weight and *ACE* (Rangel et al., 2014). Notably, the *ACE* I-allele is associated with lower serum and tissue ACE activity (Rigat et al., 1990; Costerousse et al., 1993; Woods et al., 2000). Since methylation is commonly associated with gene silencing, the association between *ACE* I-allele and higher DNA methylation suggests that *ACE* methylation may be involved in mediating decreased *ACE* expression in individuals with the I-allele.

Individuals at high altitude displayed increased [Hb] and decreased SaO_2 and $P_{ET}CO_2$ compared to low altitude. We found two CpG sites, in the gene *CLK2* and near the gene *TOR4,* that were associated with SaO2. CDC like kinase 2 or CLK2 suppresses *PPARGC1A* transcriptional activity on

gluconeogenic genes (Sahu et al., 2019) and thus downregulates hepatic gluconeogenesis and glucose output. We found *CLK2* methylation to be positively associated with SaO_2, suggesting that *CLK2* expression is potentially decreased in hypoxic conditions, given methylation is linked to gene repression. Interestingly, the CpG site in *CLK2* is upstream of the gene *PKLR* that is significantly differentially methylated in high- compared to low-altitude Quechua (Childebayeva et al., 2020). We also found a CpG site upstream of *TOR4A* (Torsin family 4 member A), which is associated with dystonia (Cascalho et al., 2017). Dystonia is linked to hypoxic exposure, more specifically cerebral anoxia/hypoxia (Kuoppamaki et al., 2002; Kern et al., 2016), and our finding might indicate a potential epigenetic mechanism playing role in the development of this condition.

Tissue types can show different methylation profiles across the body, and the degree to which they correlate varies by study design, type of sample, or age (Langie et al., 2017). For example, there is evidence of a low correlation between salivary and blood global DNA methylation (Godderis et al., 2015). Here, we analyzed saliva. Saliva is an attractive tissue for the analysis of DNA methylation in field studies given its relative ease of collection compared to blood or other tissues (Langie et al., 2017). By focusing on a singular tissue type, our results may be restricted to salivary tissue alone. However, salivary DNA methylation patterns have been shown to correlate with DNA methylation from blood (Thompson et al., 2013; Langie et al., 2016), intestinal mucosa (Hearn et al., 2019), and the brain (Smith et al., 2015). Furthermore, saliva panels have shown proteomic changes upon hypoxic exposure in cell cultures (Jain et al., 2020), suggesting the relevance of this tissue for analyzing the overall hypoxic response. Therefore, we suggest that our analysis of saliva is an important first step in identifying DNA methylation changes to acute hypoxia that may be relevant to other bodily tissues.

Overall, our data demonstrate that various pathways and systems are affected by exposure to high altitude, including the HIF pathway, RAS pathway, cognitive performance, and inflammatory systems. Moreover, we identified a significant association between SaO_2 and *ACE* I/D, and associations between *ACE* I/D and *ACE* methylation, further highlighting the connection between *ACE* and SaO_2 as well as the role of *ACE* in altitude acclimatization.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author AC, upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Syracuse University Institutional Review Board (Protocol 18-006), University of Michigan Institutional Review Board (HUM00141118), Mount Royal University Human Research Ethics Board (Protocol 100012 and 101361), and Nepal Health Research Council (Protocol 109-2017). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AC, TB, and AB: conceptualization. AC, TD, and JW: data curation. AC: formal analysis. TD and AC: funding acquisition. AC and TH: investigation. AC and AB: writing—original draft preparation. AB, AC, TB, TD, and TH: writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.660906/full#supplementary-material

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Enhanced Vasoconstriction Mediated by α_1-Adrenergic Mechanisms in Small Femoral Arteries in Newborn Llama and Sheep Gestated at Low and High Altitudes

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The authors previously demonstrated that newborn llama (NBLL) express high levels of α_1 adrenergic receptors, which provide a potent vasoconstriction response when compared with newborn sheep (NBSH) gestated at sea level. However, data regarding the impact of chronic gestational hypobaric hypoxia on α-adrenergic vasoconstriction in the neonatal life has not been studied. We evaluated if gestation under chronic hypobaric hypoxia modifies α_1-adrenergic vasoconstrictor function in NBLL and NBSH. We compared the vasoconstrictor response induced by potassium and α-adrenergic stimuli in isolated small femoral arteries of NBLL and NBSH gestated at high altitude (HA; 3,600 m) or low altitude (LA; 580 m). The maximal contraction (R_{MAX}) and potency (EC_{50}) to potassium, noradrenaline (NA), and phenylephrine (PHE) were larger in HA-NBLL than LA-NBLL. R_{MAX} to potassium, NA, and PHE were lower in HA-NBSH when compared with LA-NBSH and potency results were similar. Competitive blockade with prazosin showed that RNLL LA/HA have a similar pA_2. In contrast, NBSH had increased pA_2 values in HA when compared with LA. Finally, small femoral arteries denudated or treated with LNAME in LA and HA lacked NO or endothelium participation in response to PHE stimulation. In contrast, NBSH displayed that denudation or blockade with LNAME support NO or endothelium participation in response to PHE activation. In conclusion, HA chronic hypoxia enhances α_1 adrenergic receptor activity in small femoral arteries in NBLL to a higher degree than NBSH, implying a higher vasoconstriction function.

Keywords: newborn llama, newborn sheep, sea level, chronic hypoxia, femoral vasoconstriction, noradrenaline, phenylephrine

INTRODUCTION

When humans and animals are exposed to high-altitude (HA) environments, the low barometric pressure decreases the amount of oxygen in the body (Conkin and Wessel, 2008). Chronic hypobaric hypoxia conditions likely exerted a selective pressure on those species with ancestry at HA, which allowed them to develop cellular and physiologic defense strategies to live under low atmospheric PO_2 (Monge and León-Velarde, 1991; Llanos et al., 2007). Camelids are dwellers of the Andean plateau that have a wide distribution between 3,500 and 5,000 m above sea level, and evidence supports an ancestry of over 2 million years in this low PO_2 environment (Stanley et al., 1994). Sheep (*Ovis aries*) and llama (*Lama glama*) have been compared to evaluate how species manage to dwell at HA; the former as a paradigm of acclimatization, with only 500 years in highlands and the latter of adaptation. Both species have been compared in studies of perinatal physiology and cardiovascular adaptations to chronic hypoxia (Llanos et al., 2003, 2007; Reyes et al., 2020).

The llama is a camelid species that inhabits the Alto Andino plateau. They are often studied for their notable physiological adaptations to hypoxia (Meschia et al., 1960; Banchero and Grover, 1972; Braunitzer, 1980; Llanos et al., 2007), that is the hemoglobin mutation, allowing a higher hemoglobin oxygen affinity (Braunitzer, 1980; Moraga et al., 1996). Additionally, studies performed during fetal life show strong vasoconstriction when the fetus is submitted to acute hypoxia. This response is induced by the release of catecholamines, neuropeptide Y (NPY), and other vasoconstrictors (Giussani et al., 1996, 1999; Reyes et al., 2020). α-Adrenergic vasoconstriction is higher in sea level fetal llama than fetal sheep. It is also critical for blood flow redistribution and survival during acute hypoxia since α-adrenergic blockade elicited death in fetal llama but not in fetal sheep (Giussani et al., 1999). However, in chronically hypoxic fetal sheep, α-adrenergic peripheral vasoconstriction is also enhanced and is critical for survival (Block et al., 1984; Giussani et al., 1996, 1999). We previously demonstrated that llamas living at sea level for several generations deliver newborns with a high femoral vasoconstrictor response to acute hypoxia compared with their sheep counterparts. Also, sea level newborn llamas (NBLL) have greater femoral sensitivity and maximal contraction to norepinephrine and phenylephrine (PHE) than newborn sheep (NBSH). This sensibility was corroborated by competitive inhibition experiments that showed higher prazosin affinity in NBLL than NBSH. This indicates the presence of one high-affinity adrenoceptor, which is consistent with the greater α_{1B}-adrenoceptor transcript expression observed in the small femoral arteries of NBLL compared with NBSH (Moraga et al., 2011). In addition, α_1-adrenergic receptor activation by PHE may be counterbalanced by simultaneous NO release from the endothelium in neonatal sheep but not in neonatal llama at lowland (Moraga et al., 2011). However, the effect of chronic hypoxia on the α-adrenergic vasoconstrictor tone has not been compared between species with or without HA ancestry (Longo and Pearce, 1998; Goyal et al., 2014). We hypothesize that HA chronic hypoxia enhanced femoral α-adrenergic vascular reactivity in NBLL but not in NBSH. For this, we used pharmacological approaches to test the role of adrenergic receptors in small femoral arteries by comparing the responses of llama (NBLL) and sheep neonates (NBSH) gestated at low altitude (LA) and HA.

MATERIALS AND METHODS

All the procedures of animal care, maintenance, and experimentation were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986, and the American Physiological Society "Guiding Principles for Research Involving Animals and Human Beings" (American Physiological Society, 2002), and approved by Faculty of Medicine Ethics Committee of the University of Chile.

Animals

We studied eight NBSH and seven NBLL s gestated, born, and raised at the University of Chile farm, Santiago, located at 580 m above sea level; and six NBSH and six NBLL, gestated, born, and raised at Putre Research Station, International Center for Andean Studies (INCAS), University of Chile, located at 3,600 m above sea level (see **Supplementary Table**). The ancestry of HA maternal sheep has been estimated at over 50 generations (Herrera et al., 2007) and around 2 million of years for maternal llamas (Stanley et al., 1994). The llama and sheep mothers and their newborns were maintained with access to food and water *ad libitum* in an open yard. The shed time of both llamas and sheep was programmed so that all deliveries occurred in the same spring–summer season. The installed experimental station is in Putre, which is at 3,500 m. The measured values indicate a low relative humidity close to 20%, with thermal oscillations of 5°C at night and 16°C during the day in the spring–summer season. All the animals used for the study of small arteries were healthy and showed the expected postnatal growth for the altitude where the experiment was performed.

Ex Vivo Small Vessel Wire Myography

All the animals underwent euthanasia using sodium thiopentone (1 g I.V.). We dissected from deep femoral artery where small arteries of approximately 300–350 μm diameter and 1.8–2.0 mm length were cut and placed in ice-cold saline (see **Supplementary Table**). Arterial rings were mounted in a myograph to perform studies of isometric force (610M, Multimyograph, Danish Myotechnology, Aarhus, Denmark) by continuously recording tension using a data acquisition system connected to a computer (Powerlab/8SP; AD Instruments) (Moraga et al., 2011). The small femoral arteries were evaluated with (+E) or without (–E) endothelium. To eliminate the endothelium from the femoral arteries, a strand of human hair was gently rubbed backward and forward through the lumen of the vessel (Auer and Ward, 1998). To ensure the endothelium was denudated, we evaluated the response to the endothelium-dependent vasodilator ACh (10^{-5} M) in arteries, previously contracted with 10^{-5} M PHE. All endothelium-denudated rings lacked relaxation when exposed to the ACh bath, indicating that denudation was successful. Afterward, all small femoral

TABLE 1 | Values of CRC in presence of potassium chloride, noradrenaline (NA), phenylephrine (PHE) in NBLL and NBSH at LA and HA.

	NB Llama		NB Sheep	
	LA	**HA**	**LA**	**HA**
CCR K^+				
EC_{50} (mM)	29.8 ± 1.6	25.1 ± 1.2 *	36.6 ± 1.7†	38.7 ± 3.7†
R_{MAX} (N/m)	15.6 ± 1.2	32.5 ± 0.5 *	10.6 ± 0.9†	8.3 ± 0.3*†
CCR NA				
pEC_{50} (M)	5.34 ± 0.13	6.51 ± 0.12 *	4.88 ± 0.28	5.61 ± 0.18*†
R_{MAX} (N/m)	16.2 ± 0.40	33.0 ± 0.60 *	13.8 ± 1.20	8.60 ± 0.20*†
R_{MAX} (%K^+)	98.2 ± 3.50	101.5 ± 1.60	102.8 ± 1.60	105 ± 1.80
CCR PHE				
pEC_{50} (M)	5.52 ± 0.13	6.24 ± 0.14 *	4.72 ± 0.11†	6.08 ± 0.17*
R_{MAX} (N/m)	15.0 ± 0.30	30.1 ± 0.80 *	6.20 ± 0.23†	4.99 ± 0.21*†
R_{MAX} (%K^+)	101.3 ± 2.3	95.4 ± 0.88	56.9 ± 1.10§†	57.9 ± 2.30§†

*Means ± SEM; * $p < 0.05$ LA vs. HA, §$p < 0.05$ NA vs. PHE, and †$p < 0.05$ NBLL vs. NBSH.*

arteries (+E or –E) were incubated in Krebs Ringer Bicarbonate (KRB) at 37°C and gassed (95% O_2 and 5% CO_2). After a period of incubation (1 h), the optimal diameter was determined for each artery by stretching the artery rings in a stepwise manner until a tension equivalent to the physiological transmural pressure was obtained (Mulvany and Halpern, 1997). In this condition, a dose of 62.5 mM of potassium chloride was added to promote a contraction dependent on the degree of stretch until it gave the maximal vascular response (Moraga et al., 2011; Castillo-Galán et al., 2016). Concentration-response curves (CRC) were performed for potassium chloride by adding 6.25–125 mM of KCl, α-adrenergic agonist NA, and α_1-adrenergic agonist PHE, with concentrations ranging from 10^{-10} to 10^{-3} M as described previously (Moraga et al., 2011). The participation of endothelium in modulating the vasoconstriction mechanism was evaluated through concentration-response curves for PHE in intact (+E) and endothelium-free (–E) femoral arteries, either in the presence or the absence of the nitric oxide synthase (NOS) inhibitor L-NAME (10^{-5} M). The relative affinity of prazosin was determined by performing concentration response curves to NA in preincubated vessels for 30 min with prazosin (α_1-adrenergic receptor blocker with doses of 10^{-9}, 10^{-8} and 10^{-7} M). Each curve was evaluated in triplicate. We collected atleast 12–24 rings of small femoral arteries per animal to carry out the different assays. After finishing one curve, each artery was maintained at rest, with KRB, for at least 30 min, changing KRB solution every 10 min. Before initiating a new curve, contraction induced by 62.5 mM KCl was tested to evaluate the viability of rings. If maximal responses (R_{MAX}) was lower than 80% of contraction obtained after CRC to K^+, the artery was discarded.

Solutions and Reagents

KRB contained (in mM) NaCl 118.5, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 5.5 with a pH of 7.4. In K-KRB (125 mM K^+) NaCl was replaced by an equimolar amount of KCl. Noradrenaline, PHE, L-NAME, and prazosin were obtained from Sigma Chemical Co., USA.

Analysis

Potency (EC_{50}) and R_{MAX} to the different vasoactive agents tested were obtained by fitting the concentration-response curves to a Boltzmann function (Prism 4.0, Graphpad) as described previously (Moraga et al., 2011). R_{MAX} was expressed as tension (N/m) for K^+ and/or as a percentage relative to a submaximal dose of 62.5 mM of KCl (expressed, $\%K^+_{MAX}$) for the adrenergic agonists. Values are normalized ($\%K^+_{MAX}$), in accord with sigmoidal shape and the model is assumed to have a standard slope with a Hill slope of 1.0 (Motulsky and Christopoulos, 2003). In this sense, potency was expressed as EC_{50} (the concentration at which 50% of R_{MAX} was obtained) for K^+ or as pEC_{50} ($-\log EC_{50}$) for the adrenergic agonists. The relative affinity (pA_2) for prazosin was estimated through Schild analysis by plotting log (R – 1) values for individual vessels against the antagonist (prazosin) concentration (log [A]), where R is defined as a ratio of the EC_{50} NA plus antagonist [A] divided by EC_{50} NAalone (Arunlakshana and Schild, 1959). Slope analysis was performed in each curve, supporting the presence of a competitive antagonist. Then, pA_2 values were considered the x-intercept to the Schild slope (Motulsky and Christopoulos, 2003).

Statistical Analysis

Data are expressed as means ± S.E.M. Two-way ANOVA for repeated measures followed by the *post hoc* test of Newman–Keuls or the Student's t test for unpaired data were used to compare data, as appropriate. Linear regression analysis of the Schild plot was used to estimate pA2 (intercept) values, the slope, and the correlation coefficient of the regression line (Motulsky and Christopoulos, 2003). For all comparisons, differences were considered significant when $p < 0.05$ (Zar, 1984).

RESULTS

Ex vivo Small Femoral Arteries Wire Myography

Contractile Response to Potassium Chloride in Femoral Arteries of NB Llamas and Sheep of LA and HA

In the analysis of K^+ CRC sensitivity, NBLL showed that HA neonates are more sensitive (EC_{50}) than LA neonates. NBSH showed a similar potency (EC_{50}) at LA and HA (**Table 1**). LA and HA NBSH potency was lower than LA and HA NBLL ($p < 0.05$) (**Table 1**; **Supplementary Figure 1**).

Regarding the R_{MAX}, LA NBLL was lower than HA NBLL ($p < 0.05$). In contrast with the NBLL, the LA NBSH had a greater maximal contraction than HA NBSH ($p < 0.05$) (**Table 1**). Moreover, LA and HA NBLL had a higher maximal contraction than LA and HA NBSH femoral vessels ($p < 0.05$) (**Table 1**).

Contractile Response to Adrenergic Agonists in Femoral Arteries of NB Llamas and Sheep of LA and HA

The absolute tension of HA-NBLL had a greater R_{MAX} to both NA and PHE than their LA counterparts ($p < 0.05$). However, when normalized to potassium, the R_{MAX} for NA and PHE were similar in LA- and HA-NBLL, but their corresponding pEC_{50} values were higher in HA- than LA-NBLL ($p < 0.05$) (**Table 1**). In NBSH, R_{MAX} for either NA or PHE expressed as absolute values were lower in HA- than LA-animals, but they were similar for K^+-normalized values, while pEC_{50} was larger in HA- than in LA-NBSH (**Table 1**). When compared, the contraction to PHE was ∼57% of the tension elicited by NA in NBSH (**Table 1**). Additionally, we observed that pEC_{50} and R_{MAX} to NA (in N/m) are similar between species at LA. However, the response to PHE of pEC_{50}, R_{MAX} (N/m and $\%K^+$) in NBSH is lower than NBLL. When comparing both species at HA, we observed that pEC_{50} and R_{MAX} to NA (N/m and $\%K^+$) in NBSH were lower than NBLL ($p < 0.05$). In addition, the pEC_{50} in response to PHE was similar to NBLL and R_{MAX} (N/m and $\%K^+$) in NBSH and was lower than NBLL at HA ($p < 0.05$) (**Supplementary Figure 2**).

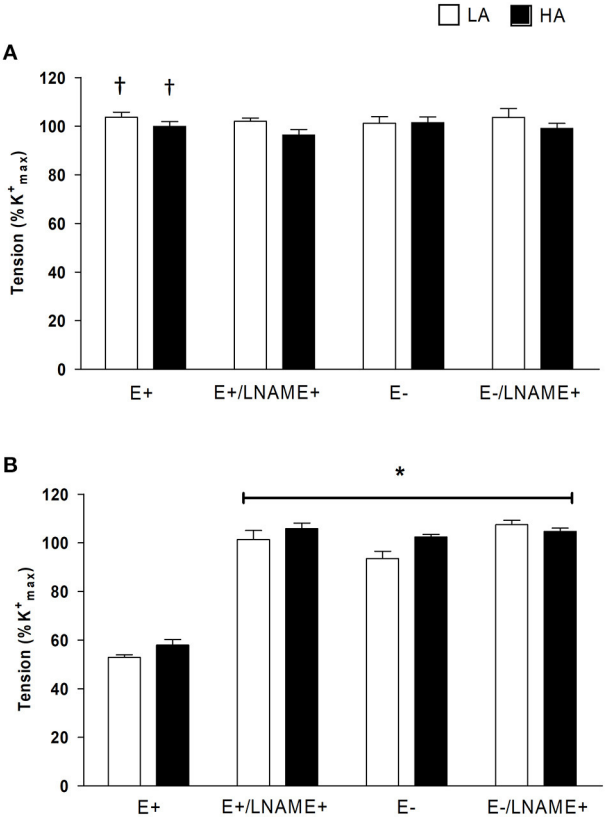

FIGURE 1 | Effect of *N*G-nitro-L-arginine methyl ester L-NAME and endothelium remove (–E) on PHE-induced contraction. **(A)** In NBLL ($n = 6$) and CRC to PHE, and PHE after 30 min of incubation with 10^{-5} M L-NAME with endothelium (+E) and without endothelium (–E). **(B)** Effect of L-NAME on PHE-induced contraction in NBSH ($n = 6$) and CRC to PHE, and PHE after 30 min of incubation with 10^{-5} M L-NAME with endothelium (+E) and without endothelium (–E), where open bar represent LA and close bar represent HA. Values are expressed as means ± SEM. Asterisk (*) represent a significant difference between NBSH (LA/HA) PHE-induce contraction (+E) vs PHE-induce contraction (+E) plus L-NAME and PHE-induce contraction (–E) ($p < 0.05$). † Represent significant difference between NBLL vs NBSH ($p < 0.05$).

The Role of (NOS) and Endothelium in the Vasocontractile Response to PHE in NBLL and NBSH of LA and HA

First, to probe the role of NOS we compared the CRC to PHE in the absence or the presence of NOS inhibition with L-NAME (10^{-5} M). In NBLL of either LA and HA, L-NAME did not modify R_{MAX} (**Figure 1A**; **Table 2**) or pEC_{50} of PHE (**Table 2**). In contrast, in NBSH of LA and HA, NOS blockade with L-NAME promoted an R_{MAX} of PHE similar to NA without modification in pEC_{50} (**Figure 1B** and **Table 2**). To examine the role of endothelium, we denudated small femoral arteries in both species and performed CRC again for PHE in the presence and the absence of L-NAME. In NBLL of either LA or HA, denudation had no effect on pEC_{50} or R_{MAX} to PHE (**Figure 1A** and **Table 2**). In contrast, in NBSH of LA and HA, denudation of small femoral arteries increased the R_{MAX} for PHE, with values similar to those in the presence of NA without modification in pEC_{50} (**Figure 1B** and **Table 2**). Lastly, we determined the role of NOS blockade with L-NAME (10^{-5} M) and arterial denudation in both species. Again, we performed CRC for PHE. In the NBLL of either LA or HA, denudation did not affect pEC_{50} or R_{MAX} to PHE (**Figure 1A** and **Table 2**). NBSH of LA and HA, blocked and with denudation of small femoral arteries, increased R_{MAX} for PHE to values similar to those observed in the presence of NA without modification in pEC_{50} (**Figure 1B**, **Tables 1**, **2**). When comparing both species at LA, we observed that pEC_{50} in NBLL was higher than NBSH in all experimental conditions. However, when comparing both species at HA, values of pEC_{50} in NBLL were similar to NBSH (**Supplementary Figure 3**).

Relative Affinity (pA_2) to NA in Femoral Arteries in NB Llama and Sheep at LA and HA

The blockade with prazosin, a selective α_1-adrenergic antagonist, promoted a concentration-dependent rightward shift of pEC_{50}

TABLE 2 | Effect of L-NAME blocked and denudation of small femoral arteries in NB LL and NBSH at LA and HA.

	NB llamas		NB Sheep	
	LA	**HA**	**LA**	**HA**
CRC PHE (+E)				
p_{EC50} (M)	5.43 ± 0.17	6.48 ± 0.15 *	4.56 ± 0.04†	6.08 ± 0.17*
R_{MAX}(%K$^+$)	103.7 ± 2.00	105.5 ± 1.60	52.8 ± 1.10§†	57.9 ± 2.30§†
CRC PHE (–E)				
p_{EC50} (M)	5.89 ± 0.15	6.68 ± 0.17*	4.88 ± 0.08†	6.00 ± 0.12*
R_{MAX}(%K$^+$)	101.2 ± 2.80	101.5 ± 1.60	96.5 ± 2.2	105.4 ± 1.10
CRC PHE (+E)+LNAME				
p_{EC50} (M)	5.90 ± 0.14	6.24 ± 0.19	4.85 ± 0.18†	5.93 ± 0.15*
R_{MAX} (%K$^+$)	102.0 ± 1.30	98.4 ± 2.20	94.9 ± 2.10†	102.0 ± 1.30
CRC PHE (–E)+LNAME				
p_{EC50} (M)	5.97 ± 0.17	6.17 ± 0.15	4.47 ± 0.17†	6.08 ± 0.12*
R_{MAX} (%K$^+$)	103.6 ± 3.60	99.4 ± 2.20	102.3 ± 3.50	104.7 ± 1.30

*Mean ± SEM; *$p < 0.05$ LA vs HA; §$p < 0.05$ Phe (+E) vs (–E), (+ L-NAME) and (–E +L-NAME); †$p < 0.05$ NBLL vs NBSH.*

for NA, in both LA- and HA-NBLL. This rightward shift is consistent with competitive blockade (**Supplementary Figure 4**). Competitive inhibition experiments modified R_{MAX} for NA, except at the highest concentration tested in NBLL at LA compared with HA. The Schild plot analysis did not show changes in pA_2 values between LA- and HA-NBLL, suggesting that the affinity of the α-adrenergic receptor for prazosin was unchanged (**Figure 2A**). In both LA- and HA-NBSH, prazosin blockade also promoted a rightward shift of pEC_{50} for NA as prazosin concentration increased. Increasing prazosin concentration did not decrease R_{MAX} for NA except at the highest concentration tested in NBSH at HA. However, the Schild analysis showed a greater pA_2 value in HA- than LA-NBSH, consistent with a greater affinity of the α-adrenergic receptor for prazosin at HA (**Figure 2B**). Further, when comparing both species at LA and HA, we observed that NBLL values for pA_2 were greater than those obtained at LA in NBSH ($p < 0.05$). However, no difference was observed in NBLL and NBSH at HA.

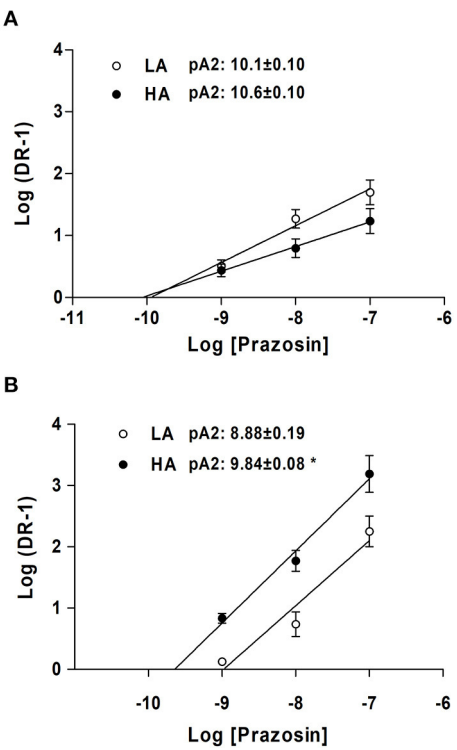

FIGURE 2 | Relative affinity (pA_2) in NBLL and NBSH of LA and HA obtained from concentration response curves to NA and NA in the presence of prazosin at 1, 10, and 100 nM and Schild plots analysis. **(A)** NBLL at LA vs HA and **(B)** NB sheep. Values are expressed as means ± SEM. Asterisk (*) represent a significant difference between NBSH (LA/HA) ($p < 0.05$).

DISCUSSION

We found that HA chronic hypoxia-induced enhanced femoral α-adrenergic vascular reactivity in NBLL compared with NBSH.

The enhanced α-adrenergic vascular reactivity found in the HA NBLL is also observed in the fetal and neonatal llama at sea level (Giussani et al., 1999; Moraga et al., 2011; Reyes et al., 2020). The importance of the α-adrenergic signaling during acute hypoxia in the fetus is vital since its blockade causes cardiovascular collapse and death (Giussani et al., 1999).

Our previous work has shown that intense peripheral vasoconstriction is essential for blood flow redistribution in response to acute hypoxia in the llama fetus and that α-adrenergic signaling is necessary for eliciting this response which is critical for fetus survival (Giussani et al., 1999). As indicated, this high peripheral vasoconstrictor tone persists in the neonatal period (Reyes et al., 2018, 2020). Moreover, we described the high femoral vascular resistance either basally or under acute hypoxia in LA-NBLL compared with LA-NBSH (Moraga et al., 2011). We also described that this higher femoral vascular resistance in

the LA-NBLL could be explained by a powerful α_1 adrenergic tone and the preferential expression of the α_{1B} adrenoceptor subtype. In contrast, the LA-NBSH preferentially expressed the α_{1A} adrenoceptor (Moraga et al., 2011). Our finding of a greater R_{MAX} to NA and PHE in HA-NBLL compared with HA-NBSH llamas are consistent with higher α_{1B} adrenoceptor subtype levels in the former.

In the present study, we have extended our previous work and have now compared the effect of HA chronic hypoxia in the femoral vasoconstrictor response in two species, llama and the sheep, with different ancestry at HA.

Vasoconstriction Mediated by Potassium in NBLL and NBSH at LA/HA

Maximum Response to Potassium Chloride

Regarding increased R_{MAX} for K^+ observed in animals with chronic exposure like NBLL at HA, there are no previous studies published on the effect of hypobaric hypoxia on femoral vessels in the NBLL. Exposure of rats to hypoxia of 10% by 24 h and 48 h decreases the contractile response in aorta rings (Auer and Ward, 1998), while pulmonary arterial bed hypoxia promotes vasoconstriction, upregulation of voltage-dependent Ca^{2+} channels, and a hyperplasic/hypertrophic structural remodeling related with increased R_{MAX} (Wan et al., 2013; Dunham-Snary et al., 2017; Reyes et al., 2018; Gassmann et al., 2021). Other mechanisms such as increased Ca^{2+} sensitization in vascular smooth muscle can also increase R_{MAX} (Jernigan and Resta, 2014). To the best of our knowledge, hypoxic remodeling, an increase of Ca^{2+} sensitization, or upregulation of voltage-dependent Ca^{2+} channels have not been described in femoral vessels; nevertheless, we cannot rule out these possibilities. The mechanism involved in the increase of femoral R_{MAX} to K^+ in HA-NBLL may improve the total peripheral contraction capacity and blood flow redistribution to withstand hypoxia in this species.

In contrast, the decreased R_{MAX} described in our study in small femoral arteries NBSH at HA is in agreement with previous observations in the same ovine neonatal model (Herrera et al., 2007) and in the carotid artery in fetal sheep (Nauli et al., 2005). Decreased sensitivity of the contractile smooth muscle machinery to Ca^{2+} could explain our results (Nauli et al., 2005). The highest function or expression of vasodilator mechanisms, like nitric oxide signaling, cannot be excluded in NBSH at HA (Herrera et al., 2008, 2019; Reyes et al., 2018).

Potency to Potassium (EC_{50})

NBLL responses increased potency at HA compared with LA. Meanwhile, NBSH showed similar potency values at LA and HA. However, there was a larger potassium potency (low values of EC_{50}) in NBLL at LA or HA than NBSH at LA and HA. In agreement with Nernst Equation, the lower EC_{50} values to K^+ described in NBLL means that it can activate a vasocontraction at a lower membrane potential (see Papamatheakis et al., 2012). This suggests that NBLL, as a species, adapted to chronic HA hypoxia over millions of years, has developed a lower threshold for calcium channel type-L activation (Ghosh et al., 2017; Ottolini et al., 2019).

Vasoconstriction Mediated by α_1-Adrenoreceptors in NBLL and NBSH at LA/HA

NBLL at LA and HA

Our study depicted the outcomes of chronic exposure at HA on vasoconstriction mechanisms mediated by α_1-adrenoceptors in NBLL compared with LA-NBLL. Vasocontraction mediated by NAor PHE in NBLL are similar to those reported previously in NBLL at LA (Moraga et al., 2011). We observed a similar pattern in NBLL at HA, resulting in increased vasocontraction when HA-NBLL was compared with LA-NBLL. Furthermore, an increased pEC_{50} was described in NBLL at HA. We did not observe any potentiation in the PHE vasoconstrictor effect using L-NAME or femoral artery denudation in LA- or HA-NBLL. This evidence supports the presence of α_1_adrenoceptors in small femoral arteries as the dominant mechanism accounting for the powerful vasoconstrictor tone in NBLL from HA or LA.

To characterize the α_1-adrenoceptor we performed competitive inhibition with increased doses of prazosin followed by CRC to NA. Schild analysis performed with NBLL showed similar pA_2 value for both LA- and HA-NBLL, indicating the presence of one α_1_adrenoceptor subtype under both conditions. In addition, $pA_2 > 9$ value, which suggests the presence of a receptor with a high affinity for prazosin (Graham et al., 1996; Moraga et al., 2011; Docherty, 2019). Nevertheless, competitive blockade was performed using prazosin, a specific α_1-adrenoceptor blocker. Therefore, the difference observed in potency to NA and PHE at HA could be explained by the expression of other α_1-adrenoceptor subtypes. NBLL at LA have preferential expression of the α_{1B}-adrenoceptor subtype over the α_{1A}-adrenoceptor subtype transcript (Moraga et al., 2011). However, further studies using specific subtype α_1-adrenoceptor blockers are required to evaluate subtype expression in NBLL at HA appropriately.

NBSH at LA/HA

R_{MAX} and pEC_{50} values for the α-adrenergic agonist (NA and PHE) described in small femoral arteries in the present work are similar to values reported in NBSH at LA (Herrera et al., 2008; Moraga et al., 2011). However, NBSH exposed to HA showed increased potency and reduced R_{MAX} to NA and PHE. The R_{MAX} values in response to PHE in NA at LA and HA show the same tendency to previously described results in NBSH at LA (Moraga et al., 2011). We can discard the vasoconstrictor role of α_2_adrenoceptor since clonidine does not promote vasoconstriction in small femoral arteries nor at LA or HA in the present study (Moraga et al., 2011). To demonstrate that α_1-adrenoceptor could promote NO production by attenuating vasoconstriction at close to 50%, we studied the contraction under NOS inhibition (L-NAME 10^{-5} M) or after removing the endothelium, both conditions permit 100% vasoconstriction recovery with PHE, similar to that observed with NA in NBSH at LA and HA. A similar pattern was described in the bronchial resistance artery in rabbits (Zschauer et al., 1997) and mesenteric resistances artery in rats (Dora et al., 2000). The proposed

mechanism suggests that PHE promotes α_1-adrenoceptor activation in endothelium, increasing NO production that opposes vasoconstriction (Tuttle and Falcone, 2001; Raj and Subramani, 2016; Marconi et al., 2020). This result suggests different subtypes of α_1-adrenoceptor with a specific location in the small femoral artery, one type of α_1-adrenoceptor present in the endothelium, and other α_1-adrenoceptors present in smooth muscle cells, as proposed by Moraga et al. (2011).

To characterize the α_1-adrenoceptor, we performed competitive inhibition experiments with increasing doses of prazosin followed by CRC to NA. Schild analysis performed to NBSH showed two different pA_2 values, suggesting a low-affinity receptor in LA-NBSH, similar to previous reports (Moraga et al., 2011) and according to the nomenclature proposed by Graham et al. (1996) and Docherty (2019). Also, the $pA_2 > 9$ value suggests the presence of a receptor with a high affinity for prazosin in HA-NBSH (Graham et al., 1996; Moraga et al., 2011; Docherty, 2019). These outcomes indicate the presence of two α_1–adrenoceptor subtypes in small femoral arteries at LA and HA-NBSH. Nowadays, low-affinity phenotype has been associated with the subtype α_{1A} adrenoceptor (Docherty, 2019). Accordingly, we previously demonstrated that NBSH at LA preferentially expresses subtype α_{1A}-adrenoceptor (Moraga et al., 2011).

In conclusion, the *ex vivo* results obtained from small femoral arteries of NBLL and NBSH gestated at HA showed that the physiology underlying this difference between species is due to the following (1) enhanced vasoconstrictor reactivity and potency to α-adrenergic agonists in NBLL; (2) an important role of NO and endothelium in NBSH in the modulation of vasoconstriction induced by α_1-adrenergic stimuli, and (3) maintenance in pA_2 in NBLL at LA/HA. In NBSH, increased pA_2 was observed at HA respect LA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Faculty of Medicine University of Chile.

AUTHOR CONTRIBUTIONS

FM, RR, and AL conceived and designed the study. FM, GE, and VL supervised the overall study. FM and GE contributed to sample data collections and statistical analysis. All authors drafted the report and contributed to the interpretation of the results, critical revision of the manuscript, and approval of the final manuscript. FM is the guarantor.

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SUPPLEMENTARY MATERIAL

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Distinct Mechanisms Underlie Developmental Plasticity and Adult Acclimation of Thermogenic Capacity in High-Altitude Deer Mice

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Developmental plasticity can elicit phenotypic adjustments that help organisms cope with environmental change, but the relationship between developmental plasticity and plasticity in adult life (e.g., acclimation) remains unresolved. We sought to examine developmental plasticity and adult acclimation in response to hypoxia of aerobic capacity ($\dot{V}O_{2max}$) for thermogenesis in deer mice (*Peromyscus maniculatus*) native to high altitude. Deer mice were bred in captivity and exposed to normoxia or one of four hypoxia treatments (12 kPa O_2) across life stages: adult hypoxia (6–8 weeks), post-natal hypoxia (birth to adulthood), life-long hypoxia (before conception to adulthood), and parental hypoxia (mice conceived and raised in normoxia, but parents previously exposed to hypoxia). Hypoxia during perinatal development increased $\dot{V}O_{2max}$ by a much greater magnitude than adult hypoxia. The amplified effect of developmental hypoxia resulted from physiological plasticity that did not occur with adult hypoxia – namely, increases in lung ventilation and volume. Evolved characteristics of deer mice enabled developmental plasticity, because white-footed mice (*P. leucopus*; a congener restricted to low altitudes) could not raise pups in hypoxia. Parental hypoxia had no persistent effects on $\dot{V}O_{2max}$. Therefore, developmental plasticity can have much stronger phenotypic effects and can manifest from distinct physiological mechanisms from adult acclimation.

Keywords: high-altitude adaptation, aerobic performance, oxygen transport, breathing, lung structure, hematology

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INTRODUCTION

Developmental plasticity is the process by which phenotypes are altered by the early life environment, and is often viewed to be irreversible and underpinned by distinct mechanisms from phenotypic plasticity in adult life (e.g., acclimation; Burggren and Reyna, 2011; Moczek et al., 2011; Burggren, 2020). The phenotypic responses to conditions experienced during development are not always the same as responses during adult life, and responses can even vary between developmental stages (e.g., prenatal vs. postnatal development; Carroll, 2003; Bavis, 2005; Ho and Burggren, 2012; West et al., 2021a). However, emerging evidence is challenging the distinction between developmental plasticity and adult acclimation, suggesting that developmental plasticity can be reversible and linked to plasticity in later life (Scott and Johnston, 2012; Beaman et al., 2016; Slotsbo et al., 2016; Burggren, 2020). However, for many

performance traits that are critical to fitness, we are just beginning to understand the importance and life-stage specificity of developmental plasticity, and how it might differ from reversible acclimation during adulthood.

Small mammals at high altitude are well suited to examining plasticity during development and adulthood. The cold and oxygen-depleted (hypoxic) environment at high altitude requires that endotherms sustain high rates of O_2 consumption for heat generation (thermogenesis) and locomotion, while facing a diminished O_2 supply. This is particularly challenging for mammals of small body size, which face greater demands for thermogenesis as a result of a larger surface area to volume ratio. As a result, some small mammals at high altitude have evolved increased aerobic capacity for thermogenesis or exercise [quantified as maximal O_2 consumption ($\dot{V}O_{2max}$) during acute cold exposure or intense running] in hypoxia (McClelland et al., 1998; Chappell et al., 2007; Schippers et al., 2012; Lui et al., 2015; Tate et al., 2017, 2020). Hypoxia-induced plasticity can also contribute to increasing $\dot{V}O_{2max}$ at high altitude (Storz et al., 2010b; Ivy and Scott, 2015). In humans, for example, developmental exposure to high-altitude hypoxia increases aerobic capacity in hypoxia (Sun et al., 1990; Frisancho et al., 1995; Brutsaert et al., 1999; Kiyamu et al., 2015a; Brutsaert, 2016). However, few previous studies have distinguished the effects of hypoxia at different life stages, such as between pre-natal and early post-natal development. These distinctions may be quite important in light of critical windows during early post-natal life for lung morphogenesis (Burri, 1984; Massaro and Massaro, 2002; Frappell and MacFarlane, 2006) and for the development of neural networks that control breathing (Wong-Riley et al., 2019). Furthermore, because high-altitude hypoxia is unremitting and unavoidable throughout life, the effects of hypoxia at early life stages might influence responses to hypoxia during adulthood. Small mammals that are native to high altitude thus provide an opportunity to examine how environmental effects across multiple distinct life stages – both early developmental and adult – might interactively affect complex adult phenotypes.

The objective of this study was to gain insight into these issues by investigating the influence of developmental plasticity and adult acclimation in hypoxia on thermogenic $\dot{V}O_{2max}$ in deer mice (*Peromyscus maniculatus*) native to high altitude. Deer mice are broadly distributed across North America and can be found from sea level to over 4,300 m elevation in the Rocky Mountains (Hock, 1964; Snyder et al., 1982; Natarajan et al., 2015). Adults from wild populations at high altitude sustain higher metabolic rates than those at low altitude (Hayes, 1989), likely to support the increased demands of thermogenesis in cold alpine environments. There can be strong directional selection for increased thermogenic $\dot{V}O_{2max}$ at high altitude (Hayes and O'Connor, 1999), which has led to evolved increases in thermogenic $\dot{V}O_{2max}$ in hypoxia in high-altitude deer mice compared to low-altitude deer mice and white-footed mice (*P. leucopus*, a congeneric species that is restricted to low altitudes; Cheviron et al., 2012, 2013, 2014; Lui et al., 2015; Tate et al., 2017, 2020). Differences in thermogenic $\dot{V}O_{2max}$ and in various respiratory and metabolic traits that underlie it become apparent ~2–3 weeks after birth in comparisons between high- and low-altitude mice raised in normoxia (Robertson et al., 2019; Robertson and McClelland, 2019; Ivy et al., 2020; West et al., 2021a). However, although the effects of adult acclimation to hypoxia on thermogenic $\dot{V}O_{2max}$ and its underlying determinants have been described (Lui et al., 2015; Lau et al., 2017; Tate et al., 2017, 2020), and life-long exposure to high altitude has been shown to increase thermogenic $\dot{V}O_{2max}$ (Chappell et al., 2007), the specific effects of hypoxia exposure during pre-natal and post-natal development has not been resolved. The current study seeks to address these knowledge gaps. We test the hypothesis that developmental hypoxia elicits a greater increase in thermogenic $\dot{V}O_{2max}$ and acts *via* distinct mechanisms as compared to hypoxia acclimation during adulthood.

MATERIALS AND METHODS

Mouse Populations and Hypoxia Exposures

Captive breeding populations were established from wild populations of deer mice native to high altitude near the summit of Mount Evans, CO, United States (39°35'18"N, 105°38'38"W; 4,350 m above sea level; *P. m. rufinus*) and white-footed mice (*P. leucopus*; a species that is restricted to low altitudes) native to the Great Plains (Nine Mile Prairie, Lancaster County, NE, United States, at 40°52'12"N, 96°48'20.3"W, 430 m above sea level). Occupation of low-altitude habitats is the ancestral condition for the phylogenetic clade containing *Peromyscus maniculatus* and *P. leucopus*, and *P. leucopus* is a good representative of this ancestral condition from which *P. maniculatus* evolved and expanded their elevational range (Velotta et al., 2018). Wild adults were transported to McMaster University (~50 m above sea level) and housed in common laboratory conditions, and were used as parental stock to produce first generation (G1) lab progeny for each mouse population. Breeding pairs were held in individual cages, the male was removed when the female was visibly pregnant, and pups were weaned and moved to separate cages at post-natal day (P)21. G1 mice were similarly used as parental stock to produce second generation (G2) progeny for each population. Experiments were conducted on three distinct families of adult G2 mice for each population. All mice were held at 24–25°C and a photoperiod of 12 h light: 12 h dark, and were provided with unlimited access to standard rodent chow and water. All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

We used a standardized breeding design to expose G2 mice to hypoxia, starting at a range of different life stages, with five different treatment groups (**Figure 1**), as previously described (Nikel et al., 2018). Each breeding pair was first allowed to raise 4 litters, in order to avoid potential effects of variation in litter size and resource allocation that may arise across the first few litters (Kirkland and Layne, 1989). Each pair then conceived and raised litters 5 and 6 in standard cage conditions

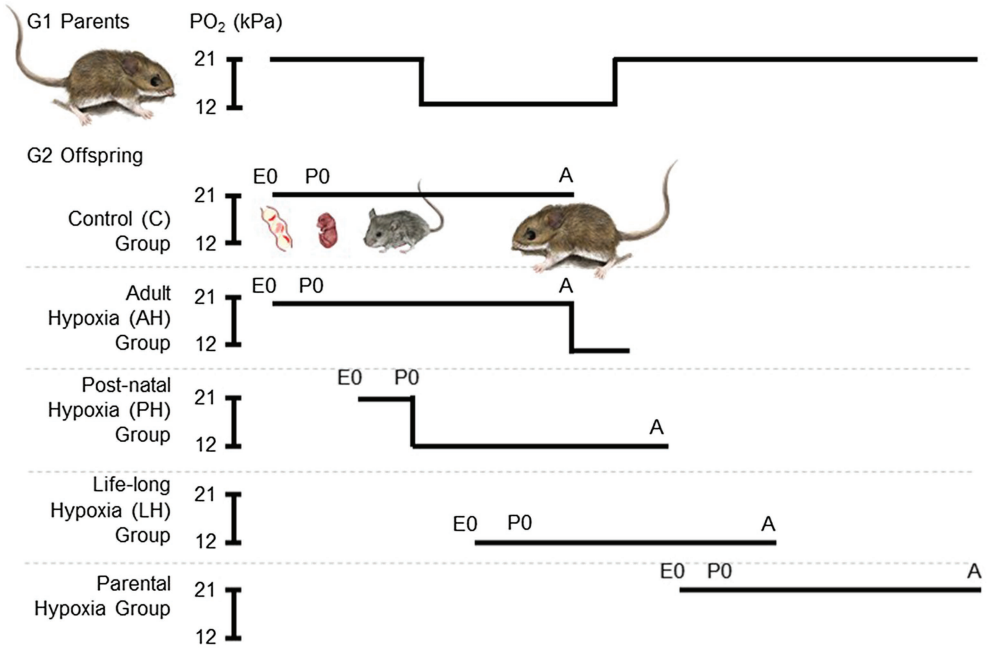

FIGURE 1 | Experimental treatment groups used to evaluate the effects of chronic hypoxia at different life stages. We used a standardized breeding design with first generation (G1) lab-raised parents to expose second generation (G2) lab-raised offspring to hypoxia starting at multiple life stages. Each breeding pair conceived and raised their 5th and 6th litters in normobaric normoxia until weaning. These progeny were split into two treatment groups, one that remained in normoxia (~21 kPa O_2; control group, C) and the other that was acclimated to hypobaric hypoxia (~12 kPa O_2) for 6–8 weeks during adulthood (adult hypoxia group, AH). Litter 7 was conceived and born in normoxia, but parents and offspring were moved to hypobaric hypoxia within 12 h of birth, and offspring were weaned and raised to adulthood in hypobaric hypoxia (post-natal hypoxia group, PH). The mother and father continued to be held in hypobaric hypoxia, and were allowed to conceive litter 8, which was born and raised into adulthood in hypobaric hypoxia (life-long hypoxia group, LH). After weaning litter 8, breeding pairs were returned to normoxia and were then allowed to conceive and raise litter 9 in normoxia (parental hypoxia group). Full details of the breeding design can be found in the Methods. E, embryonic age in days; P, post-natal age in days; A, adult; and PO_2, partial pressure of O_2.

of normobaric normoxia until weaning. These progeny from each family were split into two treatment groups, one that remained in normoxia (control group, C) and the other that was acclimated to hypobaric hypoxia (barometric pressure of 60 kPa, ~12 kPa O_2; simulating the hypoxia at an elevation of 4,300 m) for 6–8 weeks during adulthood (adult hypoxia group, AH). Litter 7 was also conceived and born in normoxia, but the family was moved to hypobaric hypoxia within 12 h of birth, and the mother and pups remained there together until weaning. After weaning, litter 7 pups continued to be raised in hypobaric hypoxia into adulthood (post-natal hypoxia group, PH). The mother and father continued to be held in hypobaric hypoxia, and were allowed to conceive litter 8, which was born and raised into adulthood in hypobaric hypoxia (life-long hypoxia group, LH). After weaning litter 8, breeding pairs were returned to normoxia and were then allowed to conceive and raise litter 9 in normoxia (parental hypoxia group). Litter sizes that survived to weaning for highlanders across treatment groups: litters 5 and 6 used for C and AH groups, 4–6 pups per litter, 13 males and 13 females in total; litter 7 for PH group, three pups per litter, two males and seven females in total; litter 8 for LH group, 2–4 pups per litter, six males and three females in total; litter 9 for parental hypoxia group, 2–3 pups per litter, three males and four females in total. Litter sizes that survived to weaning for lowlanders: litters 5 and 6, 5–7 pups per litter, 21 males and 13 females in total; litter 7, 0 pups; litter 8, 0 pups; litter 9: three pups per litter, two males and four females in total (in lowlanders, litter 9 was only conducted in two families owing to temporary suspension of research activities during the COVID-19 pandemic). Exposures to hypobaric hypoxia were conducted using specially designed hypobaric chambers (McClelland et al., 1998; Lui et al., 2015). Cages were cleaned twice a week during hypoxia exposures, which required that mice be returned to normobaria for a brief period (<20 min).

Measurements of Thermogenic $\dot{V}O_{2max}$ and Cardiorespiratory Physiology

Thermogenic $\dot{V}O_{2max}$ was measured between 6 and 8 months of age along with concurrent measurements of breathing, arterial O_2 saturation (SaO_2), and heart rate. Thermogenic $\dot{V}O_{2max}$ was measured using open-flow respirometry as the maximal rate of O_2 consumption achieved over 30 s during a 10 min exposure to acute cold (−5°C) in a heliox gas mixture containing both normoxic (21 kPa O_2, balance He) and hypoxic (12 kPa O_2, balance He) O_2 levels. Normoxic and hypoxic trials were conducted in randomized order and separated by at least 48 h. Measurements were conducted in a custom-made apparatus that allowed for simultaneous measurements of breathing by plethysmography as

well as SaO_2 and heart rate by pulse oximetry, as previously described (Tate et al., 2020). The apparatus consisted of a 530 ml respirometry chamber, in which the animal was placed, and an empty reference chamber. The respirometry chamber contained a pneumotachograph that provided a natural leak to the external environment, allowing for changes in flow relative to the reference chamber (caused by breathing) to be detected using a differential pressure transducer (Validyne DP103-18; Cancoppas, Mississauga, ON, Canada). The chamber also contained ports for incurrent and excurrent gas flows and ports for leads from the pulse oximetry collar. During measurements, heliox inflow was delivered to the respirometry chamber at 1500 ml min^{-1}, regulated using a mass flow controller (MFC-2, Sable Systems, Las Vegas, NV, United States) and precision flow control valves for oxygen and helium (Sierra Instruments, Monterey, CA, United States), and excurrent gas leaving the respirometry chamber was subsampled at 200 ml min^{-1}, dried with pre-baked Drierite, and analyzed for O_2 and CO_2 fractions (FoxBox Respirometry System, Sable Systems). The entire apparatus was held within a freezer to maintain the respirometry chamber at or slightly below −5°C (verified with a PT-6 thermocouple; Physitemp, Clifton, NJ, United States).

Thermogenic $\dot{V}O_{2max}$ trials were conducted as previously described (Tate et al., 2020). Before the start of each trial, the differential pressure transducer was calibrated, while the heliox gas mixture flowed through the empty respirometry chamber, by withdrawing and reinjecting 200 µl of gas from the respirometry chamber multiple times using a Hamilton syringe (at a flowrate similar to a mouse breathing). Baseline O_2 and CO_2 fractions were then measured, without an animal in the respirometry chamber. At the start of each trial, mice were weighed and instrumented with a collar for a MouseOx Plus pulse oximeter (Starr Life Sciences, PA, United States), which required the fur to be removed from a small area on the neck (3 days in advance). Mice were habituated to the pulse oximeter collars 1 day in advance of thermogenic trials. Mice were then placed inside the respirometry chamber for 10 min, during which time, we measured O_2 and CO_2 fractions of excurrent gas leaving the chamber, the changes in flow across the pneumotachograph that were caused by ventilation, and the pulse oximetry signals. Mice were then removed from the chamber, core body temperature was measured immediately using a mouse rectal probe (RET-3-ISO; Physitemp), and mice were finally returned to their cage in the appropriate acclimation environment. All mice had depressed core body temperature at the end of the acute cold exposure. In some cases, mice removed the pulse oximetry collar during measurement; therefore, the trial was repeated after a minimum of 48 h recovery.

Thermogenic $\dot{V}O_{2max}$ was defined as the highest O_2 consumption rate achieved over a 30 s period during the trial (generally occurring after ~ 4–6 min in the chamber), and was calculated using established formulas for baseline and excurrent measurements of O_2 and CO_2 fractions (Lighton, 2008).

$$\dot{V}O_{2\max} = \left[F_{iO_2} - \left(\frac{\left(1 - F_{iO_2} - F_{iCO_2}\right)}{\left(1 - F_{eO_2} - F_{eCO_2}\right)} \right) F_{eO_2} \right] \times FR_i$$

Where F_i and F_e denote incurrent and excurrent fraction and FR_i denotes incurrent flow rate. Breathing, SaO_2, and heart rate were then determined at $\dot{V}O_{2max}$. Tidal volume was determined using the barometric method of whole-body plethysmography, calculated from the flows that were measured across the pneumotachograph using established equations for flow-through respirometry (Drorbaugh and Fenn, 1955; Jacky, 1980). Total ventilation was the product of tidal volume and breathing frequency. Air convection requirement is the quotient of total ventilation and $\dot{V}O_{2max}$, and pulmonary O_2 extraction was calculated as $\dot{V}O_{2max}$ divided by the product of total ventilation and the O_2 concentration of inspired air. All of the above data were acquired using a PowerLab 8/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, United States). Pulse oximetry measurements of SaO_2 and heart rate were recorded using Starr Life Sciences acquisition software.

Lung Volume and Histology

Following blood collection and careful excision of the heart, lung volume was measured on a subset of mice in the N, AH, and LH groups. The trachea was intubated with PE50 tubing (BD Intramedic, FisherScientific, Mississauga, ON, Canada) and secured with 2-0-gage suture silk (Prolene, FisherScientific). The lungs were then inflated with 10% formalin at a pressure of 30 cmH_2O (Limjunyawong et al., 2015). The trachea was then tied closed and the lungs were carefully excised from the body cavity. Lung volume was measured immediately using the immersion displacement technique (Scherle, 1970). Lungs were then immersed in 10% formalin and fixed for 72 h, then stored in 70% ethanol until paraffin embedding. Embedded lungs were sectioned using a microtome at a thickness of 5 µm and sections were mounted on Superfrost Plus microscope slides (Fisher Scientific; Mississauga, ON, Canada). Sections were taken at each of 3–4 different locations along the rostrocaudal axis of both the left and right lungs. Sections were then stained for hematoxylin and eosin as follows. Sections were deparaffinized with two washes of xylene for 10 min each, incubated in two changes of 100% ethanol and one of 95% ethanol for 5 min each. Sections were washed in distilled water for 5 min, stained with Gills II hematoxylin for 2 min, washed in water for 1 min, and stained with eosin for 45 s. Sections were then rinsed in water and dehydrated using one wash of 95% ethanol and two washes of 100% ethanol for 5 min each. Sections were cleared with two changes of xylene for 10 min each, then coverslipped with Permount (Fisher Scientific).

Stained sections were imaged for analysis using an upright brightfield microscope. Images were taken at 200X magnification from three various regions within each section, yielding 12–18 images for analysis per individual. Stereological methods were then used to make unbiased morphometric measurements of alveolar surface density (surface area per volume of lung parenchyma) and the total alveolar surface area of the animal, as previously described (Mühlfeld et al., 2012; West et al., 2021a). Alveolar density was quantified as the number of distinct alveoli per imaged area of lung parenchyma.

Statistics

Linear mixed-effects models were used to test for the main effect of hypoxia treatment within each mouse population (highland and lowland) using the lme4 package in R (v. 3.6.0; Bates et al., 2015). We used a backwards model selection approach, in which initial models included body mass as a covariate and sex and family as random effects. If these terms had *p*-values above 0.1, they were removed by stepwise backward deletion (starting with the term with the highest *p* value) and the model was re-run until all random effects or covariates in the model had *p* values below 0.1. In most cases, family and sex were not significant and were thus excluded from most final models. Statistical analyses were carried out on absolute values of traits that were not corrected for body mass (because effects of body mass were accounted for in statistical models), but the $\dot{V}O_{2max}$ and ventilatory volume data presented here are expressed relative to body mass as is conventional in the literature. The full results of statistical models are included in the electronic supplementary material (**Supplementary Tables S1** and **S2**), and the salient findings are reported in the Results. Holm-Sidak post-tests were used as appropriate to make pairwise comparisons when significant effects of hypoxia treatment were detected. A value of $p < 0.05$ was considered to be significant.

RESULTS

Thermogenic $\dot{V}O_{2max}$

Hypoxia during early development enhanced thermogenic $\dot{V}O_{2max}$ in hypoxia by a greater magnitude than hypoxia acclimation during adulthood (**Figure 2**). Specifically, thermogenic $\dot{V}O_{2max}$ of highland deer mice was ~28–33% greater in post-natal hypoxia and life-long hypoxia groups compared to controls raised and held in normoxia, whereas the adult hypoxia was only ~16% greater than normoxic controls on average (**Figure 2A**). Lowland white-footed mice did not survive to weaning in the post-natal and life-long hypoxia groups (some pups were born but typically died 10–20 days after birth), but hypoxia acclimation in adulthood increased thermogenic $\dot{V}O_{2max}$ in hypoxia compared to controls (**Figure 2B**). In contrast, thermogenic $\dot{V}O_{2max}$ in normoxia was not influenced by hypoxia treatment in either highland or lowland mice (**Figures 2C,D**). Parental hypoxia had no effects on thermogenic $\dot{V}O_{2max}$ in hypoxia or normoxia in either population (**Supplementary Table S3**).

The effects of developmental hypoxia in highlanders on thermogenic $\dot{V}O_{2max}$ in hypoxia were associated with increases in total ventilation (**Figure 3**). Total ventilation was significantly higher in post-natal hypoxia (~63%) and life-long hypoxia (~100%) groups compared to normoxic controls, but total ventilation was unaltered in the adult hypoxia group (**Figure 3A**). The magnitude of variation in total ventilation equaled or exceeded that of $\dot{V}O_{2max}$ in hypoxia, as reflected by an increase in air convection requirement in the life-long hypoxia group (**Supplementary Tables S1** and **S4**). The increases in total ventilation with developmental hypoxia were driven

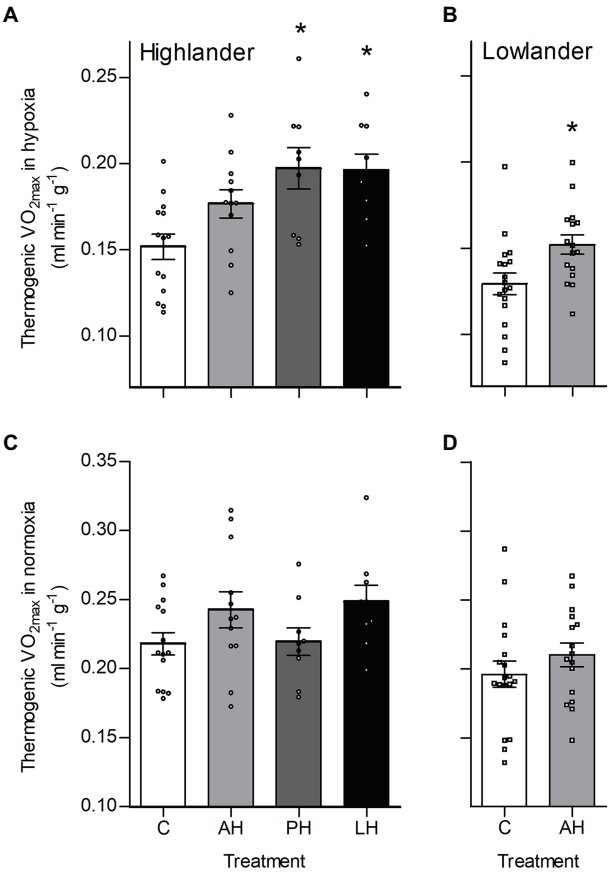

FIGURE 2 | Developmental hypoxia increased thermogenic capacity in hypoxia in deer mice native to high altitude. Thermogenic capacity was measured as the maximal rate of O_2 consumption ($\dot{V}O_{2max}$) during acute cold (–5°C) exposure in hypoxic heliox (~12 kPa O_2; **A,B**) and normoxic heliox (~21 kPa O_2; **C,D**). Lowland mice were unable to raise litters in the PH or LH groups. Bars indicate mean ± SEM and symbols represent individual values. Treatment groups are shown in **Figure 1** with N as follows: 14 control (C), 12 AH, nine PH, and nine LH for highlanders; 18 C and 16 AH for lowlanders. *Significant pairwise difference from control within each population using Holm-Sidak post-tests.

by deeper tidal volumes but no significant changes in breathing frequency (treatment effect, $p = 0.428$; **Supplementary Tables S1** and **S4**). This was reflected by the shift toward the top-right along isopleths of constant breathing frequency in plots of total ventilation vs. tidal volume (**Figure 4A**). Arterial O_2 saturation in hypoxia was ~83% in controls, but was significantly greater at ~90% in all hypoxia treatment groups (adult and developmental; **Figure 3B**). In contrast, there was no significant treatment effect on heart rate at thermogenic $\dot{V}O_{2max}$ in hypoxia ($p = 0.147$; **Figure 3C**). Ventilation, arterial O_2 saturation, and heart rate at thermogenic $\dot{V}O_{2max}$ in normoxia were not altered with developmental hypoxia treatments (**Table 1**). In contrast, whereas lowland mice exhibited a modest increase in total ventilation after adult hypoxia acclimation compared to normoxic controls (**Figure 3D**), this change was driven by higher breathing frequency (treatment effect, $p < 0.001$) and no change in

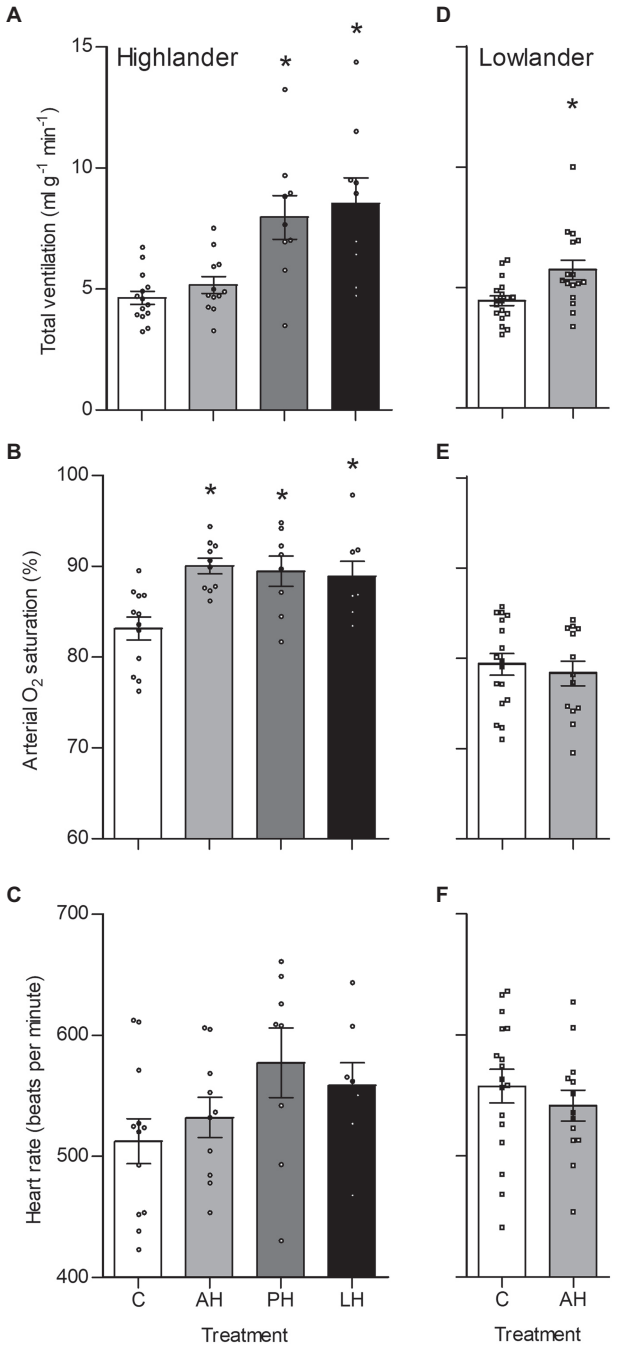

FIGURE 3 | Total ventilation **(A,D)**, arterial O_2 saturation (SaO_2) **(B,E)**, and heart rate **(C,F)** measured at thermogenic $\dot{V}O_{2max}$ in hypoxia in highland **(A,B,C)** and lowland **(D,E,F)** mice. Lowland mice were unable to raise litters in the PH or LH groups. Bars indicate mean ± SEM and symbols represent individual values. Treatment groups are shown in **Figure 1** with N as follows: 14 control (C), 12 acute hypoxia (AH), 9 post-natal hypoxia (PH), and 9 life-long hypoxia (LH) for highlanders; 18 C and 16 AH for lowlanders. *Significant pairwise difference from control within each population using Holm-Sidak post-tests.

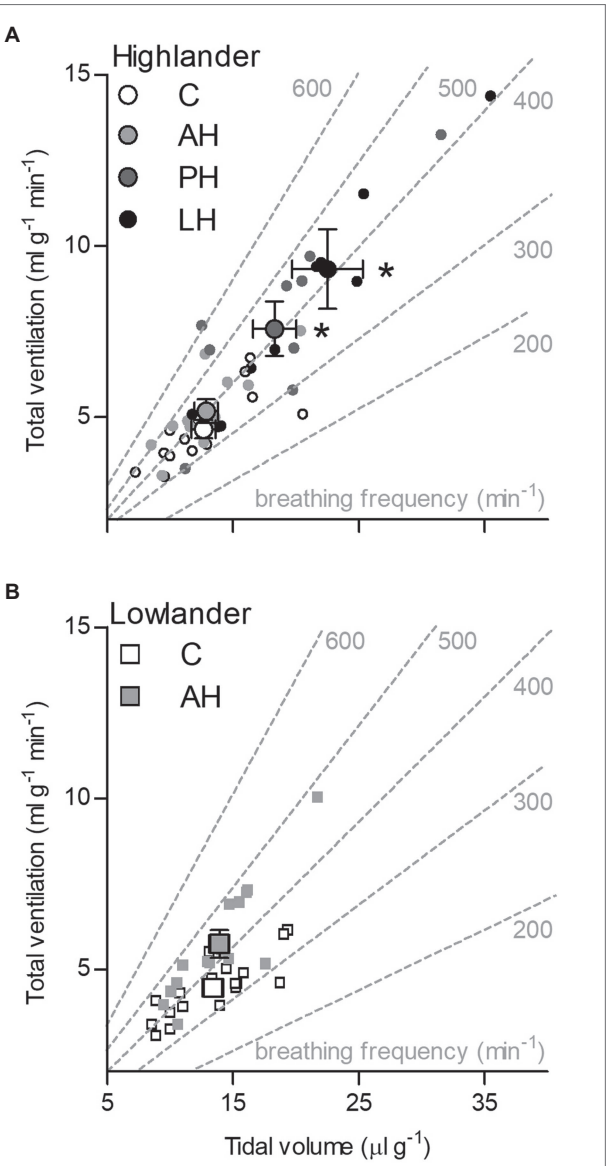

FIGURE 4 | The effects of developmental hypoxia on total ventilation in highland deer mice **(A)** arose from increases in maximal tidal volume, with no change in breathing frequency, in contrast to lowland mice **(B)**. Gray dashed lines represent isopleths of constant breathing frequency. Large symbols with error bars indicate mean ± SEM and small symbols represent individual values. Lowland mice were unable to raise litters in the PH or LH groups. Bars indicate mean ± SEM and symbols represent individual values. Treatment groups are shown in **Figure 1** with N as follows: 14 control (C), 12 acute hypoxia (AH), 9 post-natal hypoxia (PH), and 9 life-long hypoxia (LH) for highlanders; 18 C and 16 AH for lowlanders. *Significant pairwise difference from control within each population using Holm-Sidak post-tests.

tidal volume (treatment effect, $p = 0.977$; **Figure 4B**). Lowland mice acclimated to chronic hypoxia in adulthood did not increase arterial O_2 saturation (treatment, $p = 0.472$) or heart rate (treatment, $p = 0.417$) at thermogenic $\dot{V}O_{2max}$ in hypoxia (**Figures 3E,F**). During thermogenic $\dot{V}O_{2max}$ in normoxia, lowland mice exhibited similar increases in breathing as observed during thermogenic $\dot{V}O_{2max}$ in hypoxia (**Table 1**; **Supplementary Table S4**). Interestingly, despite the observation that parental hypoxia had no effects on thermogenic $\dot{V}O_{2max}$,

highland mice in the parental hypoxia group had higher arterial O_2 saturation at hypoxic $\dot{V}O_{2max}$ (90.03 ± 1.95%) compared to normoxic mice, similar to the increases observed in developmental and adult hypoxia groups. However, parental hypoxia had no other effects in highlanders, and did not affect total ventilation, arterial O_2 saturation, or heart rate in lowlanders (**Supplementary Table S3**).

TABLE 1 | Measurements at thermogenic $\dot{V}O_{2max}$ at 21 kPa O_2 for highland deer mice and lowland white-footed mice exposed to hypoxia during different stages of development.

Trait	Treatment	Highlander	Lowlander
Arterial O_2 saturation (%)	Control	98.82 ± 0.35	97.75 ± 0.50
	Adult hypoxia	97.46 ± 1.49	97.94 ± 0.45
	Post-natal hypoxia	99.26 ± 0.13	-
	Life-long hypoxia	99.41 ± 0.15	-
Heart rate (beats min^{-1})	Control	628.3 ± 17.3	640.8 ± 15.8
	Adult hypoxia	631.5 ± 21.9	655.9 ± 17.1
	Post-natal hypoxia	635.2 ± 28.4	-
	Life-long hypoxia	630.6 ± 36.8	-
Total ventilation (ml min^{-1} g^{-1})	Control	5.55 ± 0.93	4.57 ± 0.24
	Adult hypoxia	4.91 ± 0.23	5.35 ± 0.20*
	Post-natal hypoxia	5.94 ± 0.62	-
	Life-long hypoxia	7.76 ± 0.92	-
Tidal volume (μl g^{-1})	Control	13.97 ± 2.58	12.80 ± 0.84
	Adult hypoxia	11.27 ± 0.69	13.37 ± 0.59
	Post-natal hypoxia	15.36 ± 1.64	-
	Life-long hypoxia	17.97 ± 1.12	-

Data are mean ± SEM, with N as in **Figure 2**. **Significant pairwise difference from control within a population using Holm-Sidak post-tests.*

Lung Structure

Developmental hypoxia increased lung volume, but hypoxia treatment had minimal influence on other aspects of lung morphology (**Figure 5**; **Table 2**; **Supplementary Table S5**). In highlanders, lung volume increased by ~54% on average in the life-long hypoxia group compared to controls, but effects of adult hypoxia were not significant (**Figure 5A**). There were no significant changes in mean alveolar surface density (treatment effect, p = 0.406), total alveolar surface area (p = 0.276), or alveolar density (p = 0.608) between treatment groups in highlanders. In lowlanders, effects of adult hypoxia acclimation on lung volume were not significant (treatment effect, p = 0.066; **Figure 5B**), nor were there any significant changes in mean alveolar surface density (p = 0.657) or total alveolar surface area (p = 0.069).

Hematology and Right-Ventricle Hypertrophy

The effects of developmental hypoxia on hematology were similar to the effects of adult hypoxia, but developmental hypoxia had some unique effects on the right ventricle (**Table 3**; **Supplementary Table S6**). In highlanders, hematocrit increased similarly among hypoxia treatment groups (adult and developmental) compared to controls. This likely reflected erythrocyte swelling, because there were no changes in blood hemoglobin content (treatment effect, p = 0.987) and significant decreases in mean corpuscular hemoglobin content compared to controls. In lowlanders, adult hypoxia increased hematocrit, blood hemoglobin content, and mean corpuscular hemoglobin content, suggesting that erythropoiesis and/or relative reductions

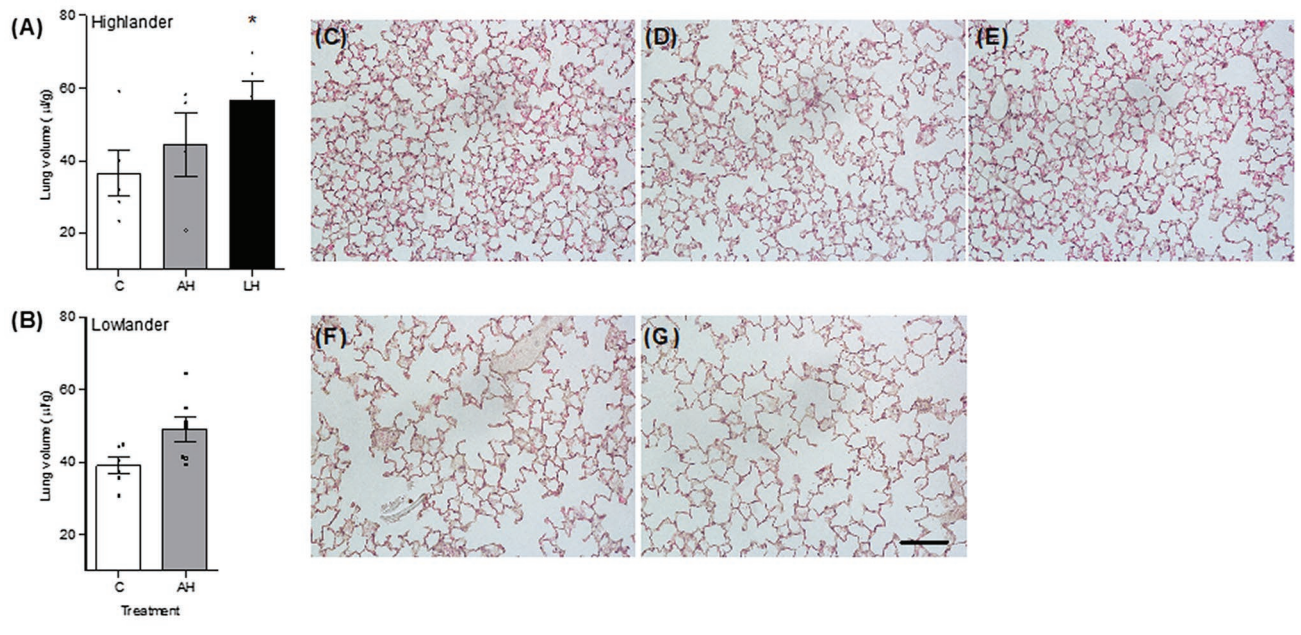

FIGURE 5 | Developmental hypoxia increased lung volume **(A,B)**. Lung parenchyma structure was preserved between control (C; **C,F**), adult hypoxia (AH; **D,G**), and life-long hypoxia (LH; **E**) treatment groups in highlanders **(C–E)** and lowlanders **(F,G)**. Bars indicate mean ± SEM and symbols represent individual values. Treatment groups are shown in **Figure 1** with N as follows: five C, four AH, and five LH for highlanders; six C and seven AH for lowlanders. Each image has the same scale, and the scale bar represents 100 μm. Lung morphometrics are reported in **Table 2**. *Significant pairwise difference from control within each population using Holm-Sidak post-tests.

TABLE 2 | Histological measurements of lung morphology.

Trait	Treatment	Highlander	Lowlander
Alveolar surface density (μm^{-1})	Control	0.108 ± 0.003	0.089 ± 0.003
	Adult hypoxia	0.102 ± 0.002	0.091 ± 0.102
	Life-long hypoxia	0.099 ± 0.006	-
Total alveolar surface area ($cm^2\ g^{-1}$)	Control	31.44 ± 5.26	27.78 ± 2.03
	Adult hypoxia	35.81 ± 6.59	35.68 ± 2.62
	Life-long hypoxia	44.18 ± 5.11	-
Alveolar density (mm^{-2})	Control	885 ± 51	770 ± 27
	Adult hypoxia	853 ± 86	661 ± 30*
	Life-long hypoxia	815 ± 73	-

*Data are mean ± SEM. N as follows: five control, four adult hypoxia, and five life-long hypoxia for highlanders; six control and seven adult hypoxia for lowlanders. *Significant pairwise difference from control within a population using Holm-Sidak post-tests.*

TABLE 3 | Tissue measurements in highland deer mice and lowland white-footed mice exposed to hypoxia.

Trait	Treatment	Highlander	Lowlander
Blood hemoglobin content ($g\ dl^{-1}$)	Control	18.96 ± 1.11	15.30 ± 0.43
	Adult hypoxia	18.64 ± 0.42	25.08 ± 2.10*
	Post-natal hypoxia	18.67 ± 0.59	-
	Life-long hypoxia	19.04 ± 1.32	-
Hematocrit (%)	Control	46.48 ± 0.65	46.22 ± 0.77
	Adult hypoxia	54.62 ± 1.19*	56.79 ± 1.06*
	Post-natal hypoxia	53.67 ± 1.28*	-
	Life-long hypoxia	58.77 ± 1.31*	-
Mean corpuscular hemoglobin content ($g\ dl^{-1}$)	Control	40.72 ± 2.14	33.12 ± 0.79
	Adult hypoxia	34.30 ± 1.10*	44.61 ± 4.16*
	Post-natal hypoxia	34.44 ± 1.12*	-
	Life-long hypoxia	32.58 ± 2.59*	-
Right ventricle mass relative to left ventricle and septum mass	Control	0.21 ± 0.01	0.25 ± 0.01
	Adult hypoxia	0.23 ± 0.02	0.34 ± 0.02*
	Post-natal hypoxia	0.30 ± 0.02*	-
	Life-long hypoxia	0.31 ± 0.03*	-

Data is mean ± SEM, with N as in **Figure 2**. *Significant pairwise difference from control within a population.*

in plasma volume were more important contributors to hematological changes than erythrocyte swelling. Right-ventricle hypertrophy did not occur in highlanders during adult hypoxia acclimation, in contrast to the robust hypertrophy that occurred in lowlanders, but hypertrophy did occur in highlanders after developmental hypoxia treatment. Parental hypoxia had no effect on hematology or right-ventricle hypertrophy in highlanders, but in lowlanders it increased blood hemoglobin content (30.0 ± 1.1 g dl^{-1}) and mean corpuscular hemoglobin content (67.3 ± 3.1 g dl^{-1}), and it increased the relative mass of the right ventricle (0.32 ± 0.02; **Supplementary Table S6**).

DISCUSSION

Developmental plasticity can help organisms respond to environmental change, but for many performance traits that are critical to fitness, we still have a poor understanding of the importance and life-stage specificity of developmental plasticity and how it relates to reversible acclimation during adulthood. Here, we show that high-altitude deer mice exposed to hypoxia during early development had augmented thermogenic capacity in hypoxia later in adult life, and the effect appeared to be larger when hypoxia exposure began at earlier life stages (i.e., pre-natal vs. post-natal life). The magnitude of developmental plasticity was much greater than the effects of hypoxia acclimation during adulthood, and it arose *via* distinct mechanisms (increases in lung ventilation and volumes). This parallels recent findings that developmental hypoxia leads to effects on resting breathing pattern that are not elicited by hypoxia acclimation in high-altitude deer mice (Ivy and Scott, 2017, 2021). Evolved characteristics of deer mice enabled developmental plasticity in hypoxia, because lowland white-footed mice could not successfully raise young in hypoxia. In light of the advantage of a high thermogenic $\dot{V}O_{2max}$ for survival (Hayes and O'Connor, 1999), developmental plasticity in hypoxia is likely adaptive and may improve fitness in deer mice native to high altitude.

Our finding that neonatal and life-long hypoxia enhanced $\dot{V}O_{2max}$ in hypoxia but not in normoxia suggests that developmental hypoxia reduced the depressive effects of hypoxia on aerobic capacity, consistent with studies in humans with high-altitude ancestry (Frisancho et al., 1973, 1995; Greksa et al., 1985; Sun et al., 1990; Brutsaert et al., 1999; Kiyamu et al., 2015a; Brutsaert, 2016). For example, among Peruvian Quechua, individuals that were born and raised to adulthood at high altitude had significantly higher aerobic capacity during exercise in hypoxia than individuals that were born and raised at sea level, in association with increased vital capacity and arterial O_2 saturation during exercise (Kiyamu et al., 2015a,b). However, the magnitude of these developmental effects in humans (≤10%) have often been smaller than those observed here in deer mice (≥30%). Unlike deer mice, developmental hypoxia often reduces absolute ventilation and/or ventilation relative to O_2 consumption (i.e., air convection requirement) during exercise in adult humans (Brutsaert, 2016). By increasing both total ventilation and lung volume, developmental hypoxia in deer mice may elicit greater increases in thermogenic $\dot{V}O_{2max}$ in hypoxia than would be achieved by increasing lung volume alone. This could arise as increased total ventilation (*via* tidal volume) augments alveolar ventilation, and thus the alveolar O_2 tension driving diffusion, while increased lung volume combined with preserved alveolar surface density augments pulmonary O_2 diffusing capacity.

The expansion of lung volume and preservation of alveolar surface density with developmental hypoxia suggests that highland deer mice overcome the detrimental effects of hypoxia during critical windows of lung morphogenesis during early post-natal life. The formation of lung alveoli occurs during early post-natal life in mice, and hypoxia exposure during this period can impede lung development by impairing septation in lowland mice (Blanco et al., 1991; Ambalavanan et al., 2008). However, we have previously shown that highland mice exposed to developmental hypoxia suffer no impairment in alveolar formation from 7 to 30 days after birth, and exhibit increased lung volume from 14 to 30 days (West et al., 2021a). Our findings here show that these patterns persist into adulthood. Similarly, colonies of domestic mice bred for many generations in captivity at 3600 m in La Paz, Bolivia exhibit increased lung volume and alveolar surface area in adulthood compared to sea level controls

(Jochmans-Lemoine et al., 2018). Avoidance of the pathological effects of hypoxia on early lung development is likely critical for maintaining high pulmonary $\dot{V}O_2$ diffusing capacity and contributing to increased thermogenic $\dot{V}O_{2max}$ in high-altitude mice.

The increases in pulmonary ventilation and volumes induced by developmental hypoxia likely interact with evolved and acclimation-induced variation in other traits across the O_2 transport pathway in high-altitude deer mice. Our findings here are consistent with previous observations that highlanders exhibit higher arterial O_2 saturation at $\dot{V}O_{2max}$ than lowlanders, which likely arise from population differences in pulmonary function and hemoglobin-O_2 affinity (Snyder et al., 1982; Storz et al., 2009, 2010a; Ivy et al., 2020; Tate et al., 2020; West et al., 2021a,b). Developmental hypoxia increases hemoglobin-O_2 affinity starting at P14–P30 (Ivy and Scott, 2021), and if this effect persists into adulthood then it might have contributed to some of the plasticity in arterial O_2 saturation observed here. Highlanders exhibit higher cardiac output (largely *via* increased stroke volume) at $\dot{V}O_{2max}$ than low-altitude deer mice and white-footed mice, and they increase cardiac output by a greater magnitude in response to hypoxia acclimation (Tate et al., 2020). Highlanders also exhibit greater capillarity, oxidative capacity, and mitochondrial density in the gastrocnemius (a large hindlimb muscle important for shivering thermogenesis) than lowlanders, but neither developmental hypoxia nor adult hypoxia acclimation affect these traits (Lui et al., 2015; Scott et al., 2015; Mahalingam et al., 2017, 2020; Nikel et al., 2018). Therefore, plasticity during development and adulthood along with evolved changes across the O_2 pathway contribute to increasing thermogenic $\dot{V}O_{2max}$ in deer mice at high altitude.

Some other observed effects of hypoxia could represent maladaptive plasticity. Our finding that lowland white-footed mice exhibit right-ventricle hypertrophy in response to hypoxia acclimation in adulthood is consistent with recent findings in low-altitude deer mice and white-footed mice (Velotta et al., 2018; West et al., 2021b) along with many other lowland taxa (Monge-C et al., 1992; Zungu et al., 2008; Jochmans-Lemoine et al., 2015). This likely represents a pathological outcome of hypoxic pulmonary hypertension (HPH), a maladaptive response to chronic hypoxia that contributes to disease in humans (e.g., mountain sickness) and other mammals (e.g., brisket disease in cattle; Monge-C et al., 1992; Rhodes, 2005). We also found that parental hypoxia exposure in lowlanders led to right-ventricle hypertrophy in offspring that were never exposed to hypoxia themselves. In contrast, in high-altitude deer mice, HPH is attenuated and right-ventricle hypertrophy does not occur during hypoxia exposure in adulthood (Velotta et al., 2018; West et al., 2021b), and parental hypoxia does not lead to persistent effects on the right ventricle. The difference between high-altitude deer mice and white-footed mice in the effects of chronic hypoxia in adulthood on right-ventricle mass is associated with appreciable differences in right-ventricle gene expression (Velotta et al., 2018). However, developmental hypoxia did lead to right-ventricle hypertrophy, suggesting that high-altitude deer mice have not completely eliminated all of the detrimental effects of life-long exposure to chronic hypoxia.

Whether developmental plasticity has evolved in high-altitude deer mice remains an open question. We have previously shown that the hypoxia acclimation response of adults is augmented in highlanders, leading to exaggerated increases in thermogenic $\dot{V}O_{2max}$ as compared to lowland deer mice and white-footed mice (Tate et al., 2017, 2020). Our results here show that developmental hypoxia elicits even greater increases in thermogenic $\dot{V}O_{2max}$ in highlanders, but it is not yet clear whether the magnitude of this developmental response has evolved. On the one hand, developmental hypoxia was intolerable to lowland white-footed mice, which were unable to raise offspring to weaning in hypoxia, as previously observed in rats (Jochmans-Lemoine et al., 2015). Therefore, evolved characteristics of deer mice enabled developmental plasticity in hypoxia by supporting offspring survival. On the other hand, the evolved differences in cardiorespiratory physiology in highland parents likely reduced the level of hypoxia experienced by their offspring during prenatal development in utero. Differences in survival and the developmental hypoxia response might therefore be explained by different levels of fetal hypoxia exposure. Whether or not developmental plasticity has evolved, our findings show that it can lead to adaptive increases in thermogenic capacity that contributes to the success of deer mice in high-altitude environments.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://dx.doi.org/10.17632/wy2xmwfc9d.1; Mendeley Data.

ETHICS STATEMENT

The animal study was reviewed and approved by McMaster University Animal Research Ethics Board.

AUTHOR CONTRIBUTIONS

GS and CI designed the study. CI carried out mouse breeding, ran and analyzed the *in vivo* experiments. HP and CW carried out histological preparation and analysis. CI and GS wrote the manuscript, and all authors edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.718163/full#supplementary-material

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Ontogeny of Carbon Monoxide-Related Gene Expression in a Deep-Diving Marine Mammal

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Marine mammals such as northern elephant seals (NES) routinely experience hypoxemia and ischemia-reperfusion events to many tissues during deep dives with no apparent adverse effects. Adaptations to diving include increased antioxidants and elevated oxygen storage capacity associated with high hemoprotein content in blood and muscle. The natural turnover of heme by heme oxygenase enzymes (encoded by *HMOX1* and *HMOX2*) produces endogenous carbon monoxide (CO), which is present at high levels in NES blood and has been shown to have cytoprotective effects in laboratory systems exposed to hypoxia. To understand how pathways associated with endogenous CO production and signaling change across ontogeny in diving mammals, we measured muscle CO and baseline expression of 17 CO-related genes in skeletal muscle and whole blood of three age classes of NES. Muscle CO levels approached those of animals exposed to high exogenous CO, increased with age, and were significantly correlated with gene expression levels. Muscle expression of genes associated with CO production and antioxidant defenses (*HMOX1*, *BVR*, *GPX3*, *PRDX1*) increased with age and was highest in adult females, while that of genes associated with protection from lipid peroxidation (*GPX4*, *PRDX6*, *PRDX1*, *SIRT1*) was highest in adult males. In contrast, muscle expression of mitochondrial biogenesis regulators (*PGC1A*, *ESRRA*, *ESRRG*) was highest in pups, while genes associated with inflammation (*HMOX2*, *NRF2*, *IL1B*) did not vary with age or sex. Blood expression of genes involved in regulation of inflammation (*IL1B*, *NRF2*, *BVR*, *IL10*) was highest in pups, while *HMOX1*, *HMOX2* and pro-inflammatory markers (*TLR4*, *CCL4*, *PRDX1*, *TNFA*) did not vary with age. We propose that ontogenetic upregulation of baseline *HMOX1* expression in skeletal muscle of NES may, in part, underlie increases in CO levels and expression of genes encoding antioxidant enzymes. *HMOX2*, in turn, may play a role in regulating inflammation related to ischemia and reperfusion in muscle and circulating immune cells. Our data suggest putative ontogenetic mechanisms that

may enable phocid pups to transition to a deep-diving lifestyle, including high baseline expression of genes associated with mitochondrial biogenesis and immune system activation during postnatal development and increased expression of genes associated with protection from lipid peroxidation in adulthood.

Keywords: hypoxia tolerance, marine mammal, gene expression, carbon monoxide, heme oxygenase, diving physiology

INTRODUCTION

While intermittent hypoxemia, ischemia, and reperfusion cause tissue damage in laboratory animals and humans, marine mammals routinely experience these conditions due to their diving lifestyle with no apparent adverse effects (Allen and Vázquez-Medina, 2019). Physiological adaptations of marine mammals to breath-hold diving include enhanced blood oxygen stores and high antioxidant capacity (Ponganis, 2011). For example, deep-diving phocid seals such as the northern elephant seals (*Mirounga angustirostris;* NES) have some of the highest reported mass-specific blood volumes and total body oxygen stores in mammals (Kooyman and Ponganis, 1998). Elevated total oxygen stores and large body sizes contribute to greater breath-hold capacity and longer dive durations (up to 90 min) in NES compared to many other pinniped species (Hassrick et al., 2010). NES spend more than 90% of their time submerged at sea, undergoing repeated apneas, peripheral vasoconstriction, and bradycardia as part of the dive response (Andrews et al., 1997; Ponganis et al., 2008). *In vivo* blood oxygen measurements in diving NES have revealed over 90% depletion of blood oxygen stores during routine dives (Meir et al., 2009), further highlighting the remarkable hypoxemia tolerance in this elite-diving species. Even when hauled out on land, NES undergo repeated sleep apneas with little to no apparent oxidative damage to tissues (Stockard et al., 2007; Vázquez-Medina et al., 2011a; Tift et al., 2013).

Despite new advances in understanding cellular and molecular mechanisms underlying hypoxia tolerance in deep-diving species, few studies have addressed their mechanistic drivers within an ontogeny framework (Weitzner et al., 2020). Among marine mammals, pinnipeds are unique in their developmental transition from birth and nursing on land to diving after weaning. This makes them ideal model systems for studying ontogeny of diving adaptation. In general, blood and muscle oxygen stores (hemoglobin and myoglobin, respectively) increase as marine mammals approach adulthood, enhancing their dive capacity (Horning and Trillmich, 1997; Dolar et al., 1999). In Steller sea lions (*Eumetopias jubatus*), mass-specific total body oxygen stores not only increase with age, but shift predominantly to muscle for primary oxygen storage (Richmond et al., 2006). Similar age-related changes in oxygen-storing capacity have been observed in other pinniped species, such as the Weddell seal (*Leptonychotes weddellii*) (Penso-Dolfin et al., 2020). Expression and activity of antioxidant enzymes were also found to increase with maturation in hooded seals (*Cystophora cristata*) and ringed seals (*Pusa hispida*) (Elsner et al., 1998; Vázquez-Medina et al., 2006, 2011b). Under basal conditions, antioxidant enzyme activity was more than fourfold higher in skeletal muscle of adult hooded seals compared to newborn and weaned pups (Vázquez-Medina et al., 2011c). High antioxidant expression and activity have also been reported in northern NES, specifically in the context of adaptive responses to apneas and prolonged fasting (Allen and Vázquez-Medina, 2019; Ensminger et al., 2021), but have not been extensively profiled across ontogeny.

One potential driver of cytoprotective gene expression in mammals exposed to chronic hypoxia is carbon monoxide (CO), a gasotransmitter produced during heme catabolism by heme oxygenase (HO) enzymes. NES and Weddell seals have levels of blood CO that are comparable to chronic human smokers and that increase with age and blood oxygen stores (Pugh, 1959; Tift et al., 2014). Humans living at high altitude in South America also exhibit elevated hemoglobin concentrations in blood, and high blood CO (Tift et al., 2020). Recent studies in humans and laboratory rodents have shown that exogenous administration of low to moderate doses of CO via inhalation or pharmacological application of CO-releasing molecules (CO-RMs) stimulates mitochondrial biogenesis, regulates inflammation, and confers cytoprotection from injuries associated with hypoxia and ischemia-reperfusion and cold ischemia during organ transplantation (Motterlini and Otterbein, 2010; Dugbartey, 2021). The cellular effects of CO are mediated in part by the p38-MAPK, soluble guanylyl cyclase, and NFkB signaling pathways and their downstream targets (Ryter, 2020). CO administration in mice increases expression of *PGC1A* and *SIRT1*, which regulate mitochondrial biogenesis, oxidative metabolism, and cell survival (Suliman et al., 2007; Rhodes et al., 2009; Kim et al., 2015; Sun et al., 2017). CO treatment under inflammatory conditions also decreases levels of pro-inflammatory cytokines TNF-α, IL-1β, and MIP-1β (*CCL4*) and inhibits TLR4 and JNK signaling, while increasing production of the anti-inflammatory cytokine IL-10 (Otterbein et al., 2000; Ryter et al., 2018). Currently, NES and Weddell seals are the only non-laboratory species confirmed to endogenously produce CO at concentrations believed to have cytoprotective effects (Pugh, 1959; Tift et al., 2014). However, the regulation of the HO/CO pathway and downstream effects of elevated CO in diving mammals have not been examined.

Carbon monoxide is produced endogenously by the heme degradation activity of two HO isoforms, HO-1 and HO-2 (encoded by the genes *HMOX1* and *HMOX2*, respectively). While *HMOX2* is considered constitutively expressed, *HMOX1* expression is induced by a number of stimuli, including hypoxia, oxidative stress and inflammation, and is regulated by the electrophile-responsive transcription factor Nrf2 (Gozzelino et al., 2010). In addition to CO, heme catabolism by HO

enzymes generates biliverdin, which is converted by biliverdin reductase (BVR) to bilirubin, a potent antioxidant at low to moderate concentrations (Jansen and Daiber, 2012; Chen et al., 2018). Moreover, HO-1 may exert direct cytoprotective effects by translocating to the nucleus and activating expression of transcription factors that regulate cell proliferation and survival (Lin et al., 2007). HO-2 has also been implicated in cellular responses to hypoxia and oxygen sensing in the carotid body in humans and rodents (Muñoz-Sánchez and Chánez-Cárdenas, 2014), and has shown evidence of positive selection in human populations adapted to high altitude (Simonson et al., 2010). However, the expression and potential role of HO enzymes in hypoxia tolerance in wild animals have not yet been studied.

Due to their high levels of endogenous CO in blood, NES are a natural study system for examining the role of CO in hypoxia tolerance. In this study, we evaluated ontogenetic changes in gene expression associated with CO production and signaling in NES skeletal muscle and whole blood, two tissues which are routinely sampled from wild NES. Skeletal muscle experiences myoglobin desaturation and some ischemia and reperfusion during apneas in NES (Ponganis et al., 2002, 2008). It also contains high abundance of hemoproteins (myoglobin and cytochromes), which could serve as potential sources of CO production. Gene expression in whole blood, which also experiences fluctuations in oxygen levels (Meir et al., 2009), reflects immune activity as it is derived primarily from peripheral blood mononuclear cells such as lymphocytes and monocytes (He et al., 2019). We measured CO levels and expression of 13 genes in skeletal muscle and 10 genes in whole blood of weaned pups, juveniles, and adult NES. Genes targeted in muscle encoded proteins associated with CO production (*HMOX1*, *HMOX2*, *BVR*), antioxidant defense (*NRF2*, *GPX3*, *GPX4*, *PRDX1, PRDX6*, *SIRT1*), mitochondrial biogenesis (*PGC1A*, *ESRRA*, *ESRRG*), and inflammation (*IL1B*). Genes targeted in whole blood included six markers measured in muscle (*HMOX1*, *HMOX2*, *BVR*, *NRF2*, *PRDX1*, *IL1B*) and four others associated with inflammation (*IL10, TNFA*, *TLR4*, *CCL4*). We examined whether baseline gene expression varied with age class, sex, and fasting state, as NES were sampled early and late in haul-out periods characterized by extensive fasting, which can impact gene activity (Champagne et al., 2012). We hypothesized that expression of genes associated with CO production, signaling, and cytoprotective effects is correlated with CO levels and increases with age and dive capacity in NES, providing a potential mechanism of ontogeny of ischemia-reperfusion tolerance in a deep-diving mammal.

MATERIALS AND METHODS

Study Subjects

All animal handling procedures were approved by Sonoma State University and University of the Pacific Institutional Animal Care and Use Committees and conducted under National Marine Fisheries Service permit nos. 19108 and 23188. Three age classes of NES were sampled at Año Nuevo State Reserve (San Mateo County, CA, United States): weaned pups (n = 15; 5 females and 10 males, spring 2020), juveniles (n = 9; 4 females, 4 males, 1 sex not recorded, fall 2017), and molting adults (n = 22; 11 females and 11 males, summer 2020 and 2021; **Supplementary Table 1**). NES pups fast for 6–8 weeks at the rookery after weaning before leaving for their first foraging trip. Adults haul out for approximately 4 weeks to molt, during which they are also fasting (Champagne et al., 2012). In contrast, juveniles (0.8–1.8 year-old) haul out for a brief period in the fall that is not characterized by extensive fasting (Jelincic et al., 2017). To examine whether any of the markers measured in this study varied with fasting, independent cohorts of weaned pups and adults were sampled at the beginning and end of their haulout periods. Seven pups were sampled early in their post-weaning fast, while 8 were sampled after 4–6 weeks of fasting. Six adult females and 5 adult males were sampled during early molting (<10% molted), and 5 females and 6 males were sampled during late molting (>90% molted, after 3–4 weeks of fasting). Blood samples were not obtained for 2 of the weaned pups, 2 of the adult females, or any of the juveniles. CO concentrations in skeletal muscle were not obtained for 2 of the weaned pups, one of the juveniles, one of the adult females, and one of the adult males.

Sample Collection

Collection of skeletal muscle and whole blood samples was conducted after study animals were anesthetized as described previously (Tift et al., 2014). Specifically, animals were chemically immobilized with 1 mg/kg intramuscular injections of tiletamine-zolazepam HCl (Telazol, Fort Dodge Animal Health, United States), and sedation was sustained with intravenous doses of ketamine and diazepam (0.25–1 mg/kg) (Fort Dodge Animal Health, IA, United States). Blood samples were obtained from the extradural vein using an 18 G, 3.25-inch spinal needle. Whole blood (2.5 ml) was drawn into PAXgene^{TM} Blood RNA Tubes (PreAnalytiX, United States), mixed, stored for ∼ 2 h at room temperature, frozen at −20°C for 24 h, and transferred to −80°C for long-term storage. Skeletal muscle samples were collected from the external abdominal oblique muscle using a 6.0-mm diameter biopsy punch (Integra Miltex, United States), frozen in a cryovial on dry ice and stored at −80°C until further processing. Muscle biopsies collected from juveniles were preserved in RNA*later*^{TM} Stabilizing Solution (∼300 mg tissue per 1.5 mL; Invitrogen, United States) for 24 h at 4°C. RNA*later*^{TM} was removed from samples prior to freezing at −80°C for long term storage. We previously showed that the integrity of RNA isolated from samples frozen in the field and those preserved in RNA*later*^{TM} were comparable and can be used interchangeably for targeted gene expression analyses (Pujade Busqueta et al., 2020).

Carbon Monoxide Measurements

CO extraction and quantification from tissues were conducted using previously established protocols (Vreman et al., 2005). The quantity of CO extracted from tissue samples was measured using a reducing compound photometer gas chromatography system (GC RCP, Peak Performer 1, Peak Laboratories LLC, United States). A certified calibration gas (1.02 ppm CO balanced with nitrogen) was purchased from Airgas® and used to generate a daily standard curve prior to experiments. Amber borosilicate

glass chromatography vials (2 mL) with gas-tight silicone septa were used for all experiments and were purged of CO using a custom catalytic converter prior to the addition of calibration gas or samples. The vial headspace was flushed with CO-free carrier gas into the gas chromatography system via a custom-made double needle assembly attached to the front of the instrument. Frozen aliquots of tissue sample were rinsed of external blood with ice-cold KH_2PO_4 buffer (pH 7.4) and placed into 2.5 mL microcentrifuge tubes. Tissues were diluted to approximately 10–45% (w/w) with ice-cold Milli-Q® water. The tissue was thoroughly diced with surgical scissors over ice and then homogenized using the Ultra-Turrax T8 grinder (IKA Works, Inc., United States) for 6 to 8 one-sec pulses followed by 6 to 8 one-sec pulses from an ultrasonic cell disruptor (Branson, Danbury, CT, United States). After completely homogenized, 10 μL of tissue homogenate and 20 μL of 20% sulfosalicylic acid were injected into the purged 2 mL amber vials with gas-tight syringes connected to repeating dispensers. The CO released into the headspace of the amber vials was measured using the system mentioned previously. Each sample was run in triplicate. CO values were reported as $pmol^{*}mg^{-1}$ wet weight tissue.

RNA Isolation

Muscle tissue (30–60 mg) was minced using a scalpel on dry ice and homogenized by bead beating in 1 mL of TRIzol Reagent (Life Technologies, United States) using a Bullet Blender Storm 24 instrument (Next Advance, United States) at max power for three two-min cycles. Tissue homogenates were further disrupted using a 1 mL syringe and 22 G needle to shear genomic DNA and centrifuged to pellet insoluble components. RNA was isolated from homogenates by phase extraction using chloroform (VWR Life Sciences, United States) following the TRIzol manufacturer's protocol. RNA was purified using the RNeasy Mini kit (Qiagen, United States) according to the manufacturer's protocol, including a 15-min on-column DNase I digest. Total RNA was isolated from whole blood using the PAXgene Blood RNA Kit Version 2 (PreAnalytiX, United States) following the manufacturer's protocol with a 15-min on-column DNAse I digest. RNA concentration was quantified using Qubit 3.0 Fluorometer Broad Range RNA Assay (Life Technologies, United States). RNA integrity was assessed using the Total RNA 6000 Pico Kit on the 2100 Bioanalyzer (Agilent Technologies, United States). Mean (±S.D.) RNA integrity numbers (RIN) for muscle and blood samples were 8.93 ± 0.37 and 5.11 ± 1.26, respectively. RIN values for RNA isolated from muscle samples that were preserved in RNA*later*™ and those that were flash frozen in liquid nitrogen were comparable as shown previously (Pujade Busqueta et al., 2020).

RT-Quantitative Polymerase Chain Reaction

Total RNA (0.5 μg input) was reverse transcribed to complementary DNA (cDNA) using SuperScript IV VILO Master Mix with ezDNase (Thermo Fisher, United States). cDNA samples were diluted 1:10 and 2 μL were used in a 20 μL real-time quantitative polymerase chain reaction (qPCR) with PowerUp SYBR Green Master Mix (Thermo Fisher, United States). qPCR was performed on a QuantStudio 5 Real-Time PCR System instrument (Thermo Fisher, United States) using the following program: 2 min at 50°C, 2 min at 95°C and 40 cycles of 15 s at 95°C and 60 s at 60°C. All samples were run in triplicate with intra-assay coefficient of variation (CV) < 0.5% and inter-assay CV < 1%. No-template and minus-RT controls were included in each run and did not show any amplification.

Sequence-specific primers were designed with NCBI Primer-Blast using NES transcriptomes (**Supplementary Table 2**) (Khudyakov et al., 2015; Deyarmin et al., 2019). Eighteen target genes were selected based on significant BLASTX hits to protein orthologs in the UniProt SwissProt database (*e*-value < 0.001) and high transcript abundance in transcriptomes (transcripts per million, TPM ≥ 20). Primers were designed to specifically target highly conserved regions of each transcript. All primers were used at 400 nM with the exception of *EF2* and *GAPDH*, which were used at 200 nM final concentration. Amplification efficiencies for each primer pair (**Supplementary Table 2**) were calculated from slopes of standard curves of serially 1:2-diluted NES cDNA (n = 6 dilutions). Primer specificity was confirmed using melt curve analysis and agarose gel electrophoresis.

Normalized gene expression values (delta C_T) were obtained by subtracting the C_T of the gene of interest from the C_T of a reference gene (Schmittgen and Livak, 2008). *EF2* was used as the reference gene for muscle, while *GAPDH* was used as the reference gene for blood after evaluation of stability using RefFinder (CV: muscle *EF2* = 2.45%, blood *GAPDH* = 2.58%) (Xie et al., 2012). Delta C_T values and primer efficiencies were calculated using Microsoft Excel 2020.

Statistical Analyses

Data analyses were conducted using R v3.6.2 in RStudio v1.3.1073 (R Core Team, 2016, 2019). Spearman correlation (r_S) was conducted using the 'corr.test' function from the *psych* package (Revelle, 2019) to evaluate associations between normalized expression levels of target genes. Gene expression data were scaled across samples and summarized using the *pheatmap* package (Kolde, 2019) with complete clustering by row and column (Euclidean distance).

Principal components analysis (PCA) of muscle and blood gene expression levels was conducted using the 'principal' function in the *psych* package (Revelle, 2019) after evaluating the appropriateness of this approach (Bartlett's test of sphericity p < 0.05, determinant > 0.00001, Kaiser-Meyer-Olkin mean sampling adequacy values > 0.7) (Field et al., 2012). PCA diagnostic data are shown in **Supplementary Table 3**. The presence of potential outliers was assessed using Mahalanobis distance. No outliers in the muscle or blood gene expression datasets were identified at the recommended chi-square cutoff of alpha = 0.01 (Leys et al., 2018). Principal component interpretability was improved using varimax rotation.

Rotated components were extracted, and general linear models (GLM) were used to examine whether they varied by age, sex, fasting state (early, late) and their interactions. The 'Anova' function in the *car* package was used to obtain type III sums of squares. We first ran full models including all main

and interaction terms, after which non-significant variables were selected for removal using the 'drop1' function. Models with the lowest Akaike information criterion (AIC) were retained. Juveniles were first excluded from full models as they were sampled during a short haul-out period that is not characterized by extensive fasting. In cases in which components did not vary with fasting in pups and adults, juveniles were replaced in the models to assess the effects of age and sex. Levene's and Shapiro-Wilk's tests were used to determine whether variables and model residuals met equal variance and normality assumptions, respectively. Variables were log-transformed as necessary to meet model assumptions. *Post hoc* comparisons between sample groups were conducted using estimated marginal means (EMM) with the *emmeans* package (adjustment = Tukey) (Lenth, 2021). GLM was also used to assess whether log-transformed CO levels varied by age, sex, and fasting. Spearman correlation was used to evaluate relationships between CO levels and rotated components.

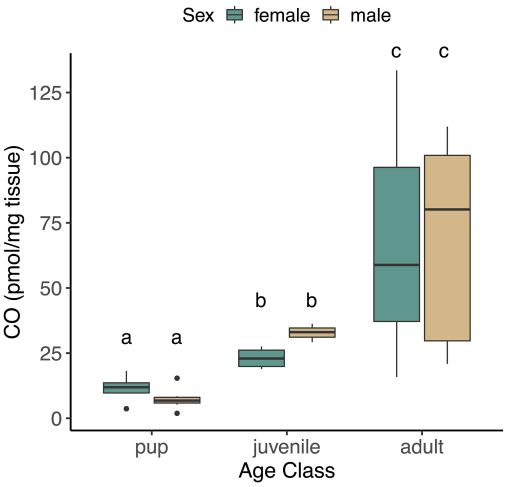

FIGURE 1 | CO levels measured in skeletal muscle of NES pups (n = 13; 4 female, 9 male), juveniles (n = 7; 4 female, 3 male), and adults (n = 20; 10 female, 10 male). Different letters denote significant differences between groups (GLM using log-transformed CO, followed by *post hoc* EMM test, $p < 0.05$).

RESULTS

Carbon Monoxide Levels in Skeletal Muscle

The concentration of CO in NES skeletal muscle was unaffected by fasting in pups or adults ($p = 0.37$), and none of the interaction terms that included fasting were significant ($p > 0.05$). These terms were therefore removed, and juveniles were included in reduced models with age, sex, and age*sex. CO concentrations in skeletal muscle varied significantly with age ($F_{2,37} = 43.15$, $p < 0.0001$), but were unaffected by sex ($p = 0.99$, **Figure 1**) or the interaction between age and sex ($p = 0.39$). Skeletal muscle CO concentrations increased with age, from 8.5 ± 4.7 (mean ± S.D.) pmol*mg^{-1} tissue in pups, to 26.7 ± 6.1 pmol*mg^{-1} tissue in juveniles, and 67.1 ± 37.3 pmol*mg^{-1} tissue in adults (**Figure 1**).

Gene Expression in Skeletal Muscle

Baseline gene expression in skeletal muscle was variable between individuals but was clustered by gene and age, with higher expression levels of most markers, with the exception of those related to mitochondrial biogenesis, in adults compared to pups and juveniles (**Figure 2**). We used PCA to reduce the dimensionality of the dataset as gene expression was highly correlated (**Supplementary Figure 1**). Muscle gene expression was described by four rotated components (**Table 1**), which in sum explained 77% of the variance in the data and showed clustering by age (young *vs.* adult, **Figure 3**).

Muscle expression of *GPX4*, *PRDX1*, *PRDX6*, and *SIRT1*, which are associated with protection from lipid peroxidation, was described by rotated component 1 (mRC1) and explained 24% of the variance in the data. *PGC1A*, *ESRRA* and *ESRRG*, which are associated with mitochondrial biogenesis, loaded on rotated component 2 (mRC2; 18% of variance explained). Expression of *HMOX1*, *BVR*, *GPX3*, and *PRDX1*, which are associated with CO production and antioxidant function, was described by rotated component 3 (mRC3; 15% of variance explained). Lastly, *HMOX2*, *NRF2*, and *IL1B*, which are associated with responses to oxidative stress and inflammation, loaded on rotated component 4 (mRC4), which explained 20% of the variance in the data. *PRDX1*, which encodes an antioxidant enzyme, loaded on both mRC1 and mRC3.

We examined whether any of the muscle-specific rotated components varied by age (pup, juvenile, adult), sex, or fasting state (early, late; in pups and adults only as juveniles were sampled during a haulout period not associated with prolonged fasting), or their interactions. None of the rotated components varied between animals sampled early and those that were sampled late in their fast in pups or adults (mRC1: $p = 0.37$, mRC2: $p = 0.10$, mRC3: $p = 0.13$, mRC4: $p = 0.42$), and none of the interaction terms that included fasting state were significant ($p > 0.05$). Therefore, these terms were removed, and juveniles were included in reduced models with age, sex, and age*sex as explanatory variables. mRC1 expression increased with age ($F_{2,42} = 5.31$, $p = 0.0088$; **Figure 4A**); it was higher in adults compared to juveniles ($p = 0.034$) and pups ($p = 0.026$) but did not differ between pups and juveniles ($p = 0.92$). mRC1 did not vary by sex ($p = 0.92$) or the interaction between age and sex ($p = 0.22$). mRC2 expression decreased with age ($F_{2,41} = 19.07$, $p < 0.0001$; **Figure 4B**); it was higher in pups compared to juveniles ($p = 0.016$) and adults ($p = 0.0002$) but did not vary between adults and juveniles ($p = 0.88$). mRC2 expression was higher in females compared to males ($F_{1,41} = 22.14$, $p < 0.0001$), with highest levels in female pups and lowest levels in adult males (**Figure 4B**); the interaction term age*sex was not significant ($p = 0.37$). mRC3 increased with age ($F_{2,39} = 14.84$, $p < 0.0001$). The interaction term age*sex was marginally significant ($F_{2,39} = 3.13$, $p = 0.055$); mRC3 expression was higher in adult females compared to adult males ($p = 0.0099$; **Figure 4C**). mRC4 expression did not vary with age ($p = 0.68$), sex ($p = 0.77$), or their interaction ($p = 0.088$; **Figure 4D**).

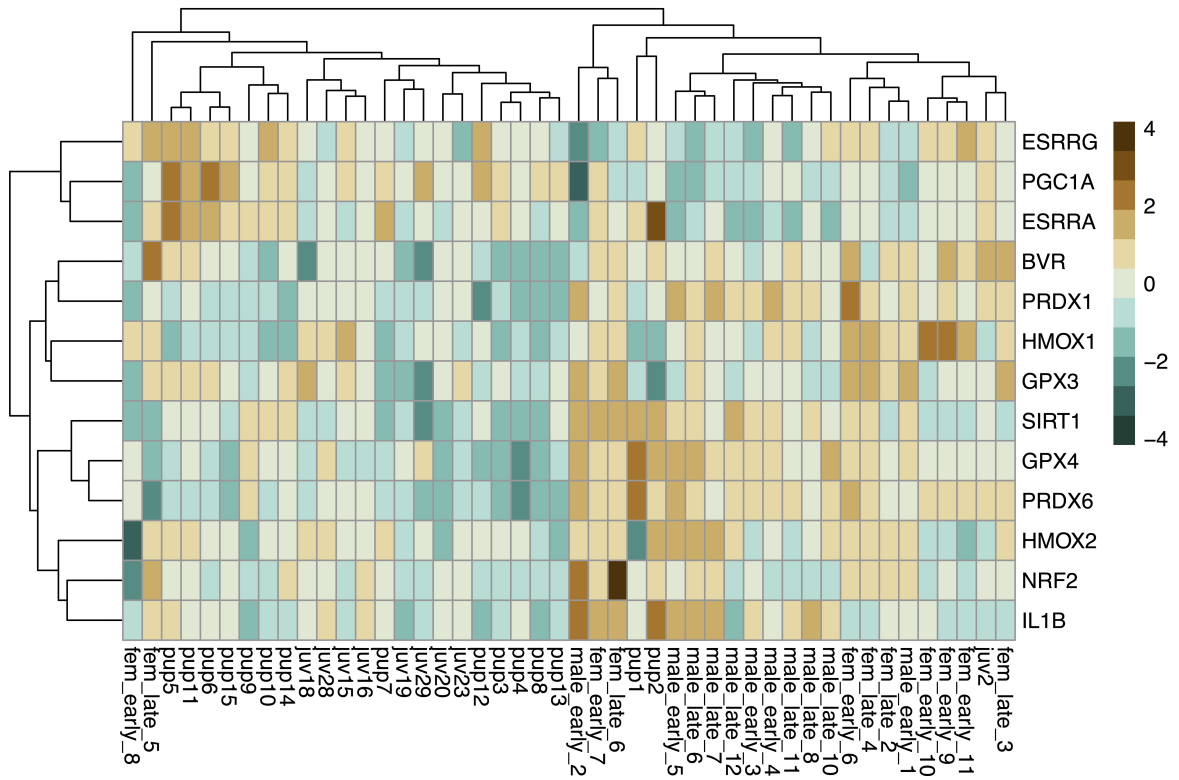

FIGURE 2 | Heatmap showing scaled baseline expression (delta C_T; higher: brown, lower: blue) of 13 genes (rows) associated with CO signaling and cytoprotection in skeletal muscle of NES pups (n = 15), juveniles (n = 9), and adults (n = 22; columns). Rows and columns were clustered by Euclidean distance.

We then evaluated relationships between CO levels and rotated component expression in muscle. Muscle CO levels were positively correlated with mRC1 (r_S = 0.43, p = 0.006; **Figure 5A**), mRC3 (r_S = 0.57, p = 0.0001; **Figure 5C**) and mRC4 (r_S = 0.33, p = 0.034; **Figure 5D**), and negatively correlated with mRC2 (r_S = −0.36, p = 0.021; **Figure 5B**).

TABLE 1 | Eigenvalues and percent of explained variance for varimax-rotated components mRC1, mRC2, mRC3, and mRC4 that describe expression of genes associated with protection from oxidative stress and lipid peroxidation, production of CO, and mitochondrial biogenesis in skeletal muscle of NES.

	mRC1	mRC2	mRC3	mRC4
Eigenvalue	3.12	2.35	1.98	2.66
% of variance	24	18	15	20
Gene	Rotated component loadings			
HMOX1	0.12	−0.34	**0.80**	−0.07
HMOX2	0.07	0.00	0.12	**0.82**
BVR	0.33	0.40	**0.66**	0.16
NRF2	0.17	−0.01	0.20	**0.86**
GPX3	−0.16	−0.05	**0.66**	0.43
GPX4	**0.89**	−0.23	−0.04	0.16
PRDX1	**0.56**	−0.23	**0.51**	0.37
PRDX6	**0.92**	−0.16	0.22	0.01
SIRT1	**0.74**	0.03	0.02	0.42
IL1B	0.40	−0.09	−0.01	**0.70**
PGC1A	−0.49	**0.73**	−0.15	−0.08
ESRRA	−0.04	**0.88**	−0.24	0.19
ESRRG	−0.16	**0.78**	0.15	−0.40

Rotated component loading scores are shown for each gene used in the analysis, with values >0.5 shown in bold.

Gene Expression in Whole Blood

We measured expression of *HMOX1*, *HMOX2*, *BVR*, *NRF2*, *PRDX1*, *IL1B* and four other markers associated with immune function, which were not detectable in muscle, in whole blood (*IL10*, *TLR4*, *TNFA*, *CCL4*). Baseline expression of these 10 genes in whole blood showed clustering by age, with higher expression levels of most markers, with the exception of *HMOX1* and *NRF2*, in pups compared to adults (**Figure 6**). Blood gene expression was described by three rotated components (**Table 2**), that in sum explained 79% of the variance in the data and showed clustering by age (young *vs.* adult, **Figure 7**). Expression of *HMOX2*, *PRDX1*, *CCL4*, and *TNFA* in whole blood, which are associated with antioxidant function and inflammation, loaded on rotated component 1 (bRC1) and explained 33% of the variance in the data. Expression of *IL1B*, *NRF2*, *BVR*, and *IL10*, which are associated with regulation of inflammation, was described by rotated component 2 (bRC2; 31% of variance explained). *HMOX1*, which is associated with CO production, loaded on rotated component 3 (bRC3; 15% of variance explained). *TLR4* loaded on both bRC1 and bRC2.

We examined whether any of the blood-specific rotated components varied by age, sex, fasting state, or their interaction

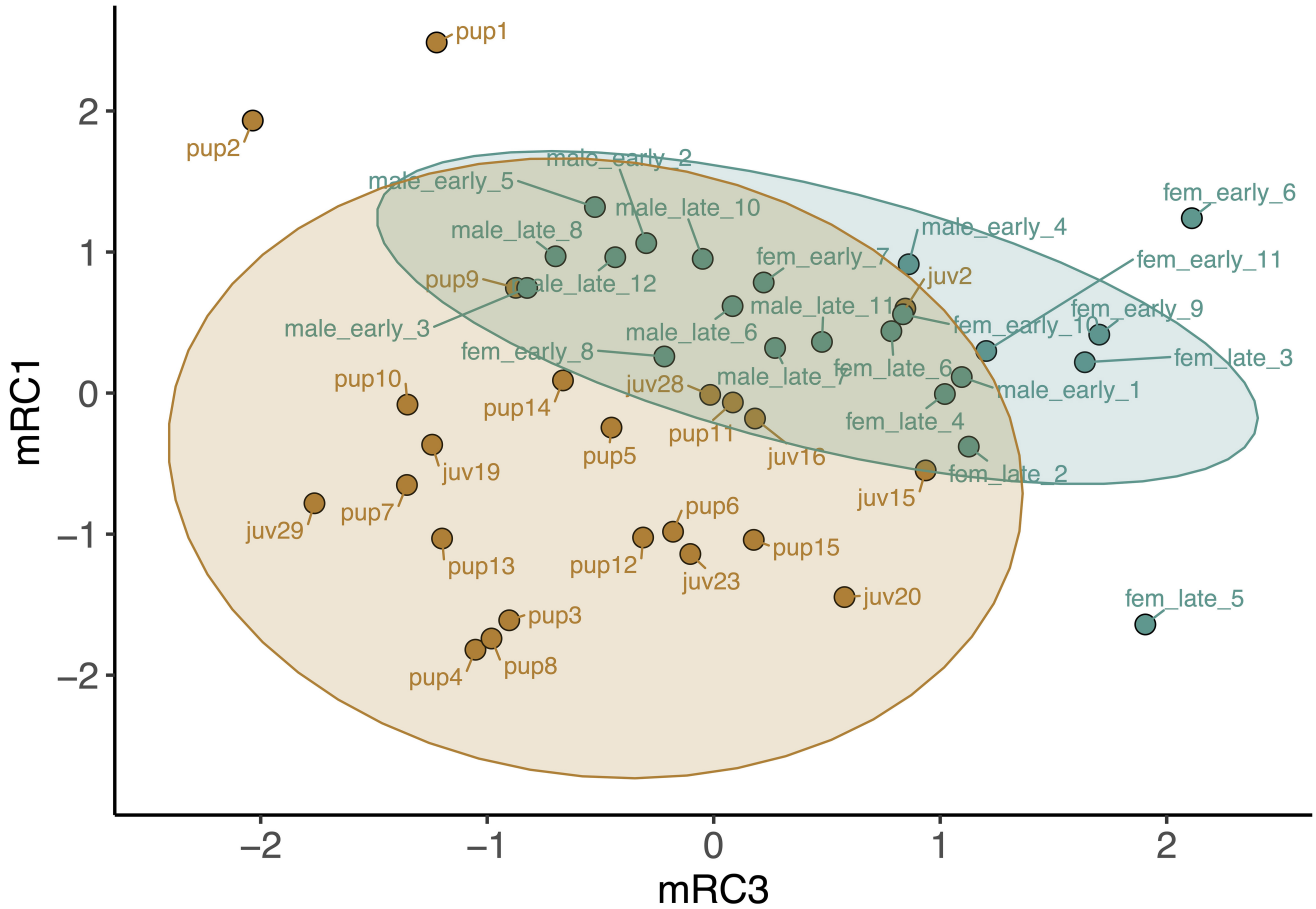

FIGURE 3 | Principal components analysis loading plot for rotated components describing baseline gene expression associated with CO signaling and cytoprotection in skeletal muscle of NES pups, juveniles, and adults. mRC1: *GPX4*, *PRDX6*, *PRDX1*, *SIRT1*; mRC3: *HMOX1*, *BVR*, *GPX3*, *PRDX1*. Ellipses show 95% confidence intervals for young (pups and juveniles, $n = 24$; brown) and adult ($n = 22$; blue) seals.

in pups and adults. None of the components varied with fasting (bRC1: $p = 0.14$; bRC2: $p = 0.48$; bRC3: $p = 0.78$), and none of the interaction terms that included fasting were significant ($p > 0.05$); these terms were subsequently removed from the models. bRC1 expression decreased with age ($F_{1,31} = 73.41$, $p < 0.0001$; **Figure 8**), but was not affected by sex ($p = 0.18$) or the interaction between age and sex ($p = 0.39$). bRC2 and bRC3 did not vary by age (bRC2: $p = 0.33$; bRC3: $p = 0.65$), sex (bRC2: $p = 0.32$; bRC3: $p = 0.12$), or the interaction between age and sex (bRC2: $p = 0.51$; bRC3: $p = 0.10$; data not shown).

Lastly, we examined potential associations between gene expression in skeletal muscle and peripheral blood. There was a significant inverse correlation between *PRDX1* expression in muscle and its expression in blood ($r_S = -0.40$, $p = 0.02$; **Figure 9**). Expression levels of *HMOX1*, *HMOX2*, *BVR*, *IL1B*, and *NRF2* in skeletal muscle and blood were not correlated (*HMOX1*: $p = 0.56$, *HMOX2*: $p = 0.31$, *BVR*: $p = 0.93$, *IL1B*: $p = 0.22$, *NRF2*: $p = 0.98$).

DISCUSSION

Carbon monoxide has long been characterized as an anthropogenic pollutant and toxic gas due to its ability to significantly alter oxygen delivery in the body. Contrary to its former reputation, endogenously produced CO is now widely accepted as a gasotransmitter that confers cytoprotection in the face of hypoxemia, ischemia, and reperfusion in humans and laboratory species (Motterlini and Otterbein, 2010; Tift et al., 2020). However, the role and regulation of endogenous CO production and its potential to exert similar effects in species that are naturally adapted to chronic hypoxia have not been studied. One of the deepest-diving marine mammals, NES, produce and maintain CO at concentrations higher than those of cigarette smokers, and avoid tissue injuries typically seen in other mammals that experience repeated exposure to hypoxemia and ischemia-reperfusion events (Tift et al., 2014, 2020). Due to the large amount of evidence demonstrating the cytoprotective

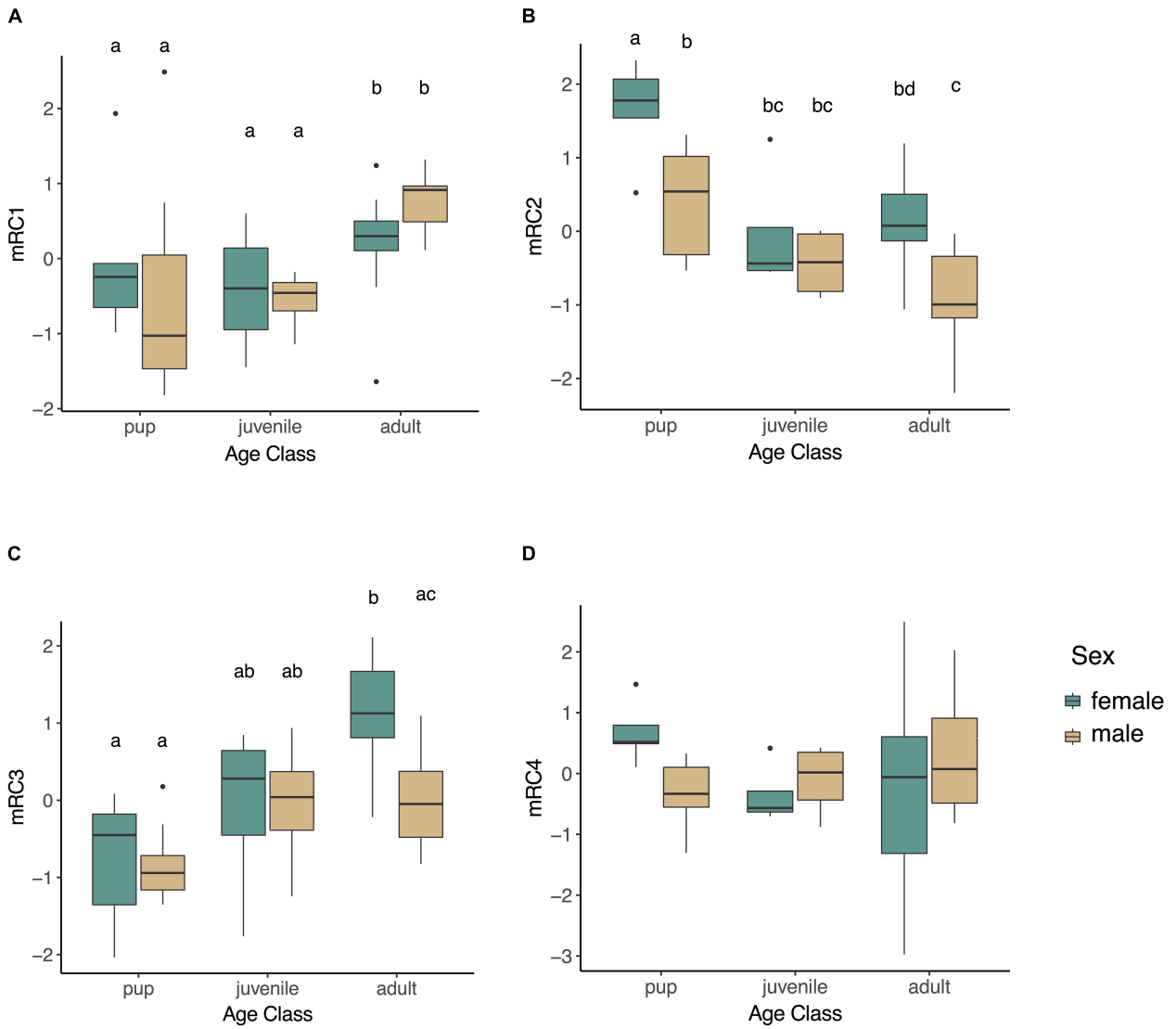

FIGURE 4 | Boxplots showing expression of rotated components mRC1 (**A**; *GPX4*, *PRDX6*, *PRDX1*, *SIRT1*), mRC2 (**B**; *PGC1A*, *ESRRA*, *ESRRG*), mRC3 (**C**; *HMOX1*, *BVR*, *GPX3*, *PRDX1*), and mRC4 (**D**; *HMOX2*, *NRF2*, *IL1B*) in skeletal muscle of NES. mRC1 varied by age ($F_{2,42} = 5.31$, $p = 0.0088$), while mRC2 and mRC3 varied by age and sex (mRC2: $F_{4,41} = 14.26$, $p < 0.0001$; mRC3: $F_{2,39} = 3.13$, $p = 0.055$). Different letters denote significant differences between groups (GLM followed by *post hoc* EMM test, $p < 0.05$).

role of low to moderate CO exposure in humans and rodents (Motterlini and Otterbein, 2010), we hypothesized that the high levels of CO previously measured in NES may be related to expression of genes associated with regulation of oxidative stress and inflammatory pathways, thus potentially contributing to hypoxia tolerance in this deep-diving species.

Our previous work demonstrated that percent carboxyhemoglobin (COHb) levels increase across ontogeny in NES, from mean (±S.D.) 7.1 ± 0.3% in pups and 7.6 ± 0.2% in juveniles to 8.7 ± 0.3% in adults (Tift et al., 2014). Therefore, we hypothesized that tissue-level CO concentrations and expression of genes associated with CO production and cytoprotection would similarly increase as NES pups transitioned from a terrestrial lifestyle to elite diving adults. We found that the concentration of CO in skeletal muscle increased across ontogeny, as predicted from previous measurements of CO in the blood of NES (Tift et al., 2014). To our knowledge, these are the first measurements of endogenous tissue CO concentrations reported in a wild mammal. The mean concentrations of CO measured in skeletal muscle of NES pups (8.5 pmol/mg) resemble mean resting levels of CO in mice (10 pmol/mg). However, the mean (67.1 pmol/mg) and maximum (133.5 pmol/mg) CO concentrations seen in adult NES skeletal muscle are more similar to mean concentrations of CO found in skeletal muscle of humans that died during fires (102 pmol/mg), and much higher than mean CO concentrations in skeletal muscle of mice that inhaled 500 ppm CO for 30 min (14 pmol/mg) (Vreman et al., 2005, 2006). We believe that there are two possible explanations

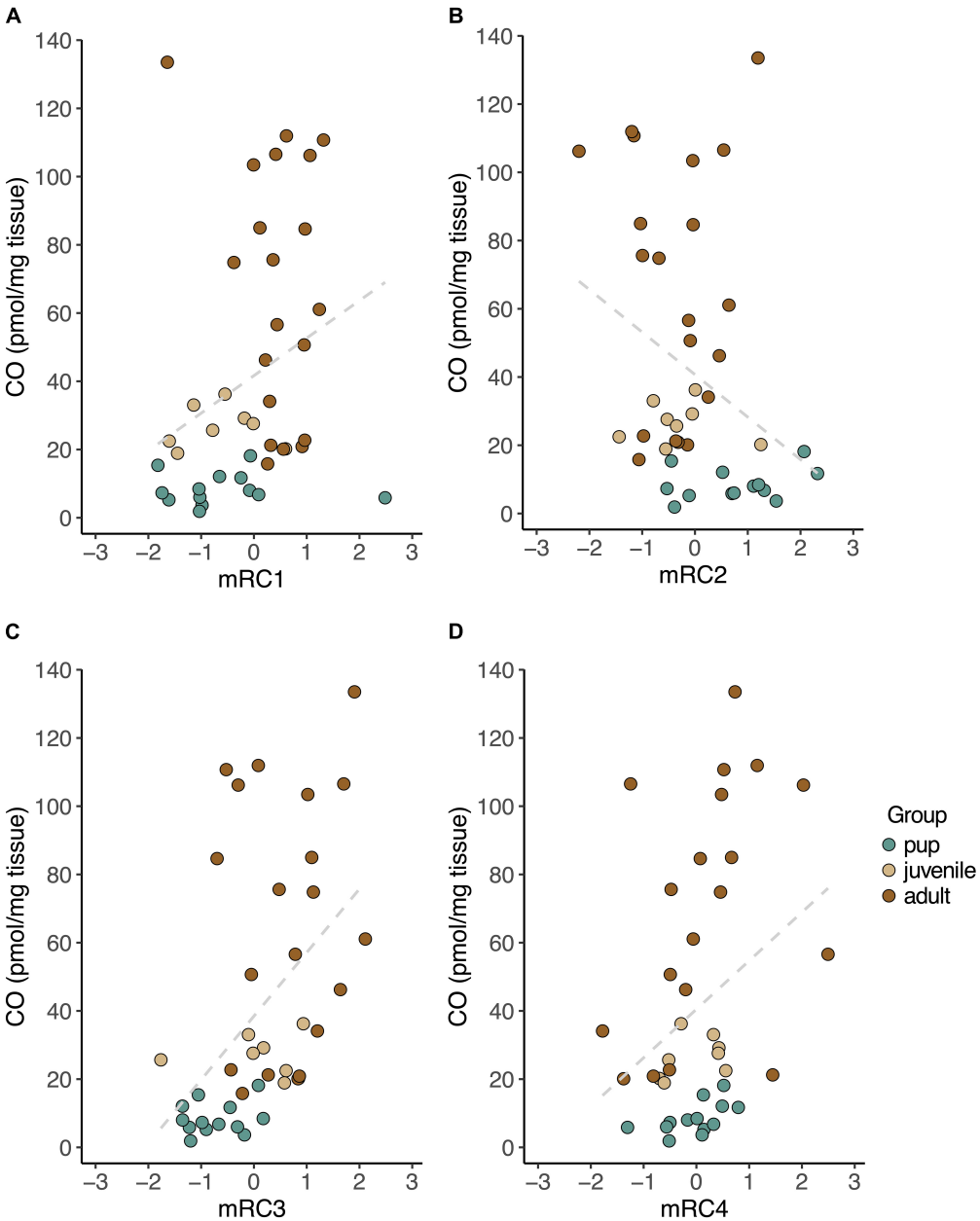

FIGURE 5 | CO levels measured in skeletal muscle were positively correlated with baseline expression of mRC1 (**A**; *GPX4*, *PRDX6*, *PRDX1*, *SIRT1*; $r_S = 0.43$, $p = 0.006$), mRC3 (**C**; *HMOX1*, *BVR*, *GPX3*, *PRDX1*; $r_S = 0.57$, $p = 0.0001$), and mRC4 (**D**; *HMOX2*, *NRF2*, *IL1B*; $r_S = 0.33$, $p = 0.034$), and negatively correlated with expression of mRC2 (**B**; *PGC1A, ESRRA, ESRRG*; $r_S = -0.36$, $p = 0.021$) in NES.

for such high CO concentrations in NES skeletal muscle. First, NES could be producing high quantities of CO directly in skeletal muscle, as a cytoprotective mechanism to alleviate potential injuries occurring in a tissue that experiences routine hypoxia and ischemia and reperfusion events. CO produced in skeletal muscle could directly be acting on other proteins (e.g., cGMP) to induce cytoprotective effects (Motterlini and Otterbein, 2010). We were unable to determine the subcellular localization of CO in NES skeletal muscle, but we suspect it is likely bound to a hemoprotein (e.g., myoglobin or cytochrome) based on the high affinity of CO for heme (Levitt and Levitt, 2015). NES pups and adults have approximately 10-fold and 17-fold higher myoglobin concentrations in skeletal muscle, respectively (Thorson and Le Boeuf, 1994; Hassrick et al., 2010), compared to humans (Möller and Sylvén, 1981). This represents a large *source* of heme for HO enzymes to produce endogenous CO. However, this quantity of myoglobin also represents a large *sink* to bind free CO delivered to the tissue, thus potentially inhibiting CO from binding to other critical hemoproteins (e.g., cytochrome-c-oxidase) (Almeida et al., 2015). A second potential

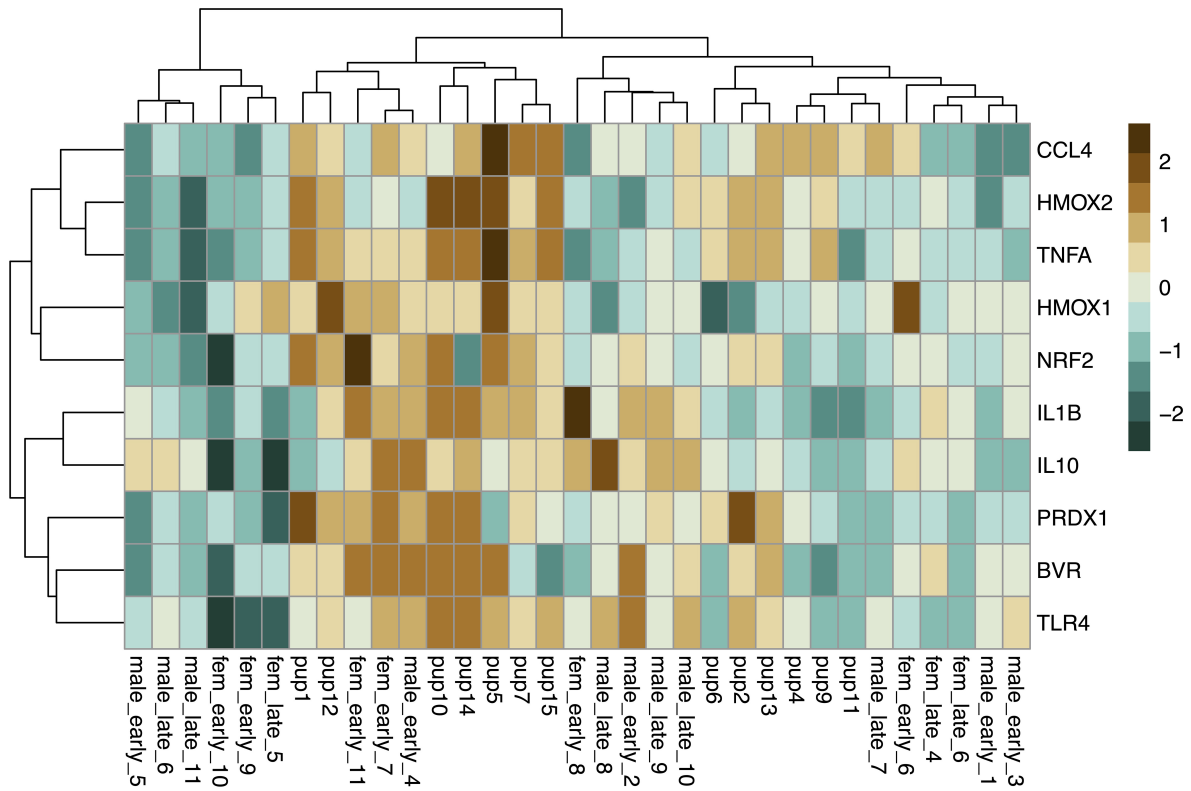

FIGURE 6 | Heatmap showing scaled baseline expression (delta C_T; higher: brown, lower: blue) of 10 genes (rows) associated with CO signaling, cytoprotection, and inflammation in whole blood of NES pups (n = 13) and adults (n = 20, columns). Rows and columns were clustered by Euclidean distance.

explanation is that CO is being produced in other tissues (e.g., spleen), and is delivered to the skeletal muscle via blood. NES have the highest mass-specific blood volume of all mammals, with extremely high hemoglobin concentrations and hematocrit values (Hassrick et al., 2010). This represents another large heme store which could be used by HO enzymes to produce endogenous CO as product of erythrocyte turnover. We have already established that NES have a significant portion of their hemoglobin consistently bound to CO (Tift et al., 2014), yet it is not known how much of the gas is being transferred between blood and muscle. Diffusion of CO from blood into tissues may enable the gas to exert its potent cytoprotective effects, but it may also have deleterious impacts on oxygen delivery to the mitochondria. Considering that NES routinely dive for over an hour and spend very little time at the surface recovering from these long duration dives, suggesting minimal production of anaerobic metabolic byproducts during dives that must be dealt with at the surface (Thorson and Le Boeuf, 1994; Hassrick et al., 2010), we do not believe their onboard CO concentrations limit adequate oxygen delivery. Instead, we believe the high quantities of intravascular and extravascular CO seen in this species likely plays a cytoprotective role to resist injuries to tissues that routinely experience hypoxia and ischemia and reperfusion events (Tift et al., 2020).

TABLE 2 | Eigenvalues and percent of explained variance for varimax-rotated components bRC1, bRC2, and bRC3 that describe expression of genes associated with inflammation, protection from oxidative stress, and production of CO in whole blood of NES.

	bRC1	bRC2	bRC3
Eigenvalue	3.31	3.13	1.49
% of variance	33	31	15
Gene	Varimax-rotated component loadings		
HMOX1	0.28	0.10	**0.91**
HMOX2	**0.89**	0.07	0.27
IL1B	0.00	**0.85**	0.34
NRF2	0.43	**0.53**	0.45
BVR	0.36	**0.70**	0.22
TLR4	**0.51**	**0.77**	−0.06
IL10	0.01	**0.90**	−0.07
CCL4	**0.78**	0.09	0.22
PRDX1	**0.71**	0.43	−0.21
TNFA	**0.88**	0.19	0.34

Rotated component loading scores are shown for each gene used in the analysis, with values >0.5 shown in bold.

We found that baseline gene expression in skeletal muscle was highly correlated by function, including (1) protection from lipid peroxidation (mRC1), (2) mitochondrial biogenesis (mRC2), (3) CO production (mRC3), and (4) regulation of inflammation (mRC4), all of which were associated with concentrations of CO

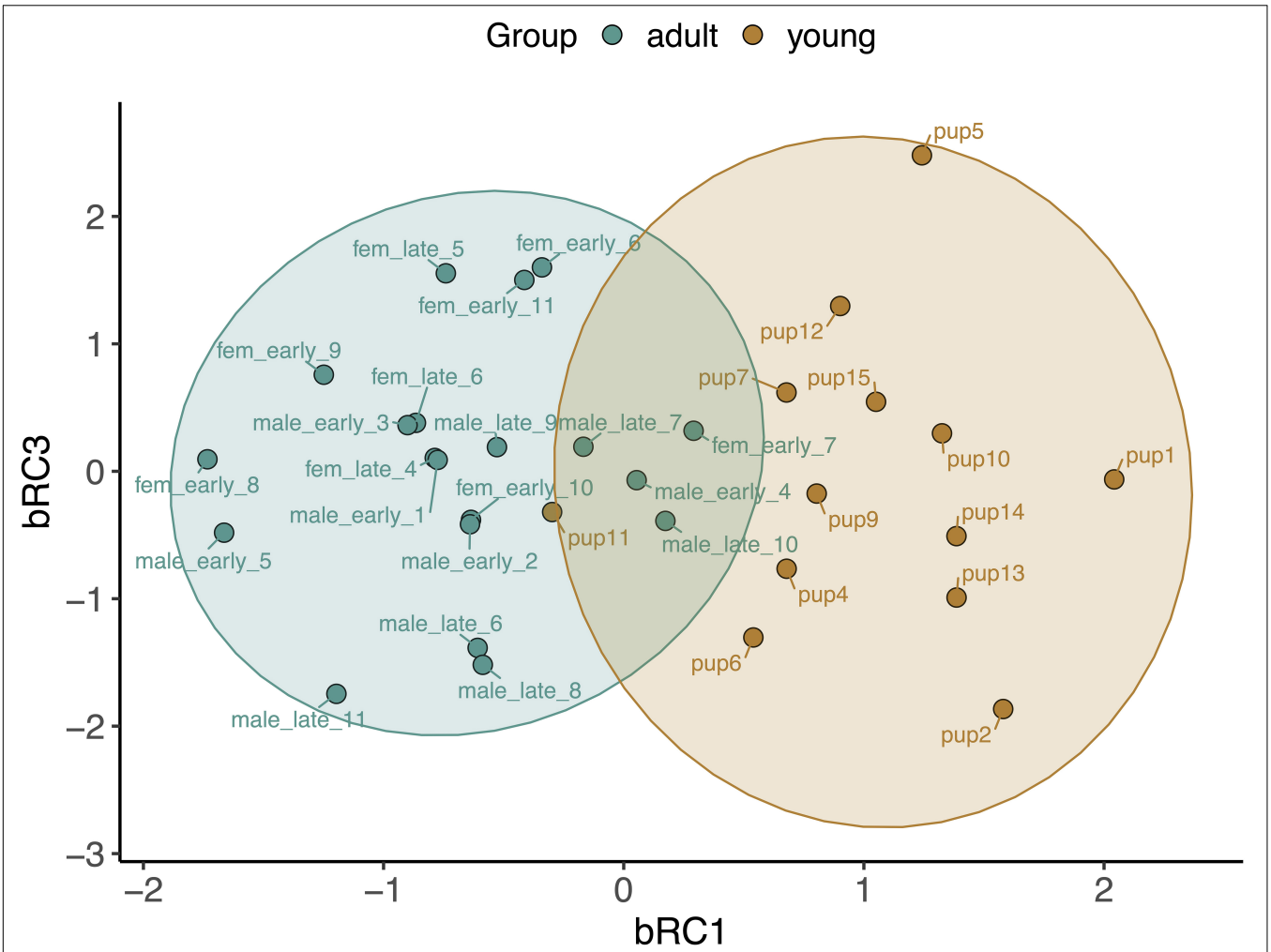

FIGURE 7 | Principal components analysis loading plot for rotated components describing baseline gene expression associated with CO signaling, cytoprotection, and inflammation in whole blood of NES pups (n = 13) and adults (n = 20). bRC1: *HMOX2*, *PRDX1*, *TLR4*, *CCL4*, *TNFA*; bRC3: *HMOX1*. Ellipses show 95% confidence intervals for the two age classes.

in skeletal muscle. Gene expression in peripheral blood cells also clustered by function, including (1) protection from oxidative stress (bRC1), (2) regulation of inflammation (bRC2), and (3) CO production (bRC3, *HMOX1*). Of the genes evaluated in this study, none varied significantly with the duration of fasting that the animals experienced prior to sample collection. This was surprising due to the potential oxidative cost of fasting and previous reports of adaptive antioxidant responses in fasting NES (Ensminger et al., 2021). Our ability to detect differences in gene expression between early and late fasted seals may have been limited by a small sample size and warrants further investigation.

In skeletal muscle, expression of genes associated with protection from lipid peroxidation (mRC1: *GPX4*, *PRDX6*, *PRDX1*, *SIRT1*) was positively correlated with CO and increased with age, with highest expression in adult male NES. GPX4 and PRDX6 are antioxidant enzymes that can reduce peroxidized phospholipids and repair damaged cell membranes, protecting cells from ferroptosis, or death caused by lipid peroxidation (Fisher, 2017; Arevalo and Vázquez-Medina, 2018; Ursini and Maiorino, 2020). PRDX6 has also been linked to myogenesis and maintenance of muscle mass during aging in humans and mice (Pacifici et al., 2020; Soriano-Arroquia et al., 2021), suggesting a potentially similar role in adult NES. PRDX1, another member of the peroxiredoxin family, has a similar function as GPX4 and PRDX6, but relies on thioredoxin instead of glutathione as a reductant (Ding et al., 2017). Consistent with the positive correlation between CO and mRC1 genes in NES, CO administration and *PRDX6* overexpression in mice upregulate *SIRT1*, a key regulator of energy homeostasis and longevity that confers protection from lipid-induced inflammation (Pfluger et al., 2008; Kim et al., 2015; Pacifici et al., 2020). High expression of these genes is likely adaptive for animals such as NES that have large hemoprotein and lipid stores, high metabolic rates, and frequently experience ischemia/reperfusion events that can generate reactive oxygen species (ROS). It may also serve to compensate for the evolutionary loss of paraoxonase-1,

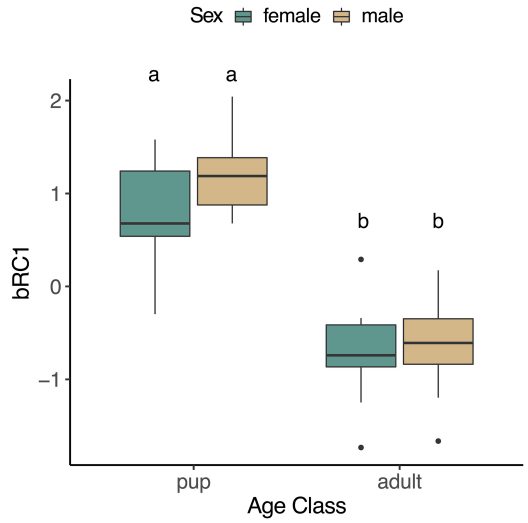

FIGURE 8 | Expression of rotated component bRC1 (*HMOX2*, *PRDX1*, *TLR4*, *CCL4*, *TNFA*) in whole blood of NES was higher in pups than adults ($F_{1,31} = 73.41$, $p < 0.05$).

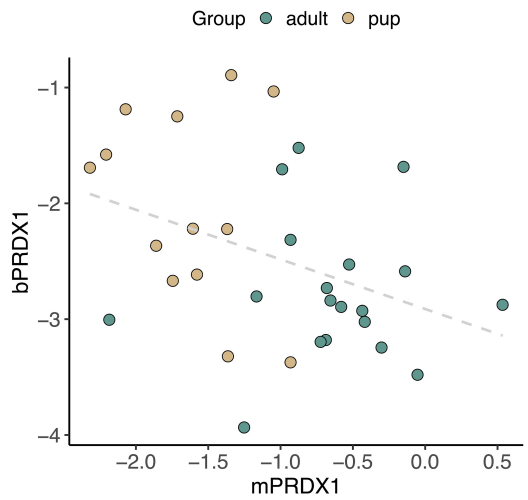

FIGURE 9 | Expression of the gene encoding antioxidant enzyme PRDX1 in NES muscle (m*PRDX1*) and blood (b*PRDX1*) was negatively associated ($r_S = -0.40$, $p = 0.02$).

a circulating antioxidant enzyme that protects lipids from oxidation, in marine mammals (Meyer et al., 2018). Compared to juvenile and adult female NES, adult males typically sustain longer diving-associated apneas at sea and higher fasting metabolic rates on land (Le Boeuf et al., 2000; Hassrick et al., 2007; Crocker et al., 2012). Due to their large body size, males also have larger hemoprotein stores, and thus have a higher potential for liberating the pro-oxidant iron during heme turnover, which also generates CO. Adult males, but not females, exhibit elevated levels of lipid peroxidation markers during prolonged fasts associated with breeding (Crocker et al., 2012; Sharick et al., 2015). Therefore, higher baseline expression of mRC1-associated genes in adult male NES may confer increased protection to animals that have greater risk for lipid peroxidation. Further work will be necessary to determine whether CO directly regulates *GPX4* and *PRDX6* expression in NES.

Muscle expression of genes involved in mitochondrial biogenesis (mRC2: *PGC1A*, *ESRRA*, *ESRRG*), which are upregulated by CO in other systems (Rhodes et al., 2009; Chan et al., 2016; Choi et al., 2016), was highest in NES pups and declined with age. This was despite age-related increases in *HMOX1* expression and CO levels in muscle and blood (Tift et al., 2014). PGC1A, a master regulator of mitochondrial biogenesis, enhances activity of the orphan estrogen-related receptors ESRRA and ESRRG, leading to increased endurance capacity and oxidative remodeling of tissues (Rangwala et al., 2010; Fan et al., 2018). In skeletal muscle, ESRRG is important for long-term exercise adaptation and ESRRA functions as a mediator of adaptive mitochondrial biogenesis, as it is co-expressed with *PGC1A* during development and under conditions of physiological stress (Villena et al., 2007; Giguère, 2008; Rangwala et al., 2010). High levels of *PGC1A*, *ESRRA*, and *ESRRG* expression may help prime rapidly developing NES for their first trip to sea, e.g., by stimulating the switch from glycolytic type II muscle fibers predominant in pups to aerobic type I muscle fibers characteristic of adults (Moore et al., 2014). Expression of mRC2 was higher in female compared to male NES, potentially due to the influence of sex hormones on mitochondrial bioenergetics (Sultanova et al., 2020). In humans, estrogen administered following trauma-induced hemorrhage increased *PGC1A* expression and mediated cardioprotection (Murphy and Steenbergen, 2007). Studies in other systems have shown that low doses of CO administration (up to 3% COHb) stimulate or activate mitochondrial biogenesis in skeletal muscle by increasing *PGC1A* mRNA expression (Rhodes et al., 2009). However, we found that mRC2 expression was inversely correlated with muscle CO levels and *HMOX1* expression in NES, which naturally experience CO levels of up to 9% COHb (Tift et al., 2014). It is possible that expression of genes associated with mitochondrial biogenesis is more responsive to rapidly increasing CO levels in pups during postnatal development than to sustained, high CO levels in adulthood. Alternatively, expression of these genes may be decoupled from the CO pathway in NES, potentially due to their unique metabolic adaptations to prolonged fasting and the role of PGC1A in promoting lipid oxidation in muscle (Gudiksen and Pilegaard, 2017). However, muscle *PGC1A* expression in NES muscle did not vary with fasting state in this or other studies (Wright et al., 2020), and its role in fasting and diving adaptations of NES requires further investigation.

Expression of genes associated with endogenous CO production (mRC3: *HMOX1*, *BVR*, *GPX3*, *PRDX1*) in muscle was positively correlated with the concentration of CO in skeletal muscle and increased with age, with highest expression detected in adult females. The breakdown of heme by HO enzymes is the primary source of endogenous CO and biliverdin, which significantly contribute to antioxidant and anti-inflammatory responses in animals (Jansen and Daiber, 2012; Canesin et al., 2020). BVR catalyzes the final step of the heme degradation

pathway, converting biliverdin into the potent antioxidant bilirubin (Canesin et al., 2020). Expression of *HMOX1*, *BVR*, and *PRDX1* in other species is induced by intracellular free heme and can confer cytoprotection from injuries associated with hypoxia and ischemia-reperfusion events (Gozzelino et al., 2010). GPX3 is a selenium-dependent antioxidant enzyme that scavenges hydrogen peroxide, organic hydroperoxides, and peroxynitrite generated during normal metabolism or oxidative stress (Chang et al., 2020). It has been shown to confer cytoprotection on muscle cells exposed to a variety of stressors (Chung et al., 2009; El Haddad et al., 2012). Similar to *PGC1A*, *GPX3* expression is increased by estrogen (Baltgalvis et al., 2010). Age-related increases in expression of *GPX3* and *PRDX1* (as well as *GPX4* and *PRDX6*) in NES skeletal muscle is consistent with studies in other phocids showing that total GPx and Prx activity increases with age under basal conditions (Vázquez-Medina et al., 2011c). This suggests that increases in expression and activity of these antioxidant enzymes is a common feature of development of dive capacity in marine mammals. As adult female NES consistently perform dives above their calculated aerobic dive limit (Hassrick et al., 2010), potentially experiencing higher rates of oxygen depletion than adult males, elevated expression of genes associated with mRC3 may enable them to avoid oxidative damage to critical tissues by increasing or simply maintaining high quantities of endogenous CO and antioxidant enzymes. The expression of genes in mRC3 was positively correlated with concentration of CO in skeletal muscle, providing a potential link between *HMOX1* expression, CO production, and expression of cytoprotective factors, which may enable prolonged, deep diving in NES (Tift and Ponganis, 2019). Further functional studies will be necessary to test this hypothesis, as elevated CO levels in skeletal muscle of adults, compared to young NES, may simply be a consequence of elevated concentrations of hemoproteins (e.g., myoglobin), which could serve as a source for CO production via HO activity or a sink where CO could bind and be stored. Furthermore, a fraction of endogenous CO is derived from non-heme sources such as lipid peroxidation and the gut microbiome (Vreman et al., 2001), which warrant further investigation.

We found that muscle expression of genes associated with regulation of inflammation (mRC4: *HMOX2*, *NRF2*, *IL1B*) was positively correlated with CO levels but did not vary with age or sex. This result was consistent with reports of constitutive *HMOX2* expression in other systems (Ayer et al., 2016). HO-2 has been shown to play a critical role in inflammatory-reparative regulation, oxygen sensing, cytoprotection, and evolutionary adaptation to high altitude in humans (Seta et al., 2006; Simonson et al., 2010; Yang et al., 2016). Knockdown of *HMOX2* expression in mice aggravated corneal inflammatory damage and impaired angiogenesis and overall HO activity (Bellner et al., 2008). In laboratory rats, CO released by the pharmacological CO-releasing agent CORM-2 alleviated chronic inflammation in the gastric mucosa by accelerating healing and repairing mechanisms, which are intracellularly mediated by Nrf2 (Magierowska et al., 2019). Nrf2 activation, in response to ROS, leads to the transcription of genes, including *HMOX1*, that are involved in protection from oxidative stress induced by inflammation (Hennig et al., 2018). Administration of CO and overexpression or activation of Nrf2, HO-1, and HO-2 have been shown to inhibit IL-1β production in mouse studies (Muñoz-Sánchez and Chánez-Cárdenas, 2014; Kobayashi et al., 2016; Dugbartey, 2021). Oxidative stress-mediated activation of the Nod-like receptor protein 3 (NLRP3) inflammasome was also associated with increased expression of *NRF2*, *HMOX1*, and *IL1B* in humans with osteoarthritis (Chen et al., 2019). The positive correlation between CO levels and *NRF2*, *HMOX2*, and *IL1B* expression in NES muscle suggests that *NRF2* and *HMOX2* may be upregulated (and, consequently, CO produced) in conditions of high *IL1B* expression, potentially as an adaptive mechanism to ameliorate oxidative damage caused by inflammation. Alternatively, these genes may serve to regulate functions other than (or in addition to) regulation of inflammation in NES muscle. For instance, IL-1β has been shown to stimulate myoblast proliferation in response to muscle injury in mice (Otis et al., 2014) and to augment glucose uptake in skeletal muscle in response to exercise (Tsuchiya et al., 2018). Nrf2 has also been shown to reduce lipid accumulation and oxidative damage in mice with hepatic steatosis (Upadhyay et al., 2020). Recent studies in humans have linked HMOX2, Nrf2, and IL-1β with insulin resistance and obesity (Li et al., 2012, 2020; Crilly et al., 2016; Tan et al., 2018; Yao et al., 2020), which are two characteristics exhibited by fasting NES (Houser et al., 2013). Evidently, more research is needed to understand the link between CO and *IL1B* expression in diving, fasting-adapted mammals. Two caveats of our study include the measurement of mRNA levels, rather than cytokine protein secretion, and the measurement of gene expression under baseline, rather than inflammatory conditions. This makes it challenging to decipher the relationship between CO and inflammatory markers in this system. Ultimately, it would be interesting to see whether CO administration would decrease IL-1β production in seal cells in functional experiments.

Our study is the first to examine gene expression in whole blood of NES, which contains primarily circulating lymphocytes and monocytes (PBMCs) (He et al., 2019). We hypothesized that exposure of PBMCs to fluctuating oxygen tension during apneas in NES would stimulate adaptive responses in this diving-adapted species (Stockard et al., 2007; Vázquez-Medina et al., 2011a; Tift et al., 2013), upregulating *HMOX1* expression and local CO production and regulating expression of pro-inflammatory cytokines. Due to the potent anti-inflammatory effects of CO reported in laboratory species, we predicted that *HMOX1* expression would be negatively correlated with pro-inflammatory markers and positively correlated with anti-inflammatory cytokines. In a previous study, exposure of mice to low CO concentrations under inflammatory conditions inhibited production of TNF-α, MIP-1β, and IL-1β and induced expression of *IL10* via a p38 MAPK-dependent mechanism (Ryter, 2020). CO also significantly suppressed lipopolysaccharide (LPS)-induced NADPH oxidase-dependent ROS generation in mouse macrophages by inhibiting *TLR4* and its downstream signaling pathways (Nakahira et al., 2006). Contrary to our predictions, *HMOX1* expression in blood did not vary by age and loaded onto a separate component (bRC3) that was not associated with any inflammatory markers. These data suggest that baseline *HMOX1*

expression in PBMCs may be low and potentially decoupled from regulation of the markers tested in this study in a hypoxia-tolerant mammal. Baseline variability in *HMOX1* expression may also not reflect HO-1 enzyme abundance, activity, and role in immune, redox, and metabolic homeostasis under conditions of hypoxia-related inflammation or injury, which are rarely experienced by marine mammals (Allen and Vázquez-Medina, 2019). Further work will be necessary to elucidate the effects of CO on inflammatory signaling in marine mammals, especially since a recent study suggested that serum from NES and Weddell seals possessed intrinsic anti-inflammatory properties, the source of which has not yet been identified (Bagchi et al., 2018).

HMOX2 expression in whole blood of NES was positively correlated with three pro-inflammatory cytokines (*TLR4*, *CCL4*, *TNFA*) and the antioxidant *PRDX1* (bRC1). Their expression was higher in pups compared to adults, despite the fact that older animals dive longer and deeper than juveniles and experience significant blood O_2 depletion during routine dives (Meir et al., 2013), a condition that would trigger inflammation in humans or rodents (Allen and Vázquez-Medina, 2019). Higher bRC1 expression in young NES may reflect preconditioning responses to diving during postnatal development. During the post-weaning period, NES pups rapidly increase the duration spent submerged in shallow water along with the duration of sleep apneas on land, increasing their exposure to hypoxia (Blackwell and Boeuf, 1993). Repeated apneas in NES pups have been shown to potentiate mechanisms associated with protection from oxidative stress, including upregulation of hypoxia inducible factors (*HIF*s) (Vázquez-Medina et al., 2011a). The correlation between *TLR4* and *PRDX1* expression in NES blood is consistent with studies in mice that have shown that PRDX1, which is upregulated in response to ischemia-reperfusion, serves as an endogenous ligand for TLR4 (Liu and Zhang, 2019). Furthermore, the interaction between PRDX1 and TLR4 in human cancer cells was shown to upregulate HIF-1α (Riddell et al., 2012), a master regulator of adaptive responses to hypoxia that is highly expressed in NES tissues (Allen and Vázquez-Medina, 2019). The negative correlation between *PRDX1* expression in skeletal muscle and blood observed in this study highlights its complex, cell type-dependent functions in animals (Hopkins and Neumann, 2019), e.g., regulation of inflammatory signaling in PBMCs and lipid peroxidation in skeletal muscle. Our data suggest that postnatal development in a deep-diving mammal may involve priming the immune system by upregulating the oxygen sensor *HMOX2* and inflammatory markers that induce adaptive responses to hypoxia.

Lastly, we found that expression of anti-inflammatory and antioxidant markers (*IL10*, *NRF2*, *BVR*) in NES blood was positively associated with expression of pro-inflammatory markers (*IL1B*, *TLR4*). While this may seem paradoxical, recent studies have suggested that relationships between pro- and anti-inflammatory responses in mammals are extremely complex (Kowsar et al., 2019). For instance, *IL10* and *IL1B* are co-expressed under pathophysiological and physiological conditions in bovine cells (Kowsar et al., 2019), and IL-10 was recently shown to possess pro-inflammatory properties (Mühl, 2013). While primarily considered a pro-inflammatory marker, IL-1β also influences insulin secretion and insulin resistance in mice (Dror et al., 2017), and could therefore play a primarily metabolic role in fasting-adapted NES, which display insulin resistance (Champagne et al., 2012). The correlation between *BVR* and *IL10* expression in NES blood is consistent with data from other studies showing that BVR upregulates *IL10* by activating PI3K-Akt (Wegiel and Otterbein, 2012). However, BVR also directly inhibits *TLR4* expression (Medzhitov, 2001; Wegiel and Otterbein, 2012), while Nrf2 is known to suppress *IL1B* (Campbell et al., 2021). Co-expression of these factors in NES blood suggests a complex interplay of hormetic responses in a hypoxia-adapted mammal that warrant further mechanistic investigation.

CONCLUSION

Our study is the first to measure tissue CO levels in any wild animal, and to report expression of genes associated with endogenous CO production and signaling in blood and muscle of a deep-diving phocid species across ontogeny. As such, it provides a number of hypotheses for further exploration of natural hypoxia tolerance in mammals. We propose that upregulation of baseline *HMOX1* expression in skeletal muscle of NES may, in part, underlie developmental increases in CO levels and expression of genes encoding cytoprotective factors such as antioxidant enzymes, several of which are involved in protection from lipid peroxidation. *HMOX2* may play a role in regulating inflammation related to ischemia and reperfusion in muscle and PBMCs of NES. Our data propose putative ontogenetic mechanisms that may enable phocid pups to transition to a deep-diving lifestyle. These include high expression of genes associated with mitochondrial biogenesis in muscle and potential immune system activation during postnatal development and age-related increases in expression of genes associated with protection from lipid peroxidation in adulthood. Functional studies, such as *in vitro* manipulations of CO levels and HO expression and activity will be necessary to determine the nature of the relationship between the CO/HO pathway and cytoprotective factors in diving mammals.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found at figshare: https://figshare.com/s/91c6c1d1d230bf7a16e0.

ETHICS STATEMENT

The animal study was reviewed and approved by Sonoma State University and University of the Pacific Institutional Animal Care and Use Committees and conducted under National Marine Fisheries Service permit Nos. 19108 and 23188.

AUTHOR CONTRIBUTIONS

MT, JK, and DC conceived and designed the study. EP, JK, DC, and AK collected the animal samples. EP, JK, and JV-M designed the gene expression assays. EP conducted the gene expression analyses. AP and MT measured carbon monoxide. JV-M, MT, and DC aided in interpreting results. EP and JK conducted statistical analyses and drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.762102/full#supplementary-material

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REVIEW
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Influence of High Hemoglobin-Oxygen Affinity on Humans During Hypoxia

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Humans elicit a robust series of physiological responses to maintain adequate oxygen delivery during hypoxia, including a transient reduction in hemoglobin-oxygen (Hb-O_2) affinity. However, high Hb-O_2 affinity has been identified as a beneficial adaptation in several species that have been exposed to high altitude for generations. The observed differences in Hb-O_2 affinity between humans and species adapted to high altitude pose a central question: is higher or lower Hb-O_2 affinity in humans more advantageous when O_2 availability is limited? Humans with genetic mutations in hemoglobin structure resulting in high Hb-O_2 affinity have shown attenuated cardiorespiratory adjustments during hypoxia both at rest and during exercise, providing unique insight into this central question. Therefore, the purpose of this review is to examine the influence of high Hb-O_2 affinity during hypoxia through comparison of cardiovascular and respiratory adjustments elicited by humans with high Hb-O_2 affinity compared to those with normal Hb-O_2 affinity.

Keywords: altitude acclimatization, high-altitude, oxygen transport, exercise, $\dot{V}O_{2max}$ (maximal oxygen uptake), high affinity hemoglobin (Hb)

INTRODUCTION

Currently, there is ongoing debate about the advantages of higher or lower hemoglobin-oxygen (Hb-O_2) affinity in humans, particularly during hypoxia (Dempsey, 2020). A decrease in Hb-O_2 affinity is often observed among humans during acclimatization to altitudes ranging from 2500 to 4500 m, presumably to facilitate O_2 off-loading and protect against tissue hypoxia (Hall et al., 1936; Aste-Salazar and Hurtado, 1944; Lenfant and Sullivan, 1971). In contrast, several animal species adapted to high-altitude environments display a higher Hb-O_2 affinity compared to that of low-land counterparts (Bartels et al., 1963; Monge and Leon-Velarde, 1991; Weber et al., 1993; Scott and Milsom, 2007; Storz et al., 2010; Storz, 2016; Natarajan et al., 2018). These divergent observations lead to the central question of this review, is higher or lower Hb-O_2 affinity more advantageous for humans during hypoxia?

Humans rely on a continuous supply of O_2 for metabolism. Oxygen binds to hemoglobin in the lungs and travels through the large arteries, arterioles, and finally the small capillaries supplying peripheral tissue (Scholander, 1960). Although *in vitro* Hb-O_2 affinity is characterized by a single curve or metric (e.g., P_{50}, as described below), the *in vivo* Hb-O_2 affinity cannot be described as

simply. Within the vasculature, alterations of modulatory factors such as temperature, pH, and the concentration of carbon dioxide (CO_2) lead to transient changes in Hb-O_2 affinity during circulatory transit, which directly impact O_2 loading at the lung and O_2 off-loading in peripheral tissue (Jensen, 2004; Winslow, 2007). Changes in Hb-O_2 affinity can be transient or chronic due to a variety of conditions such as genetic mutations, disease, altitude acclimatization, or age (Woodson et al., 1970; Humpeler and Amor, 1973; Versmold et al., 1973; Winslow, 2007). For example, evidence suggests that some groups of humans native to high altitude have a greater Hb-O_2 affinity than sea-level residents (Simonson et al., 2014; Li et al., 2018). Although the mechanisms underlying the adaptive increase of Hb-O_2 affinity among high-altitude natives are not well understood, a number of genetic mutations in hemoglobin structure that contribute to a systemic increase in Hb-O_2 affinity in humans have been identified (Mangin, 2017), predominantly among low-altitude residents. Humans with mutations resulting in high Hb-O_2 affinity may provide unique insight to the ongoing debate regarding the advantages and disadvantages of high Hb-O_2 affinity during hypoxia. Past investigations of the cardiorespiratory adjustments to hypoxic exposure at rest and during exercise suggest that high Hb-O_2 affinity may provide better maintenance of O_2 delivery and utilization in humans. Therefore, the purpose of this review is to highlight the potential advantages and disadvantages of high Hb-O_2 affinity in humans during hypoxia through examination of cardiovascular and respiratory adjustments at rest and during exercise.

To address the central question of this review, we examine available studies reporting cardiovascular or respiratory adjustments to hypoxia at rest or during exercise in humans with genetic mutations resulting in high Hb-O_2 affinity. To avoid confounding factors that may alter cardiovascular and respiratory responses, we excluded studies in which these individuals have recently undergone venesection. Studies fitting these criteria can be found in **Table 1**, including participant characteristics and experimental design. To clearly denote the "severity" of hypoxia within the discussion, we define low altitude as $<$2500 m, high altitude as $>$2500 m, and extreme altitude as $>$7000 m.

FOUNDATIONAL CONCEPTS

Hemoglobin-oxygen affinity is largely determined by the structure of hemoglobin and modulated by a variety of factors within the vasculature [temperature, pH, CO_2, 2,3-diphosphoglycerate (2,3-DPG), organic phosphates, chloride ions (Cl^-), etc.] (Mairbaurl et al., 1993). The relationship between the partial pressure of O_2 (P_{O_2}) and O_2 saturation can be described by the O_2 dissociation curve (**Figure 1**). One common metric to quantify Hb-O_2 affinity is P_{50}, defined as the P_{O_2} at which 50% of hemoglobin is saturated with O_2. A lower P_{50} corresponds to a higher Hb-O_2 binding affinity or a "left-shifted" O_2 dissociation curve. On the other hand, a higher P_{50} corresponds to a lower Hb-O_2 binding affinity and a "right-shifted" O_2 dissociation curve. In addition to P_{50}, the Hill coefficient is often used to describe the curvature of the O_2 dissociation curve (Endrenyi et al., 1975; Piiper, 1992; Riggs, 1998). However, describing the O_2 dissociation curve with the P_{50} and the Hill coefficient presents some limitations. Experimentally, the P_{50} and Hill coefficient are commonly determined using *in vitro* standardized environmental conditions [pH ~7.4, partial pressure of CO_2 (P_{CO_2}) ~40 mmHg, and temperature ~37°C], which does not account for transient changes in the *in vivo* modulation of Hb-O_2 affinity during circulatory transit (Braumann et al., 1982). Therefore, there is not "one" O_2 dissociation curve because the binding affinity and cooperativity of hemoglobin vary throughout the vasculature. Nevertheless, standardized measurements of P_{50} and the Hill coefficient allow general inter-individual comparisons of Hb-O_2 affinity, but do not account for *in vivo* modulation of Hb-O_2 affinity.

Changes in Hb-O_2 affinity throughout the vasculature optimize both O_2 loading in the lungs and O_2 off-loading to peripheral tissue. For example, byproducts of metabolism (increased temperature, increased CO_2, and lower pH) contribute to a localized decrease in Hb-O_2 affinity in exercising muscle, thereby promoting O_2 off-loading and utilization (Böning et al., 1975). Furthermore, a lower temperature and increased pH within the lung result in a localized increase in Hb-O_2 affinity and improved O_2 loading (Mairbäurl, 2013). Alternatively, long-term regulation of modulatory factors or alterations in the structure of hemoglobin can lead to systemic wide changes in Hb-O_2 affinity. For instance, hypoxia increases 2,3-DPG concentration (due to increased glycolytic activity) in red blood cells contributing to a systemic decrease of Hb-O_2 affinity (Lenfant et al., 1968). Standard teaching supports that a decrease in Hb-O_2 affinity facilitates O_2 off-loading during hypoxia (Hall et al., 1936; Aste-Salazar and Hurtado, 1944). Yet, the systemic decrease in Hb-O_2 affinity would compromise O_2 loading in the lung, particularly when O_2 availability is limited during hypoxia. At higher altitudes, a decrease in Hb-O_2 affinity would be even more disadvantageous and further compromised O_2 loading would likely impede peripheral O_2 delivery. Conversely, an increase in Hb-O_2 affinity during hypoxia promotes O_2 loading within the lungs and mitigates reductions in arterial O_2 saturation (Eaton et al., 1974; Yalcin and Cabrales, 2012). In addition, the advantages conferred by increased Hb-O_2 affinity are augmented at higher altitudes, outweighing potential limitations in O_2 off-loading (Eaton et al., 1974). Therefore, homeostatic maintenance of O_2 delivery and utilization during hypoxia is contingent on the balance between O_2 loading in the lungs and O_2 off-loading in the periphery, both of which are largely determined by the Hb-O_2 affinity. Additional discussion of hemoglobin structure and the regulation of Hb-O_2 affinity is presented below (see section "Hemoglobin-Oxygen Affinity").

Hemoglobin-Oxygen Affinity

Hemoglobin is a tetramer consisting of two α-subunits and two β-subunits (Coates, 1975). Each subunit contains a heme group that is capable of reversibly binding O_2 (Perutz, 1963). When hemoglobin is fully saturated four O_2 molecules are bound independently to each of the four

TABLE 1 | Studies examining cardiorespiratory adjustments during normoxia or hypoxia in humans with high Hb-O_2 affinity.

Study	Age (years)	Sex (*n*)	Hb type	P_{50} (mmHg)	[Hb] (g/dL)	Hct (%)	Study design
Hebbel et al., 1977	12	1M	Hb Andrew-Minneapolis	17	16	*NR*	Hypoxic ventilatory response (F_iO_2 = 0.13, ∼3800 m)
	18	1F	Hb Andrew-Minneapolis	17	17	*NR*	
Hebbel et al., 1978	12	1M	Hb Andrew-Minneapolis	17	17	48	High-altitude acclimatization (∼3100 m) and graded cycling to exhaustion
	18	1F	Hb Andrew-Minneapolis	17	17	50	
Rossoff et al., 1980	25	2M	Hb Rainier	12	*NR*	*NR*	Hypoxic ventilatory response (F_iO_2 = 0.14, ∼3300 m)
Wranne et al., 1983	30	1M	*NR*	14	19	55	Normoxic submaximal cycling
	31	1M	*NR*	14	18	54	
Länsimies et al., 1985	38 (14)	5M	Hb Linköping	16 (0.4)	19 (1)	*NR*	Normoxic graded cycling to exhaustion
	32 (8)	5F	Hb Linköping	17 (0.5)	16 (4)	*NR*	
Dominelli et al., 2019	45 (8)	3M	Hb Malmö	15 (0.2)	21 (1)	63 (3)	Hypoxic ventilatory response (F_iO_2 = 0.14, ∼3300 m)
	43 (15)	6F	Hb Malmö (*n* = 5), Hb San Diego (*n* = 1)	16 (1.1)	19 (1)	55 (3)	
Dominelli et al., 2020	45 (8)	3M	Hb Malmö	15 (0.2)	21 (1)	63 (3)	Normoxic and normobaric hypoxic (F_iO_2 = 0.15, ∼2600 m) graded cycling to exhaustion
	31 (9)	8F	Hb Malmö (*n* = 7), Hb San Diego (*n* = 1)	16 (0.9)	18 (1)	54 (2)	

The fraction of inspired O_2 and associated elevation are provided under study design.
Abbreviations: M, male; F, female; Hb, hemoglobin; P_{50}, the P_{O_2} at which 50% of hemoglobin is saturated with O_2; Hb, hemoglobin; Hct, hematocrit; F_iO_2, fraction of inspired O_2; NR, not reported. Numbers within parentheses throughout the table indicate standard deviations.

subunits of the hemoglobin molecule. Hemoglobin undergoes a conformational shift with each O_2 molecule that binds, existing in a T (tense) state when deoxygenated and a R (relaxed) state when oxygenated, commonly described by a two-state model (Monod et al., 1965). As each individual subunit becomes oxygenated, a conformational shift further increases binding affinity for O_2 (Mihailescu and Russu, 2001). This cooperativity in O_2 binding to hemoglobin gives rise to the sigmoidal shape of the O_2 dissociation curve (**Figure 1**).

Hemoglobin is subject to allosteric regulation by multiple ligands. Most notably, higher concentrations of H^+ and CO_2 reduce Hb-O_2 affinity (Riggs, 1960; Ho and Russu, 1987). This pH dependent change of Hb-O_2 affinity is termed the Bohr effect (Bohr et al., 1904). Hb-O_2 affinity is also reduced at higher temperatures (Weber and Campbell, 2011). For example, an increase of temperature from 37 to 40°C raises P_{50} from normal values of ∼27 to 30 mmHg (Hlastala et al., 1977). Additionally, the magnitude of the Bohr effect is greater at higher temperatures, further promoting O_2 off-loading from hemoglobin (Hlastala et al., 1977). The cooperative effect of a more acidic environment along with higher temperatures, as occurs during rigorous exercise, significantly reduces Hb-O_2 affinity such that P_{50} may increase up to ∼40 mmHg within the vasculature (Thomson et al., 1974). In the case of severe respiratory alkalosis, a fivefold increase in minute ventilation may reduce arterial P_{CO_2} from normal values of ∼40 mmHg to as low as 7 mmHg and blood pH may exceed 7.7 (Houston et al., 1987; West, 2006). At extreme altitudes, *in vivo* P_{50} may be reduced to less than 20 mmHg due to changes in blood P_{CO_2} and pH (West, 1984; Winslow et al., 1984).

The erythrocytic concentrations of 2,3-DPG and Cl^- are associated with more long-term modulation of Hb-O_2 affinity. In effect, 2,3-DPG and Cl^- bind to deoxygenated hemoglobin and stabilize the T state, reducing Hb-O_2 affinity (Benesch et al., 1967; Brewer, 1974). 2,3-DPG reduces Hb-O_2 affinity and increases the cooperativity of hemoglobin, which "right-shifts" the O_2 dissociation curve and steepens the slope (Tyuma et al., 1971). In addition, an influx of Cl^- into the red blood cell, coupled to the outward transport of bicarbonate, reduces Hb-O_2 affinity (Wieth et al., 1982; Perutz et al., 1994; Prange et al., 2001). These ligands elicit independent effects on Hb-O_2 affinity, and complex *in vivo* interactions between ligands give rise to the physiological P_{50} of hemoglobin. For example, 2,3-DPG and Cl^- compete for binding to hemoglobin and the effect 2,3-DPG on Hb-O_2 affinity disappears at high concentrations of Cl^- (Imai, 1982). In addition, the Bohr effect is more pronounced at greater concentrations of 2,3-DPG (Bauer, 1969). The interested reader may consult other sources for more detailed discussions on modulation of Hb-O_2 affinity (Antonini and Brunori, 1970; Mairbaurl and Weber, 2012).

The severity and duration of hypoxia is an important factor when considering *in vivo* modulation of Hb-O_2 affinity in humans. During sojourns to altitudes of ∼4500 m or less, humans demonstrate a reduced Hb-O_2 affinity due to elevated production of 2,3-DPG (Lenfant et al., 1968). At these elevations, hyperventilation reduces blood P_{CO_2} and potentially results in respiratory alkalosis (Dempsey and Forster, 1982). However, renal compensation leads to the excretion of excess bicarbonate and conservation of H^+, normalizing blood pH to sea-level values after a few days at high altitude (Goldfarb-Rumyantzev and Alper, 2014; Bird et al., 2021). At higher elevations (4500–5400 m), hyperventilation becomes so pronounced that renal compensation is insufficient and blood pH increases (West, 2006). The rise in blood pH increases Hb-O_2 affinity, counteracting the effects of an elevated 2,3-DPG production such that P_{50} approximates values observed at sea-level (Mairbaurl and Weber, 2012). As humans travel above ∼5400 m, Hb-O_2 affinity increases as the respiratory alkalosis becomes more severe (West, 1984).

Contemporary studies suggest a potential role of hemoglobin found in cells other than erythrocytes such as alveolar epithelial cells, lung cells, and mesangial cells (Du et al., 2012; Saha et al., 2014). Within these non-erythrocytic cells, the production of hemoglobin appears to be upregulated in response to hypoxia (Cheung et al., 1997; Tezel et al., 2009; Grek et al., 2011), potentially serving as a "reservoir" for O_2 (Saha et al., 2014). Therefore, a key area for future investigation is the relationship between non-erythroid hemoglobin production and hypoxia tolerance. However, there is currently minimal evidence to suggest that non-erythroid hemoglobin provides a functional impact on cardiovascular adjustments during hypoxia.

Pharmacological Induction of High Hemoglobin-Oxygen Affinity

Several pharmacological methods which transfuse 2,3-DPG depleted red blood cells into both animals and humans have allowed investigation into the role of high Hb-O_2 affinity in O_2 transport (Riggs et al., 1973; Woodson et al., 1973; Wranne et al., 1974; Bakker et al., 1976; Malmberg et al., 1979; Woodson and Auerbach, 1982; Birchard and Tenney, 1991). However, methods used to achieve 2,3-DPG depletion often alter acid-base balance and total blood volume, potentially confounding the observed cardiorespiratory adjustments (Birchard and Tenney, 1991). More recent developments of pharmaceuticals that induce high Hb-O_2 affinity allow examination of altered Hb-O_2 affinity with fewer complications (Dufu et al., 2017; Kalfa et al., 2019; Stewart et al., 2020, 2021). For example, voxelotor binds allosterically to some, but not all hemoglobin and increases Hb-O_2 affinity. Hemoglobin modified with voxelotor exhibits a reduced Bohr effect compared to unmodified hemoglobin (Pochron et al., 2019), which may limit O_2 off-loading during instances where blood pH decreases such as rigorous exercise.

In general, allosteric modifiers allow for the manipulation of Hb-O_2 affinity with less perturbations in acid-base balance associated with 2,3-DPG depletion techniques. However, in healthy humans voxelotor induces only a modest decrease in P_{50} of ∼2 mmHg (Stewart et al., 2020, 2021) compared to the greater range from 3 to 10 mmHg obtained *via* 2,3-DPG depletion (Gillette et al., 1974; Wranne et al., 1974). The ability to pharmacologically alter Hb-O_2 affinity in humans both acutely and chronically may provide additional insights on the context-dependent circumstances at which high Hb-O_2 affinity is advantageous (i.e., magnitude and duration of hypoxia).

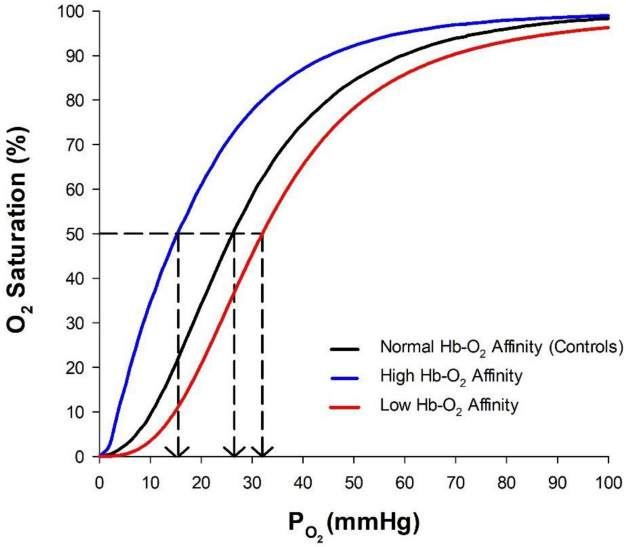

FIGURE 1 | Oxygen dissociation curve showing normal hemoglobin-O_2 (Hb-O_2) affinity (P_{50} ∼26 mmHg), high Hb-O_2 affinity (P_{50} ∼16 mmHg), and low Hb-O_2 affinity (P_{50} ∼32 mmHg). The P_{50}, denoted by the dashed lines, is defined as the P_{O_2} at which 50% of hemoglobin is saturated with O_2.

Humans With High Hemoglobin-Oxygen Affinity Hemoglobinopathies

Currently, over 200 distinct mutations resulting in high Hb-O_2 affinity have been identified (Charache et al., 1966; Mangin, 2017). By definition, high Hb-O_2 affinity is characterized by a P_{50} less than 24 mmHg (**Figure 1**; Rumi et al., 2009; Mangin, 2017). However, a majority of high Hb-O_2 affinity hemoglobinopathies examined are associated with P_{50} values ranging from 12 to 17 mmHg (**Table 1**). Both the amino acid substitution and location at which the substitution occurs within the hemoglobin molecule may affect Hb-O_2 affinity, cooperativity, and response to modulatory ligands. Within the hemoglobin mutations represented in this review (**Table 1**), all exhibit reduced cooperativity and only Hb Andrew-Minneapolis demonstrates a reduced Bohr effect (Adamson et al., 1969; Boyer et al., 1972; Nute et al., 1974; Zak et al., 1974; Wranne et al., 1983; Berlin et al., 2009). Lower cooperativity gives rise to the unique shape of the standard O_2 dissociation curve in humans with high Hb-O_2 affinity (**Figure 1**). However, the complex interactions between modulatory factors and subsequent effects on *in vivo* Hb-O_2 affinity have not been clearly elucidated in mutated hemoglobin molecules.

Due to a lower P_{50}, O_2 off-loading is likely compromised in those with high Hb-O_2 affinity. Evidence for compromised O_2 off-loading may be seen through compensatory increases in hematocrit resulting in a higher O_2 carrying capacity per unit of blood (Charache et al., 1966; Mangin, 2017; Shepherd et al., 2019). It is thought that the kidneys sense a reduction of O_2 off-loading and promote red blood cell production in response, functioning as a "critmeter" (Donnelly, 2001). In addition to an elevated hematocrit humans with high Hb-O_2 affinity likely develop skeletal muscle adaptations to compromised O_2 off-loading such as a greater percentage of non-oxidative (type II) muscle fibers than their counterparts with normal Hb-O_2 affinity (Wranne et al., 1983). Additionally, a greater accumulation of metabolic byproducts (e.g., lactate and H^+) during high-intensity exercise have been reported in humans with high Hb-O_2 affinity compared to those with normal Hb-O_2 affinity (Länsimies et al., 1985; Dominelli et al., 2020). Those with high Hb-O_2 affinity demonstrate a similar lactate accumulation at the end of exhaustive exercise during both normoxia and hypoxia, whereas controls demonstrate a reduced lactate accumulation during hypoxia compared to normoxia (Dominelli et al., 2020). A possible explanation for these observations may be that humans with high Hb-O_2 affinity obtain similar power outputs in normoxia and hypoxia and therefore demonstrate a similar metabolite accumulation between the two conditions; whereas those with normal Hb-O_2 affinity have a reduced power output and lower lactate concentrations during hypoxia compared to normoxia.

The observed differences in skeletal muscle fiber composition and utilization of metabolic pathways supporting exercise between humans with high Hb-O_2 affinity and humans with normal Hb-O_2 affinity may be due to differences in O_2 off-loading kinetics and tissue P_{O_2} (Wranne et al., 1983). In general, many physiological compensatory responses coinciding with high Hb-O_2 affinity remain uncharacterized. Key areas for future investigation include adaptations to high Hb-O_2 affinity possibly affecting capillary density, blood flow distribution, and skeletal muscle aerobic capacity (Dempsey, 2020).

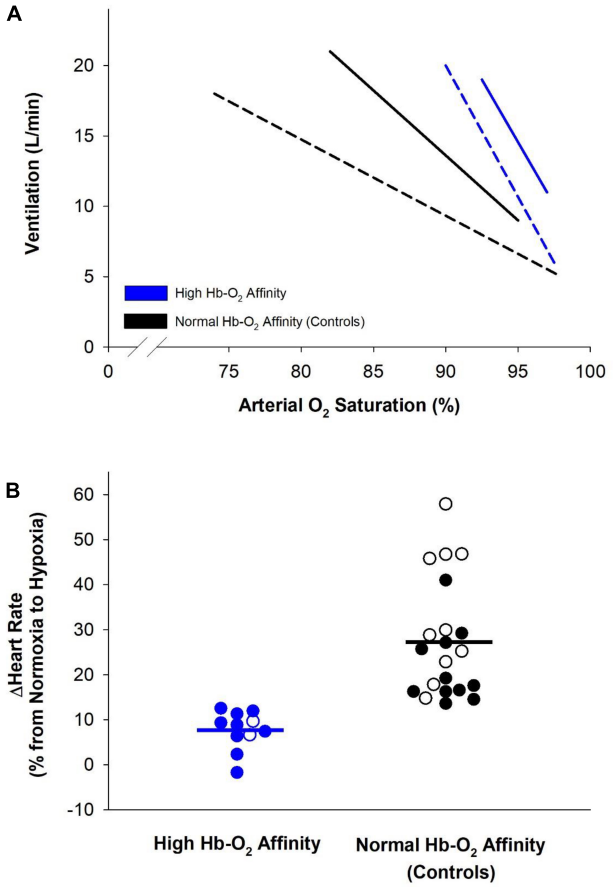

FIGURE 2 | Cardiorespiratory adjustments elicited during hypoxia by humans with high hemoglobin-O_2 (Hb-O_2) affinity (blue lines and symbols) and controls with normal Hb-O_2 affinity (black lines and symbols). **(A)** Relationship of minute ventilation and arterial O_2 saturation among humans with high Hb-O_2 affinity compared to normal Hb-O_2 affinity controls during progressive isocapnic hypoxia. Dashed lines represent data from Hebbel et al. (1977) where hypoxia was increased such that alveolar P_{O_2} was lowered from 120 to 40 mmHg over ~5 min (n = 2 humans with high Hb-O_2 affinity and n = 2 humans with normal Hb-O_2 affinity). Solid lines represent data from Dominelli et al. (2019) where hypoxia was increased such that end-tidal P_{O_2} was lowered from normal room-air values to 50 mmHg over ~12 min (n = 9 humans with high Hb-O_2 affinity and n = 12 humans with normal Hb-O_2 affinity). **(B)** Percentage increase in heart rate during progression of normoxia to hypoxia among humans with high Hb-O_2 affinity compared to normal Hb-O_2 affinity controls. Open symbols represent data from Hebbel et al. (1977) where heart rate was compared at an alveolar P_{O_2} of 100 and 40 mmHg (n = 2 humans with high Hb-O_2 affinity and n = 10 humans with normal Hb-O_2 affinity). Filled symbols represent data from Dominelli et al. (2019) where heart rate was compared at normoxia and at an end-tidal P_{O_2} of 50 mmHg (n = 9 humans with high Hb-O_2 affinity and n = 12 humans with normal Hb-O_2 affinity). Solid bars represent the average change in heart rate in both groups.

HIGH HEMOGLOBIN-OXYGEN AFFINITY AND CARDIORESPIRATORY ADJUSTMENTS DURING HYPOXIA AT REST

Acute Hypoxia

Brief periods of hypoxia require both cardiovascular and respiratory adjustments to maintain adequate O_2 delivery (Rowell and Blackmon, 1987; Bärtsch and Saltin, 2008; Naeije, 2010). One crucial immediate adjustment in response to hypoxia is increased ventilation which raises alveolar ventilation, increases arterial P_{O_2} and protects against arterial O_2 desaturation (Otis et al., 1956; Dempsey and Forster, 1982). At a given alveolar P_{O_2}, humans with high Hb-O_2 affinity have similar minute ventilation compared to humans with normal Hb-O_2 affinity (Hebbel et al., 1977; Rossoff et al., 1980; Dominelli et al., 2019). Yet, due to the left-shifted nature of their oxygen dissociation curve, those with high Hb-O_2 affinity have a higher arterial O_2 saturation at a given alveolar P_{O_2} (**Figure 2A**; Hebbel et al., 1977; Rossoff et al., 1980; Dominelli et al., 2019).

In addition to increased ventilation, hypoxia is associated with increased cardiac output, primarily through an elevated

heart rate (Brown and Grocott, 2013; Siebenmann and Lundby, 2015). As arterial O_2 saturation decreases during hypoxia, cardiac output increases and peripheral arterioles dilate to match O_2 delivery and demand (Ekblom et al., 1975; Phillips et al., 1988). These observations suggest that the change in heart rate during acute hypoxia is closely linked to systemic O_2 delivery (Casey and Joyner, 2011; Joyner and Casey, 2014; Siebenmann and Lundby, 2015). During acute hypoxia, humans with high Hb-O_2 affinity display a lesser increase in heart rate, and presumably cardiac output, likely due to better maintained arterial O_2 content (**Figure 2B**; Hebbel et al., 1977; Dominelli et al., 2019). Since arterial O_2 saturation remains fairly constant in humans with high Hb-O_2 affinity during modest reductions of P_{O_2}, as occurs at moderately high altitude, arterial O_2 content is better maintained and heart rate increases to a lesser extent compared to those with normal Hb-O_2 affinity.

Peripheral chemosensors located at both the carotid and aortic bodies respond to acute changes in arterial P_{O_2} and P_{CO_2}, such as during normobaric and hypobaric hypoxia (Lahiri and Forster, 2003). Stimulation of peripheral chemosensors during hypoxic exposure causes an increase in minute ventilation and sympathetic activity in an attempt to maintain O_2 homeostasis (Powell et al., 1998; Bernardi et al., 2001; Fletcher, 2001). Examination of humans with high Hb-O_2 affinity provides support for low P_{O_2} being a strong stimulus in the hypoxic ventilatory response, rather than arterial O_2 saturation or content (Hebbel et al., 1977; Rossoff et al., 1980; Dominelli et al., 2019). Some evidence suggests that aortic chemosensors sense changes in arterial O_2 content and heart rate is adjusted accordingly (Lugliani et al., 1971; Wasserman, 1978; Lahiri et al., 1980, 1981). Therefore, the lower heart rate during hypoxia among humans with high Hb-O_2 affinity compared to controls may be caused by decreased sensory stimulus of the aortic chemosensors (Dominelli et al., 2019). However, the mechanistic stimulation of the peripheral chemosensors requires that O_2 be dissociated from hemoglobin to be sensed (Lopez-Barneo et al., 2001). Therefore, the relationship between O_2 content and P_{O_2} sensed at the carotid chemosensors remains unclear and contention exists regarding mechanisms of O_2 sensing and regulation of systemic blood flow (Ward, 2008). Detailed discussions into the mechanism of O_2 sensing are provided elsewhere (Lopez-Barneo et al., 2001; Kumar and Prabhakar, 2012).

The observed relationship between ventilation and arterial O_2 saturation may present a disadvantage to humans with high Hb-O_2 affinity during acute hypoxic exposure. Since the stimulus for ventilation is closely linked to arterial P_{O_2} and not arterial O_2 saturation during brief periods of hypoxia (Biscoe, 1971; Guz, 1975; Weil and Zwillich, 1976), humans with high Hb-O_2 affinity have an excessive ventilatory response despite only a modest drop in arterial O_2 saturation and delivery (**Figure 2A**). Excessive ventilation increases O_2 consumption by respiratory muscles (Cherniack, 1959; Robertson et al., 1977). Although accounting for a small percentage of total O_2 consumption during rest, respiratory muscle O_2 demand increases during hyperventilation or exercise (Aaron et al., 1992; Coast et al., 1993; Dominelli et al., 2015). Thus, during exercise, there are increased and competitive demands for O_2 in metabolically active tissue including both exercising muscle and respiratory muscle (Harms et al., 2000; Sheel et al., 2001; Romer and Polkey, 2008; Dominelli et al., 2017). This competition for blood flow between respiratory and exercising muscle limits exercise tolerance at higher and extreme altitudes and is often referred to as "respiratory steal" (Pugh et al., 1964; Schoene, 2001; Helfer et al., 2016). The physiological consequences of "respiratory steal" are likely exacerbated at more extreme altitudes as hyperventilation, and thus metabolic demand of respiratory muscle, becomes more pronounced. Therefore, the excessive hyperventilation during acute hypoxic exposure may be disadvantageous for humans with high Hb-O_2 affinity due to increased O_2 consumption by respiratory muscles with minimal improvement in arterial O_2 saturation.

Chronic Hypoxia

In addition to acute hypoxic exposure, the benefits of high Hb-O_2 affinity have been observed through examination of cardiorespiratory adjustments during 10-days of residing at high altitude (Leadville, Colorado, ∼3100 m elevation) (Hebbel et al., 1978). Two humans with high Hb-O_2 affinity and two of their siblings with normal Hb-O_2 affinity were examined during the acclimatization period. Changes in arterial 2,3-DPG concentration and pH were similar during the stay at high altitude in both sets of siblings. However, peak and average heart rate during acclimatization were lower in the siblings with high Hb-O_2 affinity. During hypoxia, impaired O_2 delivery to the kidneys prompts erythropoietin production (Donnelly, 2001; Nangaku and Eckardt, 2007; Haase, 2013). Erythropoietin stimulates red blood cell production and leads to a subsequent increase in O_2 carrying capacity to compensate for impaired O_2 delivery (Erslev, 1991; Jelkmann, 2011). Humans with high Hb-O_2 affinity showed smaller increases in erythropoietin production when residing at high altitude (Hebbel et al., 1978). A lesser erythropoietin production during high-altitude acclimatization suggests that O_2 delivery is better preserved among humans with high Hb-O_2 affinity. Similarly, Hall et al. (1936) showed that mammals native to high altitude display a reduced erythropoietic response during travel from low altitude to high altitude. Combined, these findings suggest that lessened cardiovascular adjustments are needed to maintain adequate O_2 delivery during high-altitude acclimatization in humans with high Hb-O_2 affinity compared to those with normal Hb-O_2 affinity.

Marked physiological compensations are required to maintain homeostasis during sojourn to extreme altitudes (West, 2006). Hb-O_2 affinity increases at altitudes greater than ∼5400 m due to severe respiratory alkalosis with insufficient renal compensations (see section "Hemoglobin-Oxygen Affinity"). During ascent to the summit of Mt. Everest, ∼8100 m, climbers had a reduction in P_{50} from ∼26 mmHg to less than ∼20 mmHg (West, 1984). A more recent study examining blood oxygenation of four climbers reported arterial saturations ranging from 34 to 70% at the summit of Everest (Grocott et al., 2009). Without an increase of Hb-O_2 affinity due to respiratory alkalosis it is likely that humans would not be able to reach the summit without supplemental O_2.

As extreme altitude challenges the ability to transport O_2 from atmospheric air to tissue, the modulation of Hb-O_2 affinity is crucial to maintain adequate O_2 consumption. Enhanced O_2 loading in the lungs due to high Hb-O_2 affinity is even more advantageous at extreme altitude than at high altitude, where ambient P_{O_2} can fall to as low as 40 mmHg, outweighing potential limitations in O_2 off-loading (Eaton et al., 1974). The ventilatory response during hypoxia is similar between humans with genetic mutations leading to high Hb-O_2 affinity and those with normal Hb-O_2 affinity (Hebbel et al., 1977; Rossoff et al., 1980; Dominelli et al., 2019). Under the circumstances of extreme altitude, humans with high Hb-O_2 affinity may develop respiratory alkalosis to a similar degree as observed in humans with normal Hb-O_2 affinity (West, 1984; Grocott et al., 2009). In addition, some genetic hemoglobin mutations demonstrate a preserved Bohr effect, such that Hb-O_2 affinity would decrease during respiratory alkalosis by a similar magnitude compared to non-mutated hemoglobin (Adamson et al., 1969; Boyer et al., 1972; Nute et al., 1974; Wranne et al., 1983; Berlin et al., 2009). A physiological consequence of respiratory alkalosis would be further left-shifted O_2 dissociation curve adding additional protection against arterial desaturation. Therefore, humans with genetic modifications resulting in high Hb-O_2 affinity and a preserved Bohr effect may ascend to extreme altitudes with fewer physiological complications (i.e., Acute mountain sickness, high-altitude cerebral edema, and impaired cognitive function) compared to sojourners with normal Hb-O_2 affinity. However, to our knowledge no humans with genetic high Hb-O_2 affinity have been examined at altitudes greater than ∼3100 m and the proposed physiologic responses to higher and extreme altitudes are theoretical.

Groups of indigenous humans who have resided at high altitude for many generations display genotypic and phenotypic adaptations to the hypoxic environment (Beall, 2007, 2014; Moore, 2017; Tymko et al., 2019; Storz, 2021). Recent evidence has suggested an adaptive increase of Hb-O_2 affinity among high altitude natives of the Qinghai-Tibetan Plateau (>3500 m) compared to sea-level residents (Simonson et al., 2014; Li et al., 2018). However, others have reported that some high-altitude populations [Nepalese (>3800 m), Peruvian (>4500 m), and Qinghai-Tibetan (>3500 m) natives] do not show this adaptive increase in Hb-O_2 affinity (Samaja et al., 1979; Winslow et al., 1981; Tashi et al., 2014). Additional studies may improve understanding of changes in Hb-O_2 affinity observed among high-altitude natives and molecular mechanisms underlying such adaptation.

The Qinghai-Tibetan natives had a P_{50} ∼2 mmHg lower than the sea-level residents (24.5 vs. 26.2 mmHg, respectively) (Simonson et al., 2014). However, the high-altitude natives did not display improvements in pulmonary gas exchange or peak exercise capacity during hypoxia compared to the sea-level residents, suggesting no clear benefit of high Hb-O_2 affinity in the population examined. These findings, contradictory to those observed in humans with genetic mutations resulting in high Hb-O_2 affinity, could be explained by differences in the magnitude of P_{50}. The high-altitude natives studied had a P_{50} of ∼25 mmHg, in contrast to values ranging from 12 to 17 mmHg observed in humans with genetic mutations resulting in high Hb-O_2 affinity (**Table 1**). Therefore, the P_{50} observed in the high-altitude native population is probably not low enough to warrant significant alterations in pulmonary gas exchange, O_2 extraction, and exercise capacity during hypoxia. In addition, adaptations of high-altitude populations, which affect multiple steps within the O_2 transport cascade (Beall, 2007), may confound our ability to clearly dissociate the role of increased Hb-O_2 affinity in humans native to high altitude.

HIGH HEMOGLOBIN-OXYGEN AFFINITY AND CARDIORESPIRATORY ADJUSTMENTS DURING EXERCISE

Maximal Oxygen Consumption During Normoxia

Studies examining the effects of pharmacologically induced high Hb-O_2 affinity on O_2 consumption during normoxia have provided discordant results in both humans and animals (Riggs et al., 1973; Woodson et al., 1973; Wranne et al., 1974; Valeri et al., 1975; Yhap et al., 1975; Bakker et al., 1976; Malmberg et al., 1979; Ross and Hlastala, 1981; Woodson and Auerbach, 1982; Stewart et al., 2020, 2021). Recently, Stewart et al. (2021) showed that pharmaceutical induction of high Hb-O_2 affinity (only ∼2 mmHg decrease in P_{50}) using voxelotor reduced normoxic maximal O_2 consumption ($\dot{V}O_{2max}$) in humans. The decrement in normoxic $\dot{V}O_{2max}$ observed by Stewart et al. (2021) could be due to both an increase in Hb-O_2 affinity and a reduced Bohr effect: the transient reduction of Hb-O_2 affinity with decreasing pH (Pochron et al., 2019). A reduced Bohr effect in exercising muscle would further compromise O_2 off-loading, particularly during periods of high metabolic demand (Mairbäurl, 2013). Conversely, some mathematical models suggest that normoxic $\dot{V}O_{2max}$ is relatively insensitive to modest increases in Hb-O_2 affinity despite limitations in O_2 off-loading (Wagner, 1997; Shepherd et al., 2019).

In corroboration with results found through mathematical modeling, humans with high Hb-O_2 affinity show no difference in normoxic $\dot{V}O_{2max}$ values compared to similar age, sex-matched controls with normal Hb-O_2 affinity (Länsimies et al., 1985; Dominelli et al., 2020). However, there is evidence for altered metabolic processes among humans with high Hb-O_2 affinity compared to controls during exercise testing. During cycling exercise in normoxia, humans with high Hb-O_2 affinity may have greater reliance on anaerobic metabolism during heavy to maximal exercise, as evidenced by lower blood pH and pronounced lactate production compared to controls (Wranne et al., 1983; Länsimies et al., 1985; Dominelli et al., 2020). In addition, humans with high Hb-O_2 affinity seem to display a worsened exercise efficiency during cycling, i.e., higher O_2 consumption for a given power output (Dominelli et al., 2020). Theoretically, compromised O_2 off-loading due to high Hb-O_2 affinity may give rise to the greater reliance on anaerobic metabolism, which contributes to the worsened exercise efficiency observed (Dominelli et al., 2020). In brief,

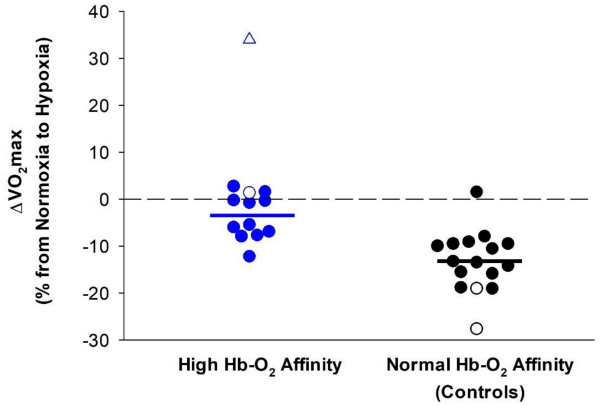

FIGURE 3 | Difference in $\dot{V}O_{2max}$ between normoxia and hypoxia in humans with high Hb-O_2 affinity (blue symbols) (–4 ± 5% without outlier) compared to normal Hb-O_2 affinity controls (black symbols) (–13 ± 6%). Open symbols represent data from Hebbel et al. (1978). The open triangle represents an outlier not included in the calculation of the mean. Closed symbols represent data from Dominelli et al. (2020). Solid bars represent the average change in $\dot{V}O_{2max}$ in both groups not including the outlier with high Hb-O_2 affinity. The dashed line provides a reference for no change.

current evidence indicates that humans with high Hb-O_2 affinity have similar normoxic $\dot{V}O_{2max}$ values despite altered metabolic processes during high-intensity exercise.

Little is known about the relationship between high Hb-O_2 affinity and compensatory mechanisms that facilitate adequate O_2 extraction. Wranne et al. (1983) demonstrated that the arterial-venous O_2 extraction was abnormally low during exercise in humans with high Hb-O_2 affinity, suggesting that O_2 off-loading may be compromised within muscle during whole-body exercise. However, humans with high Hb-O_2 affinity had a ∼25% greater O_2 carrying capacity than those with normal Hb-O_2 affinity, likely compensating for the diminished arterial-venous O_2 extraction both at rest and during exercise. The potential benefits of high Hb-O_2 affinity are likely contingent on the capacity to extract O_2 from blood (Wearing et al., 2021). The capacity of O_2 off-loading and diffusion to the mitochondria are crucial to maximize O_2 utilization in cases of high Hb-O_2 affinity, especially during peak whole-body exercise. Therefore, future research should focus on the relationship between high Hb-O_2 affinity and compensatory mechanisms which facilitate adequate O_2 extraction within peripheral tissue such as alterations in the microvascular architecture, flow of the red blood cells through the microvasculature, and the diffusion gradients driving O_2 to the mitochondria.

Maximal Oxygen Consumption During Hypoxia

Maximal O_2 consumption in humans decreases with increasing severity of hypoxia (Faulkner et al., 1968; Grover, 1970; Lawler et al., 1988; Ferretti et al., 1997; Wehrlin and Hallén, 2006; Wagner, 2010; West, 2010). However, humans with high Hb-O_2 affinity are better able to maintain $\dot{V}O_{2max}$ during hypoxia compared to those with normal Hb-O_2 affinity (**Figure 3**). As previously described, Hebbel and colleagues examined four siblings, two with high Hb-O_2 affinity and two with normal Hb-O_2 affinity, during 10 days of high-altitude acclimatization (Leadville, Colorado, ∼3100 m elevation). At high altitude $\dot{V}O_{2max}$ decreased by ∼28 and 19% compared to sea-level values in the two siblings with normal Hb-O_2 affinity (Hebbel et al., 1978). On the other hand, the two siblings with high Hb-O_2 affinity did *not* demonstrate a reduction in $\dot{V}O_{2max}$ at high altitude compared to low altitude (Hebbel et al., 1978).

Similarly, experiments using acute normobaric hypoxia showed that humans with high Hb-O_2 affinity had better maintained $\dot{V}O_{2max}$ during hypoxia compared to humans with normal Hb-O_2 affinity (Dominelli et al., 2020). In addition, peak power output during cycling exercise was better preserved in those with high Hb-O_2 affinity (Dominelli et al., 2020). At both high altitude and normobaric hypoxia, there was no difference in maximal heart rate during exercise in humans with high Hb-O_2 affinity compared to those with normal Hb-O_2 affinity (Hebbel et al., 1978; Dominelli et al., 2020). Previous studies indicate that an increase in blood viscosity associated with an elevated hematocrit, common in humans with chronic high Hb-O_2 affinity, may limit blood flow and maximal cardiac output in humans (Richardson and Guyton, 1959; Schumacker et al., 1985; Çınar et al., 1999). On the contrary, some studies suggest that systemic blood flow at rest and during exercise within animals is not reduced at a hematocrit of ∼50–60% (Gaehtgens et al., 1979; Schumacker et al., 1985; Lindenfeld et al., 2005). However, hematocrits greater than 60% likely result in a substantially elevated blood viscosity such that systemic blood flow is restricted (Weisse et al., 1964; Gaehtgens et al., 1979; Schumacker et al., 1985). Therefore, it is unclear whether cardiac output and systemic blood flow is limited among humans with high Hb-O_2 affinity where hematocrit often ranges from ∼55 to 65%.

The reduction of $\dot{V}O_{2max}$ during hypoxia is directly related to the degree of arterial desaturation (Hughes et al., 1968; Calbet et al., 2003a). As such, a higher arterial O_2 saturation in humans with high Hb-O_2 affinity for a given level of hypoxia likely contributes to the preservation of hypoxic $\dot{V}O_{2max}$ (**Figure 3**). In humans with normal Hb-O_2 affinity at high altitude, hypoxic $\dot{V}O_{2max}$ is less than values measured at sea-level and hypoxic $\dot{V}O_{2max}$ either remains the same or progressively increases during acclimatization (Saltin et al., 1968; Calbet et al., 2003b). Despite acclimatization, hypoxic $\dot{V}O_{2max}$ does not reach values previously measured at sea-level (Calbet et al., 2003b). In contrast, humans with high Hb-O_2 affinity have a better maintained hypoxic $\dot{V}O_{2max}$ upon transition to high altitude, but it is unknown how humans with high Hb-O_2 affinity may acclimatize to high altitude and subsequent effects on hypoxic $\dot{V}O_{2max}$.

CONCLUSION

High Hb-O_2 affinity has been identified as a potentially advantageous adaptation to high altitude in several animal species. From a cardiorespiratory perspective, we suggest that high Hb-O_2 affinity is advantageous for humans when exposed

to hypoxic environments both at rest and during exercise. During hypoxia, humans with high Hb-O_2 affinity exhibit lessened increases in heart rate, reduced erythropoietin production, and higher arterial O_2 saturation at rest compared to those with normal Hb-O_2 affinity. In addition, $\dot{V}O_{2max}$ and work capacity are better maintained during hypoxia compared to normoxia in humans with high Hb-O_2 affinity. The advantages associated with high Hb-O_2 affinity are likely potentiated as the degree of hypoxia becomes more severe. In addition, high Hb-O_2 affinity confers physiological disadvantages at less severe magnitudes of hypoxia such as reduced O_2 off-loading and unwarranted hyperventilation when arterial O_2 saturation is fairly well-preserved. However, current understanding on the effects of high Hb-O_2 affinity during hypoxia is largely limited to normobaric hypoxia. Future research warrants the investigation into the influence of high Hb-O_2 affinity during both short- and long-term periods of high-altitude acclimatization. In addition, long-term adaptations to pharmaceutically induced high Hb-O_2 affinity in humans remains largely unexamined. Regardless, the influence of high Hb-O_2 affinity on cardiorespiratory adjustments to environmental hypoxia is of key interest in human adaptation to environmental hypoxia, particularly during bouts of exercise.

AUTHOR CONTRIBUTIONS

MJ and CW conceived the concept for this review. KW, JS, and CW drafted the manuscript. PD, SB, JS, SK, and MJ provided critical revision of the manuscript for important intellectual content. All authors approved the final version of the manuscript.

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Long-Term Hypoxia Negatively Influences Ca^{2+} Signaling in Basilar Arterial Myocytes of Fetal and Adult Sheep

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Cerebral arterial vasoreactivity is vital to the regulation of cerebral blood flow. Depolarization of arterial myocytes elicits whole-cell Ca^{2+} oscillations as well as subcellular Ca^{2+} sparks due to activation of ryanodine receptors on the sarcoplasmic reticulum. Previous evidence illustrates that contraction of cerebral arteries from sheep and underlying Ca^{2+} signaling pathways are modified by age and that long-term hypoxia (LTH) causes aberrations in Ca^{2+} signaling pathways and downstream effectors impacting vasoregulation. We hypothesize that age and LTH affect the influence of membrane depolarization on whole-cell intracellular Ca^{2+} oscillations and sub-cellular Ca^{2+} spark activity in cerebral arteries. To test this hypothesis, we examined Ca^{2+} oscillatory and spark activities using confocal fluorescence imaging techniques of Fluo-4 loaded basilar arterial myocytes of low- and high-altitude term fetal (~145 days of gestation) and adult sheep, where high-altitude pregnant and non-pregnant sheep were placed at 3,801 m for >100 days. Ca^{2+} oscillations and sparks were recorded using an *in situ* preparation evaluated in the absence or presence of 30 mM K^+ (30K) to depolarize myocytes. Myocytes from adult animals tended to have a lower basal rate of whole-cell Ca^{2+} oscillatory activity and 30K increased the activity within cells. LTH decreased the ability of myocytes to respond to depolarization independent of age. These observations illustrate that both altitude and age play a role in affecting whole-cell and localized Ca^{2+} signaling, which are important to arterial vasoreactivity and cerebral blood flow.

Keywords: arterial myocytes, calcium oscillations in living cells, calcium sparks, ontogeny, high altitude

INTRODUCTION

In an age where cardiovascular health is so heavily scrutinized, we still struggle to reduce deaths from cardiovascular diseases. With the identification of modifiable risk factors such as smoking, diet, exercise, and obesity, we are now able to help prevent a significant portion of the morbidity and mortality associated with cardiovascular disease; however, environmental exposures that we have little or no control over are important to cardiovascular development and health

(Yang et al., 2017). What is more, previous research illustrates that many cardiovascular diseases have origins in fetal development and that gestational stressors have complex effects and impacts on vascular health (Ducsay et al., 2018).

Gestational long-term hypoxia (LTH) from living at high altitude or placental insufficiency of various etiologies has a marked effect on fetal cerebral vascular development that can have repercussions throughout an individual's lifetime (Ducsay et al., 2018). Decreasing oxygenation during the fetal period plays a significant role in vascular development, affecting vessel structure and function. Low oxygen exposures cause significant changes in the regulation of cellular phenotype, which influences vasoreactivity (Adeoye et al., 2015; Ducsay et al., 2018). Cerebral vascular dysfunction can result in an increased risk of cerebral edema, hemorrhage, and ischemic stroke. Furthermore, there are correlations between brain blood flow, developmental neuropathies, and impaired learning in children as well as psychological disorders such as clinical depression (Koenigs and Grafman, 2009) and dementia (Roher et al., 2012). These interrelationships illustrate the importance of considering the role of dysregulated vessel structure and function in neurological disorders.

Traveling to high altitude has significant effects on cerebrovascular blood flow (CBF). Cerebral arteries dilate in response to acute hypoxia, allowing for distribution of a greater portion of cardiac output to the brain in an attempt to maintain oxygen delivery despite reduced oxygen tensions (Hunter et al., 2003; Lee et al., 2009; Giussani, 2016). However, the reduced cerebral arterial contractility impairs the autoregulation of CBF (Tweed et al., 1983, 1986) and increases the risk of hemorrhage of the germinal matrix leading to varying brain disorders including epilepsy, cerebral palsy, and intellectual disability (Volpe, 1997; Nelson, 2003; Ferriero, 2004; Stoll et al., 2010). Vasodilation of cerebral vessels in response to acute hypoxia is partially regulated by an increase in large conductance potassium (BK_{Ca}) channel activation, which is responsible for membrane hyperpolarization and consequent vasorelaxation (Gebremedhin et al., 1994; Brenner et al., 2000; Ledoux et al., 2006; Thorpe et al., 2017). Long-term hypoxia, in comparison to acute hypoxia, can lead to compensatory responses that reduce CBF toward control levels. Studies done on adult animals as well as humans show that a large component of this compensation is due to increased ventilation and hematocrit, both of which serve to maintain cerebral O_2 delivery (Brugniaux et al., 2007). Prior to these adaptations and acclimatization, there is a correlation between hypoxia and increased basilar blood flow due to increases in arterial diameter (Jansen et al., 2002; Ainslie and Subudhi, 2014). Studies in fetal animals have shown that gestational LTH leads to a sustained increase in the proportion of cardiac output serving the brain (Ducsay et al., 2018). However, there is an impaired ability to increase cerebral blood flow in response to superimposed acute hypoxia (Pena et al., 2007), suggestive of altered cerebral vasoactivity. This is consistent with evidence that chronic hypoxia alters many of the pathways that regulate calcium signaling, and thus vascular tone, in fetal cerebral arteries (Pearce, 2006).

The current series of studies were designed to interrogate the effects of animal age and long-term hypoxia on calcium signals in basilar arterial myocytes under resting conditions and in response to membrane depolarization. We hypothesized that long-term hypoxia due to high altitude exposure would alter calcium signaling in ways that may restrict BK_{Ca} channel activation. We theorized that in arteries from low altitude fetuses, myocyte depolarization would increase whole-cell Ca^{2+} oscillations and localized rapid calcium signals (Ca^{2+} sparks) but that LTH would blunt these responses. We further hypothesized that postnatal development would magnify the increases in whole-cell and localized Ca^{2+} responses to membrane depolarization. This was examined by interrogating Ca^{2+} signals in basilar cerebral arterial myocytes of fetal and adult sheep housed at low- or high-altitude.

MATERIALS AND METHODS

Experimental Animals

Surgical and experimental procedures were performed in accordance with the regulations of the Animal Welfare Act, the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and "The Guiding Principles in the Care and Use of Animals" approved by the Council of the American Physiological Society and by the Institutional Animal Care and Use Committee of Loma Linda University. Pregnant (n = 7) and non-pregnant ewes (n = 7) of a mixed Western breed were divided into low altitude (normoxic) and high-altitude long-term hypoxic (LTH) groups. All ewes were obtained from Nebeker Ranch in Lancaster, CA, United States at an elevation near sea level, 720 m. Normoxic control ewes were maintained near sea level at 720 m for the duration of their gestation. Animals for the LTH groups were held at Nebeker Ranch under normoxic conditions until 30 days gestation at which time the pregnant and non-pregnant ewes were transported to the Barcroft Laboratory, White Mountain Research Station in Bishop, CA, United States at an elevation of 3,801 m. Previous work shows that residing at the Barcroft laboratory results in a maternal arterial PO_2 of 60 ± 3 Torr and a fetal arterial PO_2 of 19 ± 2 Torr (Kamitomo et al., 1993). Animals were held at elevation for the remaining ∼110 days of gestation for pregnant ewes and acclimatization for the non-pregnant ewes. Following this acclimatization period, ewes were transported (∼6 h drive) to Loma Linda University (LLU) for study at an elevation of 346 m. Once at LLU, LTH ewes were surgically instrumented with arterial and tracheal catheters. Based on frequent arterial blood gas sampling and adjustment of the rate of N_2 flow through the tracheal catheter, arterial PO_2 level in the adult sheep was maintained at ∼60 Torr for 2–4 days, mimicking the conditions of the effects of high altitude until the day of study (Kamitomo et al., 1993). After induction with thiopental sodium (10 mg/kg iv), the ewes were intubated and anesthesia was maintained via inhalation of 2–3% isoflurane in O_2 for the duration of the surgery. The fetuses were delivered via hysterectomy at a male to female ratio of ∼1:1. Following delivery, fetal sheep were euthanized with an overdose

of Euthasol (pentobarbital sodium, 100 mg/kg) and phenytoin sodium (10 mg/kg).

Artery Isolation

Non-pregnant females and mixed sex, near term fetal brains were removed and placed in iced Balanced Salt Solution (BSS) of the following composition (mM): 126 NaCl; 5 KCl; 10 HEPES; 1 $MgCl_2$; 2 $CaCl_2$; 10 glucose; pH 7.4 (adjusted with NaOH). Basilar arteries then were quickly dissected under normoxic conditions in BSS. Basilar arteries were selected from the same anatomical locations in both fetal and adult sheep to maintain segments of similar function and embryological origin. Because of this, there was a significant difference in diameter between fetal and adult arteries (∼200 μm vs. 300 μm, respectively) as described previously (Lin et al., 2003; Tao et al., 2015). All experiments were performed under normoxic conditions at room temperature (∼22–24°C).

Cytosolic Ca^{2+} Imaging

Intracellular Ca^{2+} of basilar arterial myocytes was measured *in situ* with a Ca^{2+} sensitive fluorescent dye (Fluo-4 AM, Cat No F14201, Invitrogen, Carlsbad, CA, United States) using a Zeiss 710 NLO laser scanning confocal imaging work station (Thornwood, NY, United States) with an inverted microscope (ZEISS Axio Observer), using procedures based on previous studies (Hadley et al., 2012; Harraz et al., 2014; Shen et al., 2018). Fluo-4 AM was dissolved in DMSO creating a 1 mM stock solution. Arteries were placed in BSS and exposed to a Fluo-4 concentration of 10 μM with 0.1% Pluronic F-127 Cat No P6867 (Invitrogen) from a 20% w/v stock solution in DMSO for 1 h in the dark at room temperature. These arterial segments were subsequently washed with BSS for 30 min to facilitate dye esterification and were then cut into linear strips for testing. These strips were pinned to Sylgard *en face* (Ellsworth Adhesives, Germantown, WI, United States) with fine insect dissecting pins and placed into an open bath imaging chamber (Warner Instruments, Hamden, CT, United States). Myocytes were illuminated at 488 nm via a Krypton-argon laser. Emitted light was captured with a photomultiplier tube with a band limited spectral grating of range 493–622 nm in both full frame and line scan imaging studies.

Whole-Cell Ca^{2+} Recordings

A time series of 300 full frame Fluo-4 fluorescence images of 512 × 512 pixels were made over 234 s (roughly 780 ms/frame). In order to make sure that the smooth muscle intracellular Ca^{2+} was recorded, the pinhole was set at an imaging depth of 5.4 μm, which is roughly the depth of an individual smooth muscle cell based on examination of both fixed and live preparations (Hadley et al., 2012; Shen et al., 2018). This thicker optical sectioning of samples was performed to mitigate the effects of sample ruffling, allowing for visualization of more myocytes than would otherwise be possible at other depths. The sample was focused just below the internal elastic lamina to center on the myocytes and avoid the significant autofluorescence of this layer when excited at 488 nm. Images were taken using 12-bit sampling and a water immersion 63X Plan Apochromat, 1.4 NA objective. Under basal conditions a time series was made to assess the Ca^{2+} oscillatory behavior of individual myocytes. After the video recording 30–50 line scans of 18.9 s were collected as detailed below, which were used to measure Ca^{2+} spark activity. Careful effort was made to not duplicate imaging regions for the line scans, which ensured proper sampling of individual cells in the arterial wall and reduced the potential for photobleaching and laser induced toxicity. Following basal recordings, BSS with 30 mM K (30K) through equimolar replacement of potassium for sodium was added to the tissues to depolarize the plasma membrane of the myocytes (Hadley et al., 2012; Harraz et al., 2014; Shen et al., 2018). 5 min later we performed another time series recording, followed by series of line scan recordings.

Whole-Cell Ca^{2+} Analysis

Regions of interest for full frame fluorescent imaging were automatically detected using the LCPro plug in for Fiji (ImageJ) (Francis et al., 2012; Schindelin et al., 2012), following 8 bit image conversion, image registration with StackReg using rigid body settings and cropping the images to 490 × 490 pixels to remove any erroneous pixels due to image registration (Thévenaz et al., 1998; Francis et al., 2012; Shen et al., 2018). LCPro was used to calculate fractional fluorescence intensity for automatically detected regions of interest, based on user defined region sizes, which were circles of 1.56 μm (six pixels). This region size was chosen as it provides analysis within individual cells as opposed to larger regions, which often overlap with adjacent cells.

The program performs analysis of the fluorescent intensity increases for each of the detected regions of interest above statistical noise, which was set to a threshold of $P < 0.05$. The program then analyzes the signal in the time and space domains for several parameters. In the current study, examinations were made of the following parameters: Amplitude (F/F_0), which is the maximum amplitude of the signal transient as defined by the global maximum of the F/F_0 curve within the time-period of the signal. Duration (s), which is the time interval defined by the period from one-half of the maximal amplitude values before and after the signal peak. Rise Time (s), which is written as "attack" in the program output and is the time interval defined by the period from one-half of the maximal signal amplitude value preceding the signal peak to the signal peak. Decay (s), which is the time interval defined by the time at the maximum amplitude to the time at one-half of the maximal amplitude following the peak. Area under curve (AUC) (F^*s/F_0), which is the discrete right-sided Riemann sum of the signal area during the period that is above one-half of the maximum signal amplitude (Francis et al., 2012, 2014, 2016; Shen et al., 2018).

Within the recordings from basilar arterial myocytes several different types of calcium events were observed that could be discriminated based on their temporal signaling characteristics, which were also observed in our previous studies performed in pulmonary arteries (Shen et al., 2018). These include rapid events that were between 0 and 4 s and that were scored as calcium sparks, medium duration oscillations that were defined as being between 4 and 40 s, and finally long duration oscillations that were defined both by being 40 + s and by their unique plateau at their peak fluorescence (Shen et al., 2018). Visual analysis of

each data record was used to clarify the divisions between groups and to remove false positives. The number of cells with Ca^{2+} oscillations were based on visual examination of myocytes in 1,002 μm^2 regions of interest with replicates performed in three separate regions per recording for each animal (Hadley et al., 2012; Shen et al., 2018).

Spatiotemporal Ca^{2+} Signaling Analysis

Spatiotemporal characteristics of the whole-cell Ca^{2+} oscillatory events were analyzed by performing a cross correlation analysis in time and space for the regions of interest detected by LCPro (Shen et al., 2018). The program is utilized to gain a deeper understanding of local and distant signaling networks within smooth muscle cells as well as to better determine the methods of Ca^{2+} trafficking between cells. This program combines a custom set of physiology analysis tools written within python that enable us to compare and observe correlative Ca^{2+} oscillations within a data set. A correlation coefficient of $r = 0.8$ was used for all data sets based on parameter space searches for correlating oscillations utilizing r values ranging from $r = 0.1–1.0$ (Shen et al., 2018). "Friends" were determined to be calcium transients that had a correlation coefficient greater than $r = 0.8$. "Neighbors" were defined as calcium transients occurring in nearby myocytes. A radius of 100 pixels (~23 μm) was chosen as the critical spatial cutoff because this distance allowed for the inclusion of most all neighboring cells.

Line Scan Ca^{2+} Recordings and Analysis

Fluo-4 AM loaded basilar arterial myocytes were recorded with a Zeiss LSM 710 NLO laser scanning confocal imaging workstation on an inverted platform (Zeiss Axio Observer Z1). After being loaded with Fluo-4, the arteries were prepared and imaged as detailed above. Line scan images were captured at 529 lines per second and recordings were made for a duration of 18.9 s. The lateral pixel size ranged from 0.0148 to 0.0911 μm per pixel and the pinhole was adjusted so that the cells were imaged to a depth of 2.5 μm, which is about 50% of the width of a myocyte based on prior morphological studies in live cells (Hadley et al., 2012; Shen et al., 2018).

Line scan recordings were analyzed for the percentage of cells with Ca^{2+} sparks, their frequency, amplitude, and other spatiotemporal characteristics via *SparkLab* 4.3.1 (Harraz et al., 2015; Shen et al., 2018). Before analysis, background fluorescence was subtracted from each recording assuming a homogenous level of background noise, and the fluorescence recordings normalized (Harraz et al., 2015; Shen et al., 2018). The threshold for spark detection was set to 2 standard deviations above this background level of fluorescence. 3–4 animals were used per group for both subcellular Ca^{2+} spark and whole-cell Ca^{2+} oscillation analysis. Refer to the figure legends for specific n values associated with each set of data.

Experimental Protocol

Fluo-4 loaded basilar arteries placed *en-face* in the imaging chamber were handled akin to our previous imaging studies of cerebral, pulmonary and uterine arteries (Hashad et al., 2017; Shen et al., 2018; Hu et al., 2020). Once a region of interest was identified in the control solution a video recording was made followed by line-scan images of individual myocytes in the arterial wall. These recordings required roughly 1 h. Upon completion of the video and line-scan recordings, the imaging chamber was removed from the microscope workstation and placed on the platform of a stereomicroscope. The control bathing solution was then gently removed with a pipette, and replaced with a 30 mM K bathing solution. To ensure that the bathing solution was fully exchanged the procedure was performed a total of three times. The tissues were then allowed to recover for a minimum of 5 min before resuming imaging to ensure that the cytosolic calcium reached a dynamic equilibrium.

Drugs and Chemical Reagents

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States).

Statistical Methods

Data analysis and the production of graphs was performed using GraphPad Prism 9.1.2 (La Jolla, CA, United States). The graphs present the data as means ± SD. Continuous variable data was tested for normality prior to analysis. These datasets were not normally distributed and as such they were evaluated using non-parametric statistical tests. A Kruskal–Wallis ANOVA with a Dunn's multiple comparisons test was performed on non-parametric datasets when making examinations between the experimental groups based on animal age, altitude, and treatment condition. The density of cell firing in video recordings was evaluated by drawing 3 boxes of a known size in each recording and counting the number of cells with events inside the box, with each cell only being counted once. For calcium spark analysis the numbers of cells were evaluated for those cells with or without events. The number of cells in videos or lines-scan recordings that had events was summarized. Statistical comparisons of this discrete data was made by performing contingency analysis between groups using Chi-Square analysis to evaluate potential changes in the incidence of Ca^{2+} oscillatory and spark events in whole-cell and line-scan recordings. A P-value of $P < 0.05$ was considered significant and were further broken down to $P < 0.01$ and $P < 0.001$ where appropriate.

The specific test performed for each data set are depicted in the figure legends. Sample sizes were determined by several different measurements including the number of animals studied, the number of regions of interest showing Ca^{2+} oscillations, the number of line scans examined for Ca^{2+} sparks, and number of Ca^{2+} events. The percentage of cells firing with Ca^{2+} sparks was determined by the number of line scans containing Ca^{2+} events through visual observation relative to the total number of line scans observed.

RESULTS

Whole-Cell Ca^{2+} Oscillations in Basilar Arterial Myocytes

The first series of studies were designed to test the hypotheses that membrane depolarization would increase Ca^{2+} oscillatory

activity and that long-term hypoxia (LTH) would impair oscillatory events in basilar arterial myocytes. **Figure 1A** shows a maximum intensity projection of Fluo-4 fluorescence in myocytes of the basilar arterial wall from a time series recording. A video corresponding to this image is provided in **Supplementary Material Video 1**. The image illustrates that the myocytes are spindle shaped and closely associated with one another and consistent with myocytes from other vascular beds and species that we have examined, including pulmonary and uterine arteries of sheep as well as cerebral arteries of rat and mesenteric arteries of mouse (Hadley et al., 2012; Harraz et al., 2014, 2015; Shen et al., 2018; Hu et al., 2020). **Figure 1B** shows the Fluo-4 fluorescence over time recorded from two regions of interest (ROI's) in individual myocytes that were automatically detected with *LCPro* (Francis et al., 2012; Shen et al., 2018), a custom analysis program and subsequently analyzed for spatial and temporal aspects to the Ca^{2+} signals. These "medium duration" events were the most common in the whole-cell Ca^{2+} recordings, with the oscillations spreading relatively uniformly through the myocytes and lasting between 4 and 40 s.

The recordings of Fluo-4 fluorescence (**Figure 2**) show that there were a variety of different Ca^{2+} oscillatory events in addition to the medium duration events described in **Figure 1**, which were akin to those we identified in pulmonary arterial myocytes (Shen et al., 2018). Results from these imaging studies are presented in **Figures 2–7**. **Figure 2** shows that some Ca^{2+} events were very rapid, and had features that were reminiscent of Ca^{2+} sparks in that they were localized in subcellular regions (**Figure 2A**a) and were fast, being roughly 1–2 s in duration (**Figure 2A**b), being at the edge of detectability with the full-frame recording techniques used for whole-cell Ca^{2+} recordings (Shen et al., 2018). The corresponding **Supplementary Video 2** recording for **Figure 2A**a illustrates that there are multiple types of Ca^{2+} events in the arterial myocytes. The inability to record rapid events with enough fidelity to perform analysis using full-frame recording techniques made at 1.28 Hz is exemplified in the exploded view of a single Ca^{2+} spark event. These rapid Ca^{2+} signaling events were scored as Ca^{2+} sparks, which are classically due to coordinated activation of ryanodine receptors on the sarcoplasmic endoplasmic reticulum (SER) that release Ca^{2+} into the cytosol (Jaggar et al., 1998a,b; Hadley et al., 2012; Hashad et al., 2017; Shen et al., 2018). These data also illustrate the need to use high-speed line-scan recordings to examine the Ca^{2+} spark events more thoroughly (Shen et al., 2018), results of which are presented in **Figures 8–10**.

Infrequently, we observed a third category of "long-duration" Ca^{2+} oscillations with large spatial spread through the myocytes (**Figure 2B**a) and that lasted over 40 s. **Figure 2B**b shows that these events had a unique quality in that they had rapid increases in cytosolic Ca^{2+} that plateaued and were sustained for an extended period followed by a prompt relaxation of the Ca^{2+} back to basal levels. The **Supplementary Material Video 3** provides the recording associated with **Figure 2B**.

For analysis purposes, the oscillations were broken into three discrete categories: short-duration Ca^{2+} sparks, which lasted less than 4 s, medium-duration Ca^{2+} oscillations of 4–40 s, and long-duration Ca^{2+} oscillations that lasted more than 40 s (Shen et al., 2018). Acutely, the subcellular Ca^{2+} spark events are likely to regulate the activity of voltage and calcium activated potassium channels that give rise to spontaneous transient outward currents and subsequent vasodilatory responses (Jaggar et al., 1998b; Hu et al., 2011, 2012; Hashad et al., 2017). The whole-cell Ca^{2+} oscillation events in comparison are likely responsible for causing arterial contraction as well as regulating a variety of other cellular processes including metabolism or transcription (Wilson et al., 2005; Goyal et al., 2011; Papamatheakis et al., 2011; Ureña et al., 2013; Tykocki et al., 2017).

The percentage of cells with medium-duration Ca^{2+} oscillation events were impacted by animal age and LTH, with activity being enhanced by treatment with 30 mM potassium (30K), data that is summarized in **Figure 3**. The potassium in the extracellular bathing solution was raised to 30 mM in order to depolarize the plasma membrane and increase activation of voltage gated calcium channels. Increased calcium influx across

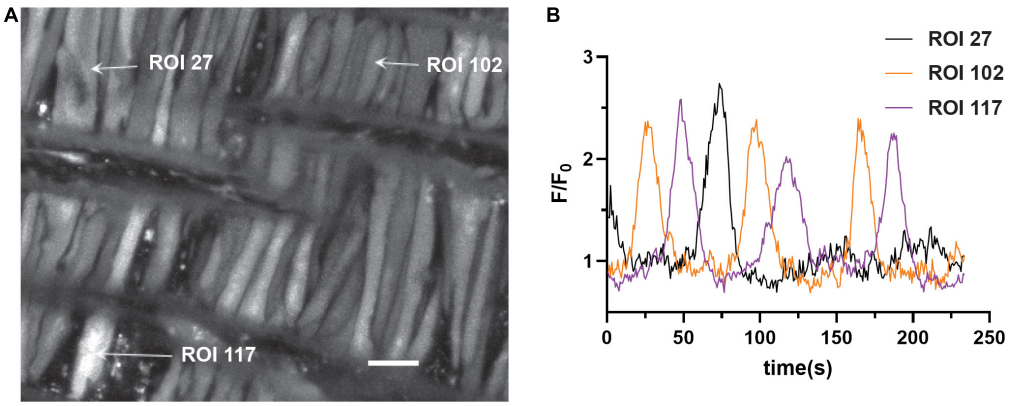

FIGURE 1 | Representative Ca^{2+} responses in basilar arterial myocytes from an adult sheep recorded *en face* under 30K conditions. **(A)** maximum intensity projection for Fluo-4 fluorescence of recorded cells using laser scanning confocal microscopy. Arrows point to regions of interest in individual myocytes shown in **(B)**; fluorescence intensity tracing showing spontaneous Ca^{2+} oscillations in two ROIs. Scale bar (white) = 10 μm.

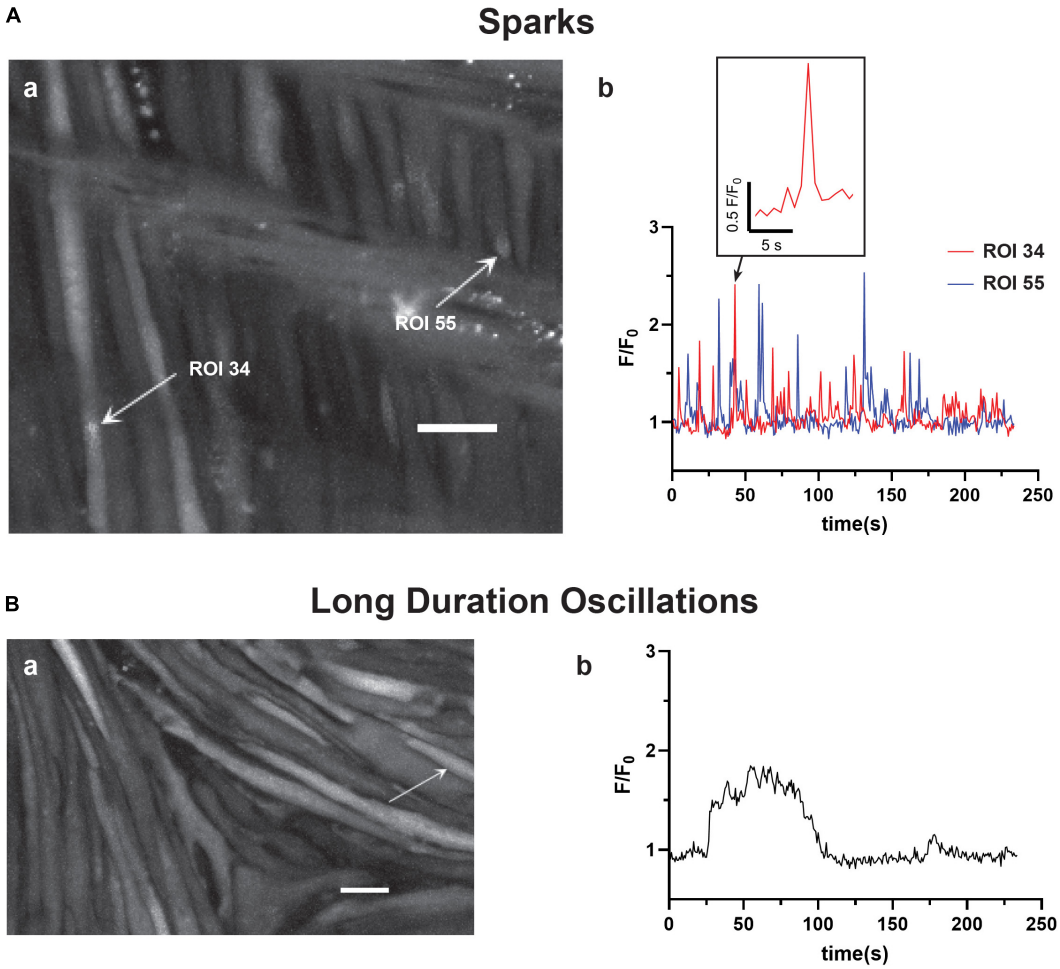

FIGURE 2 | Basilar arterial myocytes show additional distinct forms of Ca^{2+} signals. The figure shows (a) maximum intensity projections with arrows pointing to regions of interest that correspond to the fluorescence intensity tracings shown in (b). This includes **(A)** short duration Ca^{2+} "spark" events (0–4 s) and **(B)** long duration Ca^{2+} oscillations (40+s). The labeled time-series tracings of F/F_0 for these two types of Ca^{2+} responses were recorded using Fluo-4 and then detected and evaluated *post hoc* with LCPro. Scale bars (white) in (Aa and Ba) = 10 μm.

the plasma membrane leads to more calcium in the junctional space between the plasma membrane and SER. The elevated junctional calcium subsequently activates clusters of ryanodine receptors on the SER, which gives rise to subcellular events (Ca^{2+} sparks, see **Figures 8–10**) and when combined with other sources of calcium there are pronounced calcium oscillations that spread through the myocyte (Jaggar et al., 1998b; Janiak et al., 2001; Tykocki et al., 2017).

The percentage of cells with calcium events was similar in the fetal and adult groups and unaffected by LTH in the control bathing solution with low (5 mM) extracellular potassium. Membrane depolarization with 30K increased calcium oscillations in normoxic fetal and adult basilar arterial myocytes, an effect that was more pronounced in adult as compared to fetal sheep. Following long term hypoxia, however, membrane depolarization failed to increase cellular calcium oscillations in both fetal and adult basilar arterial preparations.

The kinetics and magnitude of the medium-duration Ca^{2+} oscillations were then quantified, with summarized data shown in **Figure 4**. Overall, animal age, LTH, and membrane depolarization had only mild effects on the Ca^{2+} oscillations. **Figure 4A** shows that under basal conditions the area under the curve (AUC) of Ca^{2+} oscillations were unaffected by maturation. However, in adult normoxic myocytes the AUC was slightly increased relative to that in the adult hypoxic period. Membrane depolarization caused a modest increase in the AUC in adult hypoxic myocytes. The AUC was unaffected by membrane depolarization in fetal normoxic and hypoxic groups; however, the AUC was lower in myocytes from fetal hypoxic and adult normoxic relative to fetal normoxic animals following membrane depolarization. The amplitudes of the Ca^{2+} responses are shown in **Figure 4B**. Event amplitudes recorded from myocytes of the adult hypoxic and fetal normoxic groups were reduced relative to those from normoxic adults under basal conditions. Membrane depolarization increased Ca^{2+} event amplitude in

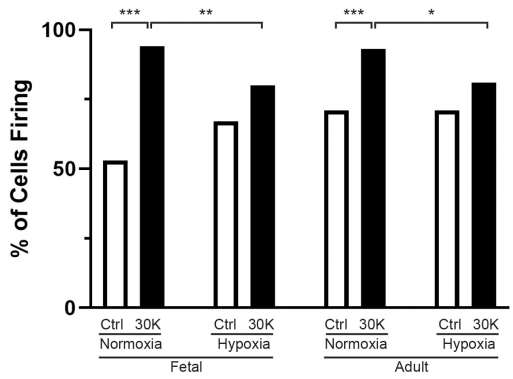

FIGURE 3 | Long-term hypoxia reduces the percentage of basilar arterial myocytes with depolarization mediated Ca^{2+} oscillations in fetal and adult sheep. Each bar represents the percentage of cells with Ca^{2+} oscillations under control (clear) or with treatment of 30K (black) based on an examination of myocytes in 1,002 μm^2 regions of interest. Replicates were performed in 3 separate regions per recording for fetal normoxic (3 animals along with 79 control and 71 30K myocytes), fetal hypoxic (4 animals with 91 control and 74 30K myocytes), adult normoxic (3 animals with 69 control and 68 30K myocytes), and adult hypoxic (4 animals with 61 control and 68 30K myocytes) animals. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate significance based on a chi-square test.

the fetal normoxic and adult hypoxic groups. Event amplitude was also elevated in adult hypoxic relative to adult normoxic myocytes following membrane depolarization. The duration of the Ca^{2+} events is shown in **Figure 4C** and again there were only modest differences between the groups. There was no impact of animal age on event duration under basal conditions, however, LTH caused a shortening of the oscillatory duration in adult myocytes. Membrane depolarization had no direct influence on the oscillatory duration in any of the four groups, though fetal normoxic myocytes had longer durations as compared to events from fetal hypoxic and adult normoxic myocytes. **Figure 4D** shows that the Ca^{2+} rise time during the oscillations was unaffected by LTH or animal age under basal conditions. Membrane depolarization shortened the rise time modestly in only the fetal LTH group. Following membrane depolarization, the rise time was longer in fetal normoxic myocytes as compared to fetal hypoxic and adult normoxic myocytes. **Figure 4E** shows the decay time for the Ca^{2+} signal. Under basal conditions, LTH modestly shortened the time for the decay of the oscillatory signal in both fetal and adult myocytes. Membrane depolarization shortened the decay in myocytes from adult normoxic animals, which caused the decay duration to become equivalent with that from LTH adult animals. Following membrane depolarization, the time for Ca^{2+} decay was also shorter in LTH fetal myocytes relative to the normoxic counterparts.

Long-Duration Ca^{2+} Oscillations

Long-duration Ca^{2+} oscillations were significantly less common than medium duration oscillations or Ca^{2+} sparks as illustrated by the reduced number of events. The effects of animal age, LTH, and membrane depolarization on these events are presented in **Figure 5**. The most notable influences were observed in the event amplitudes. Adult normoxic animals under basal conditions had increased event amplitudes (**Figure 5B**), decreased rise times (**Figure 5D**), and increased decay times relative to normoxic fetuses (**Figure 5E**). **Figure 5B** also shows that long-term hypoxia increased event amplitude in fetal animals but decreased the amplitude in adults under control conditions. Membrane depolarization decreased event amplitude in fetal hypoxic and adult normoxic animals but increased the amplitude in adult hypoxic animals.

Spatiotemporal Ca^{2+} Signaling

The interactions of the Ca^{2+} oscillations among smooth muscle cells were then analyzed for temporal correlations between the Ca^{2+} transients, or "friendship," between oscillations at the various ROIs. The larger the correlation coefficient, the greater the level of event "friendship." Temporally related correlations between the Ca^{2+} events were then identified by those events that had a correlation coefficient of $r \geq 0.8$ (Shen et al., 2018). Results from one representative sample are provided in **Figure 6**. **Figure 6A** shows an image projection with overlaid regions of interest that have varied levels of correlations, while **Figure 6B** shows the average fluorescence (black line) along with the fluorescence intensity values for individual regions of interest that were correlated. Spatial correlations between events are shown in **Figure 7B**, a relationship characterized as "neighbors" as delineated by those ROIs that were within a 100 pixels ($\sim$23 μm) radius of any individual ROI, as delineated by the larger white circle on the image in **Figure 6B** (Shen et al., 2018). Animal age, LTH and membrane depolarization had diverse effects on the numbers of friends, with summary data provided in **Figure 7**. **Figure 7** shows that relative to fetuses there was a decrease in the number of friends in adult arterial myocytes under control conditions. LTH decreased the number of friends in fetal arterial myocytes but increased them in adults under control conditions. Membrane depolarization increased the number of friends in adult normoxic myocytes. As illustrated in **Figure 7B**, the number of neighbors was also influenced by animal age, LTH, and membrane depolarization. Under basal conditions, there was a decrease in the number of neighbors in adults relative to fetuses. Under basal conditions, LTH increased the number of neighbors in adults but this was unaffected in fetuses. Membrane depolarization increased the number of neighbors in normoxic fetuses and adults, but LTH blunted the responses in myocytes of both age groups. **Figure 7C** delineates the distance of temporally correlated events (friends). The distance between friends was shorter in normoxic adults relative to fetuses under control and depolarized conditions. Long-term hypoxia resulted in a shortening of the distance only in fetuses under control conditions. **Figure 7D** provides the percentage of friends who are neighbors, illustrating the spatial and temporal relationships between Ca^{2+} oscillations. Overall, a majority of temporally correlated oscillatory events (friends) are closely associated (neighbors), however, there were some subtle effects of animal age, LTH, and membrane depolarization. There was a slight increase in the percentage of adult normoxic myocyte ROIs who were neighbors relative to those in fetal normoxic myocytes. Although there were fewer Ca^{2+} oscillatory events in fetal

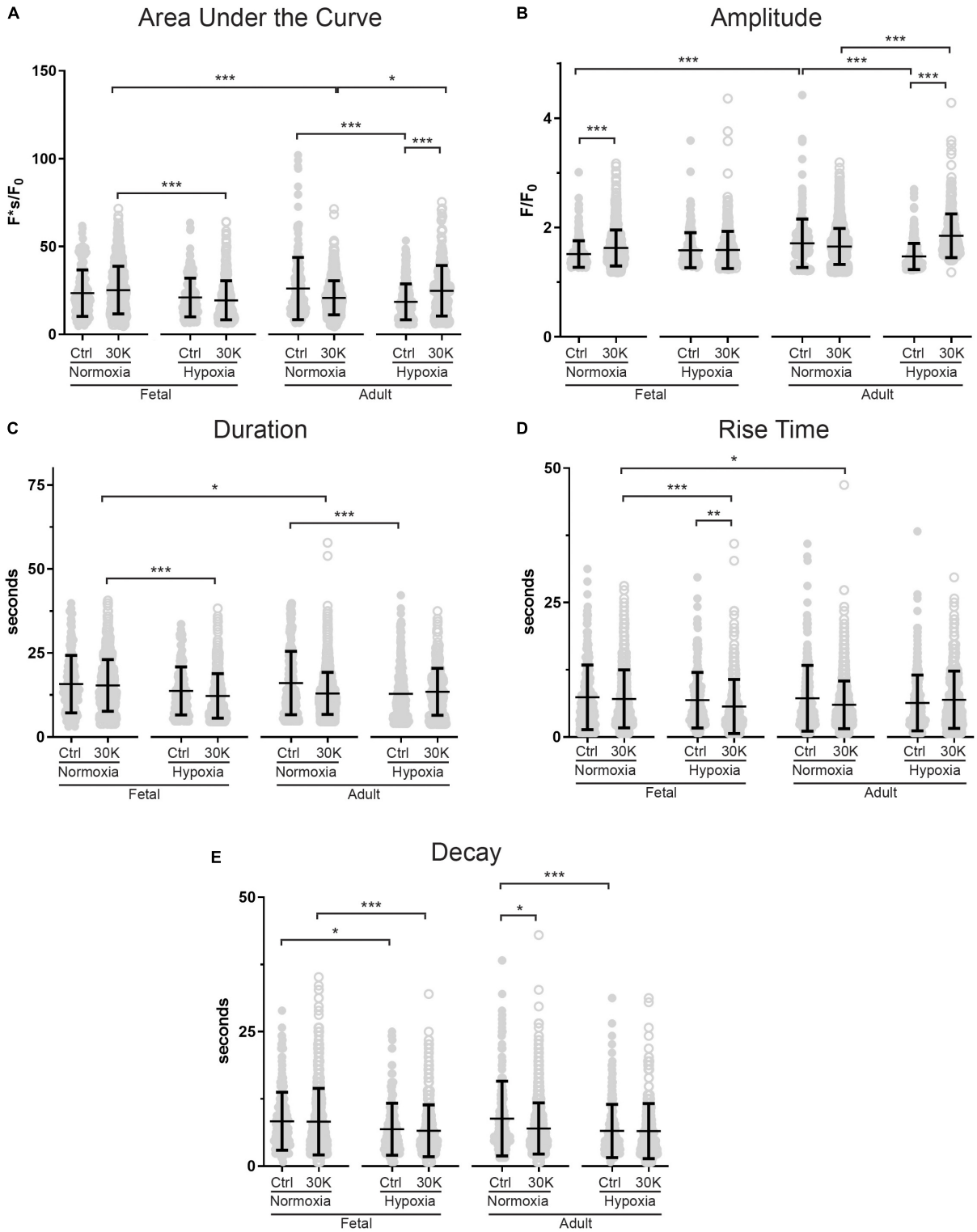

FIGURE 4 | Long-term hypoxia has minimal effect on the magnitude or temporal aspects of medium duration Ca^{2+} oscillations in fetal and adult sheep. Effects of membrane depolarization with 30 mM K, long-term hypoxia, and animal age on **(A)** area under the curve, **(B)** amplitude of the fractional fluorescence, **(C)** duration of the event, **(D)** rise time, and **(E)** decay time in cytosolic fractional fluorescence. Bars represent mean ± SD for each parameter; closed circles specify control conditions, and open circles treatment with 30K. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate significance based on a Kruskal–Wallis ANOVA with a Dunn's multiple comparisons test based on ranks. Control recordings were made in 207/3 FN, 201/4 FH, 225/3 AN, and 325/4 AH ROIs and animals, respectively. 30K recordings were made in 642/3 FN, 394/4 FH, 1204/3 AN, and 312/4 AH ROIs and animals, respectively.

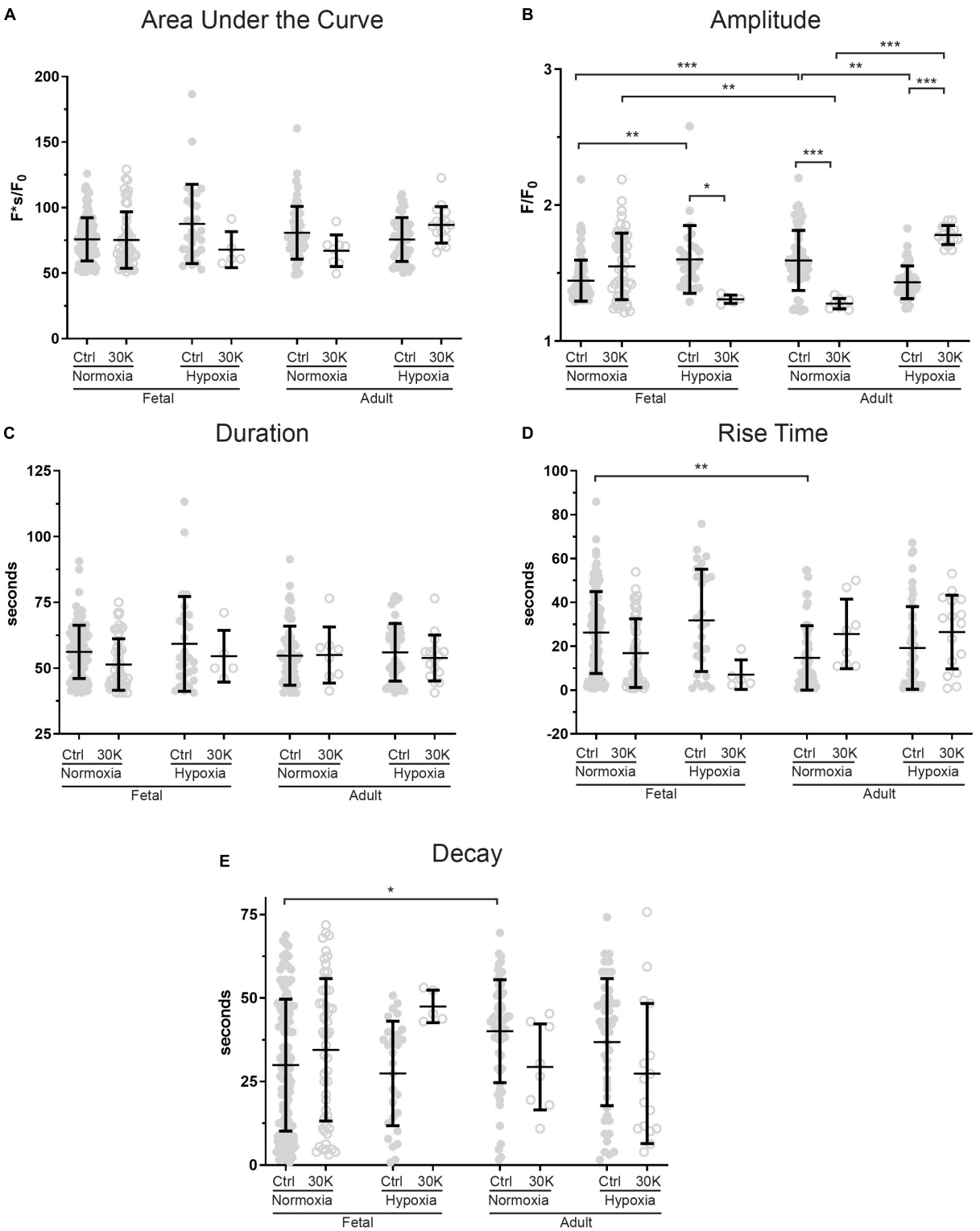

FIGURE 5 | Long-term hypoxia has minimal effect on the magnitude or temporal aspects of long duration Ca^{2+} oscillations in fetal and adult sheep. Effects of artificial membrane depolarization with 30 mM K, long-term hypoxia, and animal age on **(A)** area under the curve, **(B)** amplitude of the fractional fluorescence, **(C)** duration of the fluorescent event, **(D)** rise time for fractional fluorescence in the cytosol and **(E)** decay time for cytosolic fractional fluorescence. Bars represent mean ± SD for each parameter; closed circles specify control conditions, and open circles treatment with 30K. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate significance based on a Kruskal–Wallis ANOVA with a Dunn's multiple comparisons test based on ranks. Control recordings were made in 116/3 FN, 28/4 FH, 61/3 AN, and 48/4 AH ROIs and animals, respectively. 30K recordings were made in 52/3 FN, 5/4 FH, 8/3 AN, and 16/4 AH ROIs and animals, respectively.

hypoxic myocytes than their normoxic counterparts, a greater proportion were neighbors under control conditions. Membrane depolarization preferentially increased the percentage of friends who were neighbors in normoxic myocytes independent of animal age. **Figure 7E** presents the percentage of neighbors who were friends, which provides an index of the temporal relationship between Ca^{2+} oscillatory events that are spatially related. Unlike the tight spatial relationship between temporally related events, events that occur in nearby regions are not necessarily temporally correlated. Even still, there were influences of LTH and membrane depolarization. Long-term hypoxia preferentially reduced the percentage of neighbors who were friends in fetal myocytes under control conditions. Membrane depolarization preferentially reduced the percentage of neighbors who were friends in myocytes of fetal and adult normoxic animals.

Activation of Ca^{2+} Sparks

The last series of studies examined the impact of membrane depolarization and hypoxia on the activation of Ca^{2+} sparks in myocytes from fetuses and adults. **Figure 8** shows representative Ca^{2+} spark tracings from line scan recordings for control groups analyzed with *SparkLab* (**Figures 8A–D**; Shen et al., 2018). Our lab and others have shown that membrane depolarization with extracellular K^+ can enhance Ca^{2+} spark activity and vascular contractility, which is why we chose to depolarize the membrane with 30 mM K^+ (Jaggar et al., 1998a; Hadley et al., 2012; Papamatheakis et al., 2012; Harraz et al., 2014; Shen et al., 2018). Membrane depolarization with 30K increased the prevalence of myocytes with Ca^{2+} spark activity in all groups and the frequency of activation as shown in **Figures 9A,B**. Under control conditions the prevalence and frequency of Ca^{2+} sparks in myocytes from normoxic adults were greater than in the fetal period. LTH did not impact the prevalence or frequency of Ca^{2+} sparks in fetuses, but reduced spark activity in adult myocytes to fetal levels. Membrane depolarization increased Ca^{2+} spark activation in adult animals while LTH preferentially blunted spark activation in adults. Overall, these findings illustrate that LTH impairs spark activity in basilar arteries but support the general influence of membrane depolarization on Ca^{2+} spark activity that we have shown in our previous studies on pulmonary and uterine arteries from sheep as well as in middle cerebral as well as mesenteric arteries from rats and mice (Hadley et al., 2012; Harraz et al., 2014, 2015; Shen et al., 2018; Hu et al., 2019).

The magnitude and kinetics of Ca^{2+} sparks were quantified in basilar arterial myocytes with the summary results shown in **Figures 10A–D**. Overall, there was little impact of animal age, LTH, or membrane depolarization on the quality of the Ca^{2+} sparks. When comparing events in myocytes from fetuses and adults, the full width of half maximum was narrowed slightly in normoxic control conditions. Long-term hypoxia increased the full width of half maximum of Ca^{2+} sparks in adult myocytes under control conditions. Membrane depolarization modestly increased the full width of half maximum of Ca^{2+} sparks in adult normoxic myocytes and there was no impact on the exponential decay time constant (Tau) of the Ca^{2+} spark events.

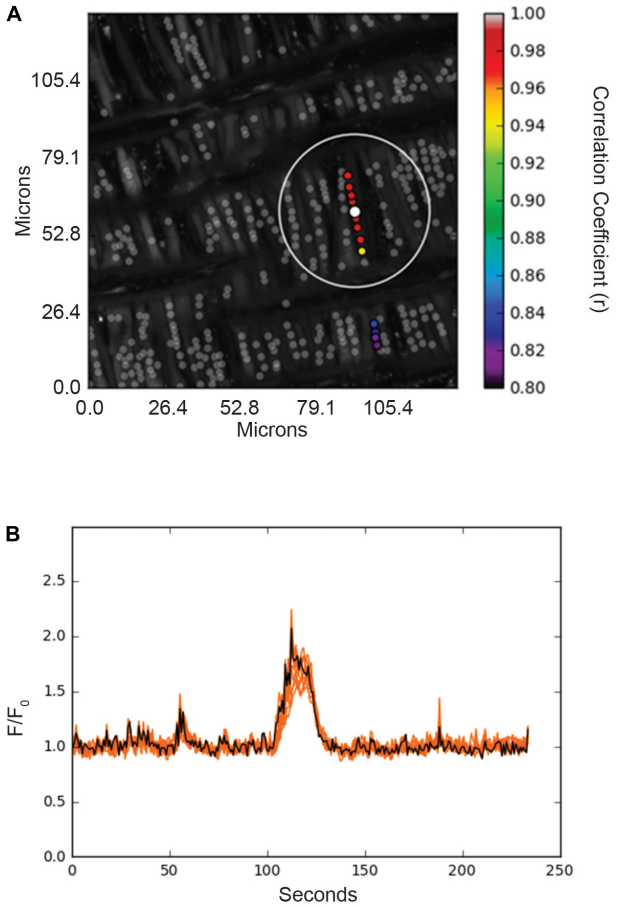

FIGURE 6 | Spatial and temporal correlations of Ca^{2+} responses in basilar arterial myocytes from an adult sheep recorded *en face* under basal conditions. **(A)** Maximum intensity projection for Fluo-4 fluorescence of recorded cells using laser scanning confocal microscopy. Highly correlated events are colored and plotted around the center (reference) region of interest (ROI, white dot). Gray dots show ROIs of spontaneous Ca^{2+} oscillations that showed less than an 80% temporal correlation with the white ROI. The large white open circle shows a ~23 μm boundary that was used for deriving spatial correlation measurements shown for the spatial and temporal analysis in **Figure 7**. **(B)** Fluorescence intensity tracing showing spontaneous Ca^{2+} oscillations, with the black line being the average tracing and the orange lines being the individual ROIs. ROIs were plotted using correlation coefficients with greater than 80% temporal correlation plotted surrounding the center ROI (white dot in **A**). Key shows the degree of correlation of each ROI relative to the reference ROI.

DISCUSSION

The current study was designed to examine the impact of animal age and high altitude induced LTH on cytosolic Ca^{2+} oscillations and sparks in basilar arterial myocytes. These studies were performed because of the integral role basilar arteries have in regulating vascular reactivity and providing blood flow to the brain stem, which is critical to autonomic cardiorespiratory functions that are impacted by LTH. Basilar arterial myocytes had robust Ca^{2+} oscillations and the number of event sites were increased by membrane depolarization. The data also illustrate

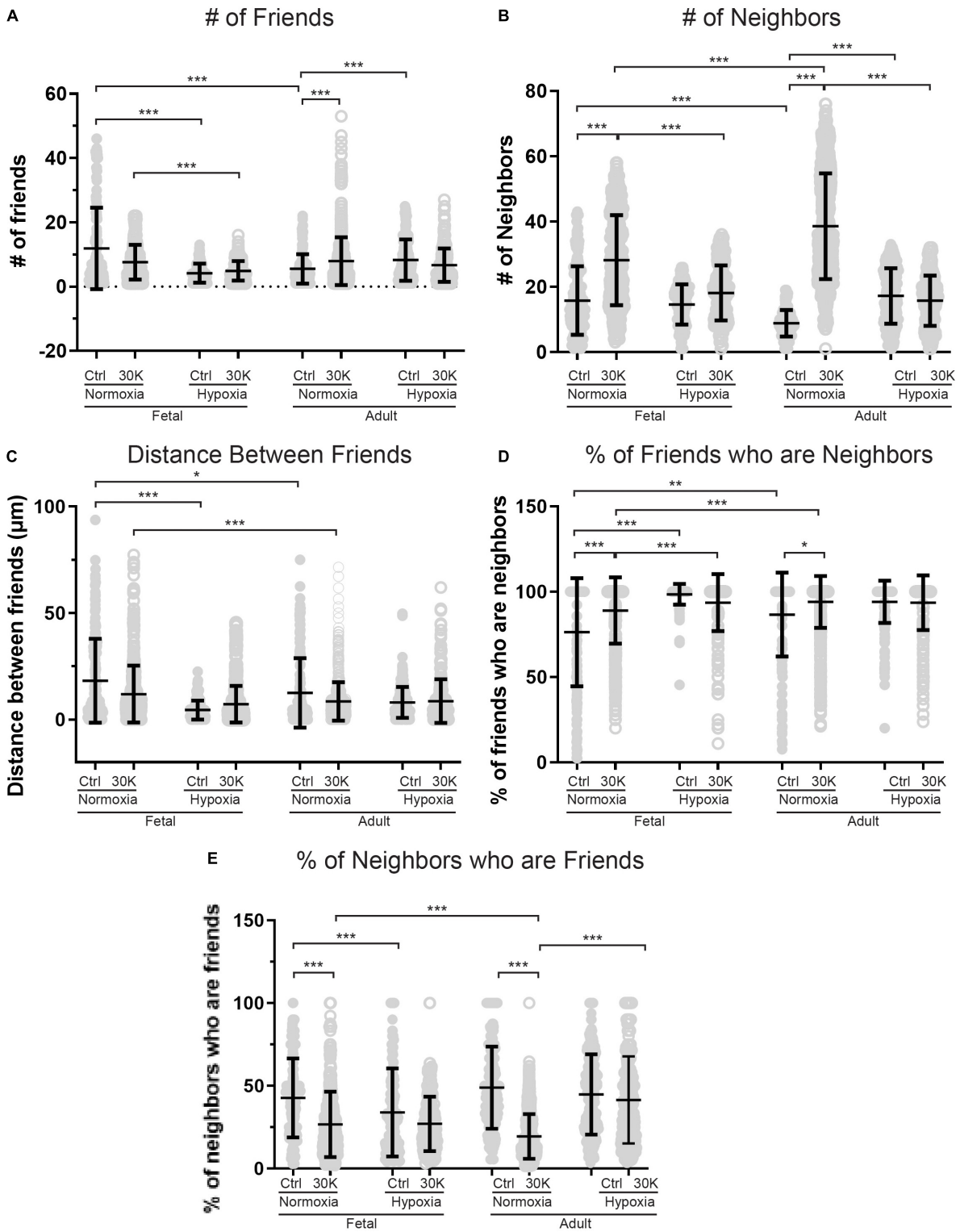

FIGURE 7 | Long-term hypoxia mitigates the influence of 30K mediated membrane depolarization on friends and neighbors. **(A–E)** Arteries were analyzed for number of correlated ROIs (friends), distance between correlated ROIs, percentage of nearby ROIs (neighbors) that were correlated, and percentage of correlated ROIs that were in nearby cells. Bars represent mean ± SD for each condition; closed circles specify control conditions, and open circles treatment with 30K. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate significance based on based on a Kruskal–Wallis ANOVA with a Dunn's multiple comparisons test based on ranks. Control responses were obtained from 178/3 AN, 235/4 AH, 175/3 FN, and 163/4 FH ROIs and animals, respectively. 30K responses were obtained from 844/3 AN, 244/4 AH, 537/3 FN, and 303/4 FH ROIs and animals, respectively.

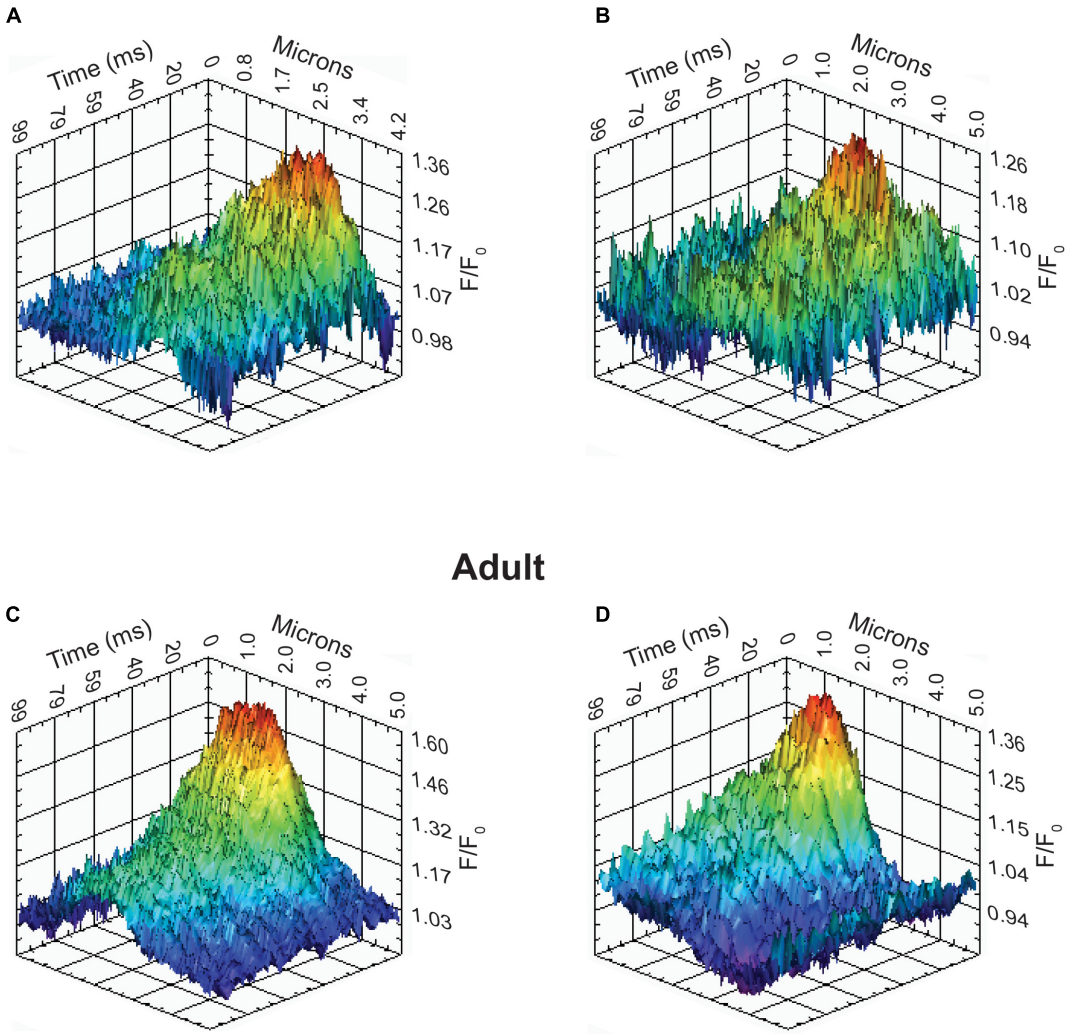

FIGURE 8 | Representative Ca^{2+} spark tracings for basilar arterial myocytes from fetal and adult sheep. **(A–D)** Fluo-4 fluorescence tracings recorded from basilar arterial myocyte line scans of fetal and adult sheep under normoxic and hypoxic conditions analyzed with *SparkLab 4.3.1*.

that animal age and LTH modify the coupling of membrane depolarization to the activation of whole-cell Ca^{2+} oscillations as well as the generation of localized Ca^{2+} sparks.

Whole-Cell Ca^{2+} Oscillations

Restriction of whole-cell intracellular Ca^{2+} oscillations by LTH is not unique to the cerebral vasculature. Previously, we reported that Ca^{2+} oscillatory activity was reduced by LTH in pulmonary arterial myocytes (Hadley et al., 2012; Shen et al., 2018). The parallel changes in Ca^{2+} signals in basilar and pulmonary arterial myocytes due to LTH suggest there may be commonalities in the mechanisms underlying the changes in oscillatory activity following LTH. Myocyte cytosolic Ca^{2+} oscillations are dependent on a functional SER (Mufti et al., 2010) and $InsP_3$ receptor activation is important to the generation of oscillatory signals (Adebiyi et al., 2010, 2011; Mufti et al., 2015; Tykocki et al., 2017). These relationships lead to the potential that losses in Ca^{2+} oscillatory activity and other temporal signaling aspects by LTH may be coupled to impairments in $InsP_3$ signaling. Indeed, previous work from our group shows that LTH reduces $InsP_3$ receptor expression in both fetal and adult middle cerebral arteries (Longo et al., 1996; Ueno et al., 1997). Depression in $InsP_3$ signaling may therefore also underlie the suppression in membrane depolarization induced Ca^{2+} oscillations we report here. With regards to the current findings that LTH restricts cellular Ca^{2+} responses, our previous data illustrate that the SER Ca^{2+} stores of sheep pulmonary arteries are largely intact regardless of age or hypoxic stress (Hadley et al., 2012), although there is significant SER stress as evidenced by structural changes in the SER of pulmonary arterial myocytes as

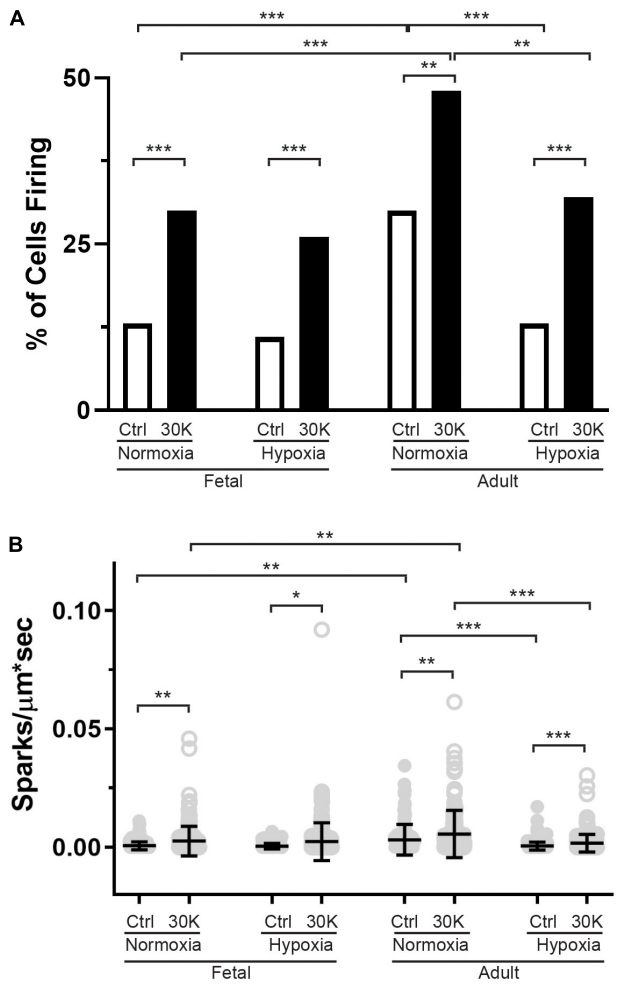

FIGURE 9 | The incidence of Ca^{2+} sparks activated by membrane depolarization is reduced in fetuses and by hypoxia in adults. **(A)** percentage of cells with or without Ca^{2+} sparks and **(B)** Ca^{2+} spark firing frequency. Bars represent mean ± SD for each condition. Clear bars and closed circles specify control conditions, while black bars and open circles treatment with 30 K. Data in A were analyzed by a chi-square test while data in **(B)** were analyzed by Kruskal–Wallis one-way ANOVA with Dunn's multiple comparison test based on ranks for each group $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Recordings were made in FN control (161/3) and 30K (172/3), FH control (171/4) and 30K (185/4), AN control (150/3) and 30K (155/3), and AH control (207/4) and 30K (217/4) lines (cells)/animals, respectively.

well as increases in markers of SER stress (Leslie et al., 2021). Such evidence suggests that the major aberrations in Ca^{2+} responses are more likely due to dysregulation of pathways critical to regulating Ca^{2+} signaling that may involve SER stress as opposed to fundamental disruption of the storage capabilities of the intracellular Ca^{2+} stores.

Membrane depolarization dependent increases in Ca^{2+} oscillatory activity was expected as this is common in smooth muscle (Iino, 1990; Hadley et al., 2012; Shen et al., 2018). Increases in oscillatory frequency with membrane depolarization may be the result of changes in the speed of Ca^{2+} release and uptake at the SER as well as increase Ca^{2+} flux across the plasma membrane (Keizer et al., 1995; Wilson et al., 2002, 2005; Goyal et al., 2009). The increases in the oscillatory amplitude of cells from fetal animals suggests that membrane depolarization may increase oscillatory signals either through increased activation of $InsP_3$ or ryanodine receptors on the SER or through enhanced recruitment and coupling to L-Type Ca^{2+} channels (Jaggar et al., 1998b; Blood et al., 2002; del Corsso et al., 2006; Ureña et al., 2013) or other Ca^{2+} permeable plasma membrane ion channels (Jaggar et al., 1998b; Janiak et al., 2001; Earley et al., 2005; Adebiyi et al., 2010, 2011; Dahan et al., 2012; Ureña et al., 2013; Harraz et al., 2014). In this regard, the picture that emerges is that the impact of LTH on basilar arterial Ca^{2+} signaling is complex and mediated through modification of multiple pathways important to the regulation of intracellular Ca^{2+}.

The finding that whole-cell Ca^{2+} oscillations were reduced in basilar arterial myocytes from normoxic fetuses compared to adults builds from our previous evidence of age-related changes in Ca^{2+} signaling of cerebral arteries (Ducsay et al., 2018). One possibility is that these differences are coupled to developmental related effects on the role of SER and Ca^{2+} influx pathways to contractility. Indeed, contractions of cerebral arteries from fetal sheep have reduced reliance on release of intracellular Ca^{2+} stores and increased dependence on Ca^{2+} influx as compared to adults (Long et al., 2000). These changes in arterial contractions were shown to be coupled to greater L-type Ca^{2+} channel protein expression in fetus and responsiveness to L-type Ca^{2+} channel activation (Long et al., 2000; Blood et al., 2002). Even still, Ca^{2+} oscillations are due to sequential filling and release of internal Ca^{2+} stores in smooth muscle, with modulation through extracellular Ca^{2+} influx pathways (Janiak et al., 2001; Wilson et al., 2005; Hume et al., 2009). Given that we did not fully assess the role of extracellular Ca^{2+} entry or release of intracellular Ca^{2+} stores to the oscillatory activity in basilar arterial myocytes, we still do not know what role age has to the process of Ca^{2+} handling.

The influence of membrane depolarization on the temporal and spatial aspects to the Ca^{2+} oscillations in normoxic animals are compelling. When the tissue was depolarized, there were significantly more events during the recordings in the arterial myocytes as demonstrated by the uptick in spatially related events (neighbors), however, there were fewer temporally related events (friends). On closer examination the neighboring events mainly occurred within individual cells as opposed to adjacent cells. While we have not explored this deeply in the current study, the findings suggest that when myocytes are depolarized the Ca^{2+} oscillations do not propagate from cell to cell, but rather, the number of events increase through recruitment of unrelated cells across the arterial wall. One explanation for this apparent lack of cell-to-cell communication is that membrane depolarization recruits additional L-Type Ca^{2+} channels on individual cells in a stochastic manner, which then increases Ca^{2+} responses in separate cells. Further, the data suggest gap junction connections between myocytes are not being activated, which limits cell-to-cell propagation of Ca^{2+} signals (Hald et al., 2014; Welsh et al., 2018; Zechariah et al., 2020). Such findings are not unfounded as physiological studies and computational models suggest there is poor electromechanical coupling between arterial myocytes

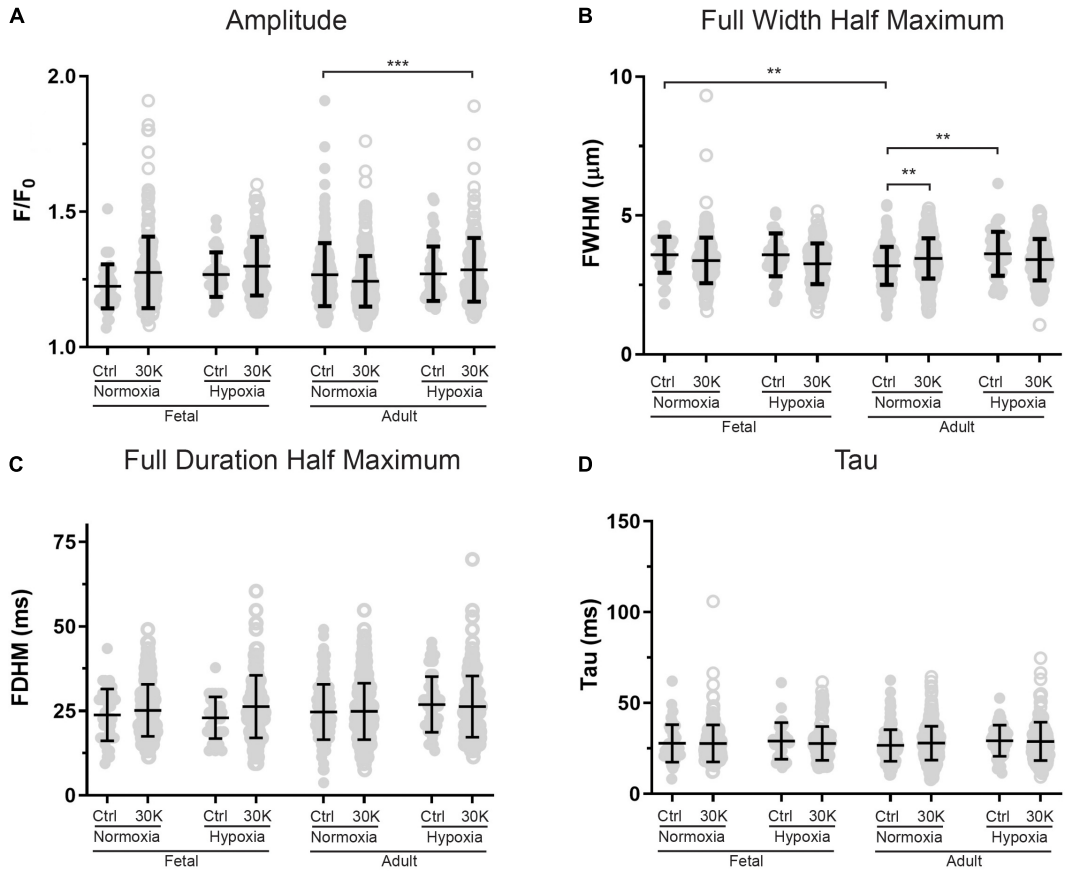

FIGURE 10 | Magnitude and kinetics of Ca^{2+} sparks were minimally influenced by animal age, LTH, or membrane depolarization. **(A–D)** amplitude, full width at half-maximum, full duration at half-maximum and tau exposed to control (closed circles) or 30 K (open circles) for Ca^{2+} spark events of arterial myocytes from adult and fetal sheep under normoxic and hypoxic conditions. Bars represent means ± SD for each parameter. Data were analyzed by a Kruskal–Wallis one-way ANOVA with Dunn's multiple comparison test based on ranks for each group $^{**}P < 0.01$, $^{***}P < 0.001$. Recordings were made in FN control (41/161/3) and 30K (199/172/3), FH control (30/171/4) and 30K (136/185/4), AN control (173/150/3) and 30K (365/155/3), and AH control (52/207/4) and 30K (177/217/4) Ca^{2+} sparks/lines/animals, respectively.

but propagation is spread more easily through the vascular endothelium (Hald et al., 2014; Tykocki et al., 2017; Welsh et al., 2018).

Ca^{2+} Spark Activity

Previous evidence illustrates that the localized rapid release of calcium, coined Ca^{2+} sparks, in cerebral vascular myocytes is due to coordinated activation of ryanodine receptor clusters (Jaggar et al., 1998a,b). These Ca^{2+} spark events are important as they are coupled to vasodilation through activation of large conductance potassium channels (BK_{Ca}). Depolarization of the plasma membrane is well regarded to enhance Ca^{2+} spark activity and the ensuing repolarization of the membrane and vasodilation acts as a negative feedback regulator of vasoreactivity. With regards to the effects of age and LTH, basilar arterial myocytes from adults had far greater Ca^{2+} spark activity relative to those from fetuses. Furthermore, LTH reduced spark activity in adults to fetal levels and suppressed the depolarization mediated increase in Ca^{2+} spark activity in adult myocytes. The inability of depolarization to increase Ca^{2+} spark activity in adults following LTH is interesting and reminiscent of the impact of LTH on fetal pulmonary arterial myocytes (Hadley et al., 2012; Shen et al., 2018). Much like the impact on whole-cell Ca^{2+} oscillations, this parallel effect of LTH on basilar and pulmonary arterial myocytes suggests there may be a common underlying mechanism whereby LTH suppresses depolarization induced activation of Ca^{2+} sparks. Presumably, this would be due to a loss in communication between plasma membrane channels and ryanodine receptors on the SER, possibly involving SER stress such as we have shown in fetal pulmonary arterial myocytes (Leslie et al., 2021). These losses in communication potentially include L-type Ca^{2+} channels but may also involve disruption in T-type Ca^{2+} channels or TRPV4, which are also important to Ca^{2+} spark activation in vascular myocytes (Earley et al., 2005; Harraz et al., 2014, 2015).

The impact of age and long-term hypoxia on the role of the Ca^{2+} spark events to feedback activation of BK_{Ca} is not fully resolved. Previous evidence from our group, however, illustrates that BK_{Ca} from fetal basilar arteries have increased activity in response to changes in the cytosolic Ca^{2+} (Lin et al., 2003)

mediated through enhanced protein kinase G (Lin et al., 2005, 2006). Further, LTH increases membrane expression of BK_{Ca} and Ca^{2+} dependent channel activity in both fetal and adult basilar arterial myocytes (Tao et al., 2015). Together these data suggest that even though fetal basilar myocytes have reduced Ca^{2+} spark activity relative to adults there may be preservation of BK_{Ca} channel activity and vasodilatory capacity through altered channel regulation. Similarly, vasodilation may be maintained following LTH in adults even though there is suppression in Ca^{2+} spark activity through enhanced Ca^{2+} dependent activation of BK_{Ca}. Unraveling the full impacts of age and LTH on the regulation of basilar arterial reactivity and the influence this has on brain blood flow will require further interrogation.

Perspectives

The impact of long-term hypoxia on cerebral vascular function remains poorly understood, especially with regards to the differing influences on fetuses and adults. The current data begin to shed light on the complexity of the effects of LTH on cellular Ca^{2+} signals depending on animal age that are important to vascular reactivity. The adaptations in Ca^{2+} signaling to LTH reported here are potentially juxtaposed by modifications in the regulation of BK_{Ca} channels, which are critical components in the feedback regulation of vascular reactivity and brain blood flow. These effects lead us to speculate that LTH-induced dysregulation in vascular function prompts compensatory responses that act to preserve brainstem nutrient and oxygen delivery.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Loma Linda University Health Institutional Animal Care and Use Committee. All study procedures adhered to the Animal Welfare Act, the National Institutes of Health Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf), "The Guiding Principles in the Care and Use of Animals" approved by the Council of the American Physiological Society.

AUTHOR CONTRIBUTIONS

SW, CW, AB, and LZ contributed to conception and design of the study. MR and NO performed laboratory experimentation. CW and JLP designed customized software for data analysis. SBC, MR, NO, and CR performed data and statistical analysis. CR wrote the first draft of the manuscript. SC wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.760176/full#supplementary-material

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An Erythropoietin-Independent Mechanism of Erythrocytic Precursor Proliferation Underlies Hypoxia Tolerance in Sea Nomads

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The Bajau Sea Nomads were recently demonstrated to have evolved larger spleens as an adaptation to millennia of a marine foraging lifestyle. The large-spleen phenotype appears to derive from increases in thyroid hormone (TH) production as a result of reduced expression of phosphodiesterase 10A (PDE10A), though the exact mechanism remains unknown. Through pharmacological inhibition of PDE10A using the selective inhibitor MP-10 in mice, we were able to mimic the Bajau adaptation and show that treated mice had significantly larger spleens than control animals. This difference appears connected to an excess of early stage erythrocytes and an apparent increase in red blood cell (RBC) precursor proliferation in response to increased TH. However, we determined that the stimulation of RBC production in the mouse model *via* TH is Erythropoietin (EPO)-independent, unlike in the altitude (chronic hypoxemia) response. We confirmed this using human GWAS data; although the Bajau PDE10A variants are significantly associated with increased TH levels and RBC count, they are not associated with EPO levels, nor are other strongly thyroid-associated SNPs. We therefore suggest that an EPO-independent mechanism of stimulating RBC precursor proliferation *via* TH upregulation underlies the increase in spleen size observed in Sea Nomad populations.

Keywords: hypoxia, red blood cell production, adaptation, evolution, spleen

INTRODUCTION

Thyroid hormones affect a wide variety of metabolic and physiological processes, and were recently linked to increased spleen size as an apparent adaptation to breath-hold diving (Ilardo et al., 2018). However, the spleen-thyroid relationship is not new; as early as 1,927 hyperthyroidism was reported in medical contexts to be associated with large spleen size (Baldridge and Peterson, 1927), and at one time splenomegaly was used as a diagnostic tool. The exact mechanisms by which thyroid hormones may affect spleen size are yet unknown. Here, we report an erythropoietin-independent mechanism of red blood cell (RBC) production as a consequence of thyroid hormone stimulation *via* the pharmacological inactivation of Phosphodiesterase 10A (PDE10A).

Phosphodiesterase 10A is a phosphodiesterase capable of hydrolyzing both cAMP and cGMP, thus regulating cyclic nucleotide signaling (Fujishige et al., 1999). It was recently discovered that PDE10A expression has been genetically reduced in a population called the Bajau as a consequence of natural selection acting on the ability to sustain repeated bouts of apneic diving (Ilardo et al., 2018). This reduction in expression has resulted in two apparent phenotypes, the first of which is increased thyroid hormone levels. Although thyroid hormones have not been directly measured in the Bajau population, a genome wide association scan (GWAS) for Thyroid Stimulating Hormone (TSH) levels (a proxy for thyroid hormones with high sensitivity), revealed a strong association between SNPs in PDE10A as well as in related phosphodiesterase PDE8B, which encodes a high affinity cAMP-specific phosphodiesterase (Arnaud-Lopez et al., 2008; Soto-Pedre et al., 2017). The second phenotype associated with reduced PDE10A expression is splenomegaly. Spleen size has been proposed to affect dive capacity owing to the spleen's role in the mammalian dive reflex, in which it contracts during diving in order to expel a bolus of oxygenated RBCs thus expanding available oxygen (Thornton et al., 2001). The large spleen phenotype observed in the Bajau has been proposed to enhance their dive capacity, though the mechanisms underlying the adaptations are unknown.

Here we use a pharmacological approach to inhibit PDE10A in mice in order to investigate the molecular mechanisms by which thyroid hormones are connected to an increase in spleen size in the Bajau. We utilized the highly selective PDE10A inhibitor MP-TRA 10 (MP-10), which has previously been shown to potentiate thermogenesis, suggesting a thermoregulatory role (Hankir et al., 2016). Through daily injections, we were able to mimic the genetic effect of the Bajau adaptation in mice. We subsequently observed a significant increase in spleen size compared to control animals, as well as a corresponding increase in RBC count originating in an excess proliferation of erythrocytic precursors in the spleen, which in mice, unlike in humans, is a site of erythropoiesis. Further, we demonstrate that this stimulation of proliferation is likely erythropoietin (EPO) independent, suggesting a direct role of thyroid hormones on erythrocytic precursor development. These data suggest the benefits of Bajau PDE10A adaptation are two-fold; first, the increased RBC count would increase oxygen capacity allowing for longer dives on a single breath, and secondly the corresponding increase in spleen size creates a larger reservoir in which the oxygenated red blood cells may be stored.

RESULTS

We chose to divide our pharmacological model into two studies conducted over different time courses; a long-term study with lower dosage to represent chronic, mild inhibition of PDE10A, and a short-term study with higher dosage to represent more acute inhibition. For both studies there is precedent in the literature (Hankir et al., 2016), and both provide different perspectives to inform us of the mechanisms of response to PDE10A inhibition. While the long-term study may more closely represent inhibition in humans with the Bajau variant in PDE10A, the short term study may more closely resemble a knockout model. For our long-term study, we injected 4-week old male mice daily *i.p.* with a low (10 mg/kg) dosage of MP-10 over a period of 9 weeks. We observed lower body weights in treated mice compared to control mice (**Figure 1**). These differences are consistent with previous observations in mouse studies that administered similar dosages of MP-10 (Hankir et al., 2016), and we believe are likely a consequence of increased basal metabolic rate (BMR) resulting from thyroid hormone stimulation. Following 0, 4, and 9 weeks of treatment, we measured spleen size *in vivo* using a small animal ultrasound. We found that after 4 and 9 weeks of treatment the treated mice had larger spleens *in vivo* than the control animals ($p = 0.0634$, and $p = 0.0374$, respectively), consistent with the large spleen phenotype observed in the Bajau (**Figure 1**; Ilardo et al., 2018). We repeated the experiment at a higher dosage (30 mg/kg) for a shorter time course of 1 week. Following 1 week of treatment, treated mice had gained significantly less weight than controls ($p = 0.0019$) (**Figure 2**). Through *ex vivo* spleen measurements of length and width, we determined that the treated mice again had significantly larger spleens than the control mice ($p = 0.0059$, **Figure 2**). However, the spleens of treated mice were also significantly less dense than those of the control animals ($p < 0.0001$, **Figure 2**), as calculated by a ratio of measured splenic area and splenic mass. Spleen sections were stained with H&E, and differences were clearly visible and appear to arise from increased distance between cells (**Figure 3**). We also observed that the treated mice had higher RBC count, hematocrit (Hct), and hemoglobin (Hgb) (**Figure 4**). While our hematological values were consistently low across all animals, they were within the normal range for young animals (Wozniak et al., 2021). These findings are consistent with the spleen becoming stretched, and thus less dense, suggesting that they may serve as a reservoir for a greater number of red blood cells. It is, however, difficult to say whether this increase is indeed due to a greater RBC mass because of the potentially confounding effect of changes in blood volume. Such a determination would require chromium labeling or CO rebreathing to accurately determine RBC mass. Using GWAS data from the UK Biobank (accessed using the Global Biobank Engine, Stanford, CA,[1] December 2019), we identified consistent results in humans of European ancestry. Humans carrying the Bajau variant at previously identified SNPs known to influence PDE10A expression were also found to have significantly higher RBC count than those carrying the ancestral variant (**Table 1**). We have not, however, confirmed this association in the Bajau population.

Through flow cytometry, we were able to attribute the difference in hematological measures to differences in erythrocytic precursor populations (**Figure 5**). We found a significant increase in proliferation at nearly every stage of cell proliferation (**Figure 5** and **Table 2** for a summary of *p*-values), however late stages (III and IV, representing late basophilic erythroblasts and orthochromatophillic erythroblasts, respectively) were also found to have a significant increase in

[1] https://biobankengine.stanford.edu/

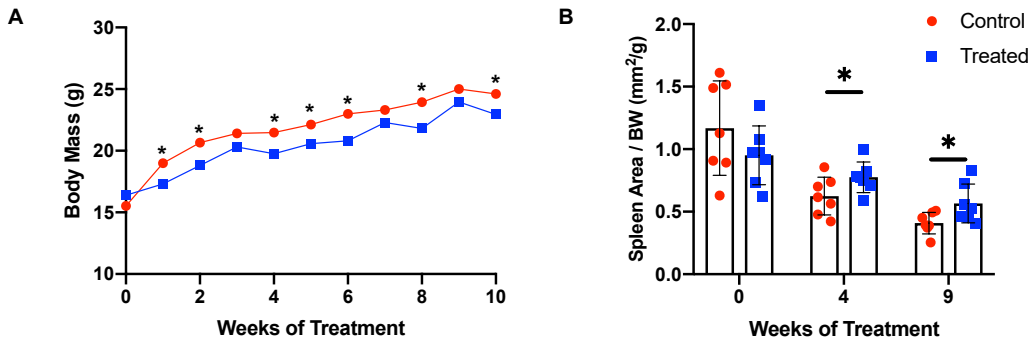

FIGURE 1 | MP-10-treated mice have lower body weights and larger spleens than control animals *in vivo*. **(A)** MP-10-treated (10 mg/kg) mice demonstrate consistently lower body weights than control mice, with a significantly lower change in body weight over the period ($p = 0.029$) ($n = 14$). **(B)** Mice receiving MP-10 (10 mg/kg) had larger spleen area as measured using a small animal ultrasound machine ($n = 14$). All comparisons were performed using an unpaired Student's *t*-test and are plotted with SD error bars. *$p < 0.05$.

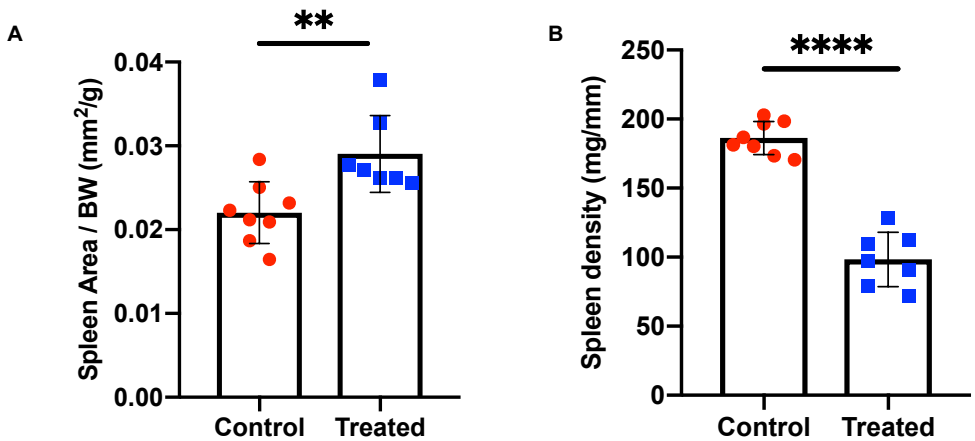

FIGURE 2 | Spleen area and density in control and MP-10 treated mice. **(A)** Mice treated with a short course of MP-10 (1 week, 30 mg/kg) displayed larger spleens ($p = 0.0038$) that were also **(B)** less dense ($p < 0.0001$) ($n = 15$). All comparisons were performed using an unpaired Student's *t*-test and are plotted with SD error bars. **$p \leq 0.01$ and ****$p \leq 0.0001$.

apoptosis, resulting in an overall lower proportional abundance of cells in these stages. This balancing effect may be mediated by thyroid hormones and serve to ensure a normal number of reticulocytes is released.

In order to investigate whether the underlying cause of increased RBC precursor proliferation could be thyroid hormone induced stimulation of erythropoietin (*Epo*), we used qPCR to measure *Epo* mRNA in kidney. We found no difference in *Epo* expression between control and MP-10 treated mice ($p = 0.48$). Using GWAS data from Prevention of REnal and Vascular ENd (PREVEND), we confirmed this result in humans of European ancestry. In spite of a clear, significant association with thyroid hormone levels previously reported in Ilardo et al. (2018), there is no association between the Bajau PDE10A SNPs and *Epo* levels (**Table 1**). We investigated additional SNPs in other genes known to be strongly and significantly associated with thyroid hormone levels in European populations, none of which were found to be associated with Epo levels (**Table 3**). We have yet to confirm these results in Bajau individuals.

DISCUSSION

Through a pharmacological approach in mice, we were able to simulate the Bajau large spleen adaptation to breath hold diving. We administered daily injections of the selective PDE10A inhibitor MP-10 daily *via ip* injection at low (to measure chronic inhibition) and high (to measure acute inhibition) dosages for 9 weeks and 1 week, respectively. Using a small-animal ultrasound, we determined that the treated mice had significantly larger spleens *in vivo* than the control animals, confirming the

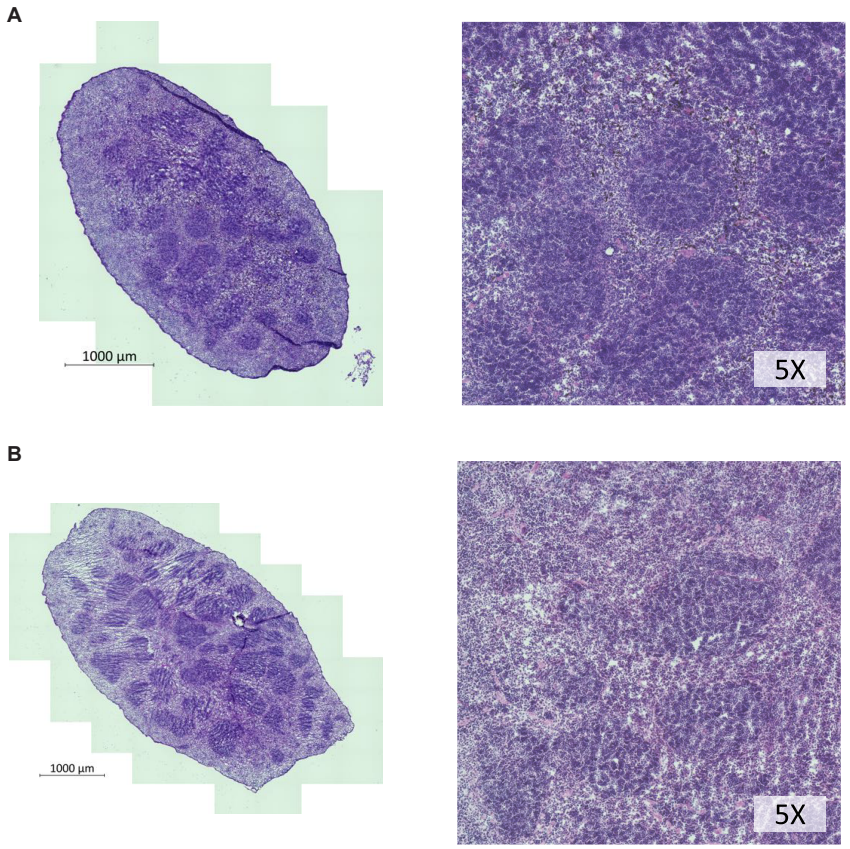

FIGURE 3 | H&E staining of spleen cross sections of whole spleens shows lower cellular density in spleens of MP-10 treated mice. **(A)** Control mice and **(B)** MP-10 treated mice from the short-term study (30 mg/kg). Representative data from n = 15 mice per group.

phenotype observed in the Bajau. This phenotype was replicable *ex vivo*, however the spleens of treated mice were significantly less dense than those of the control animals. We attribute this difference in splenic density to a stretching of the tissue to accommodate a greater quantity of RBCs, apparent through hematological testing.

We attribute the increase in RBC count to an observed significant excess of early stage erythrocytic precursors in treated mice, which has been previously identified as a response to thyroid hormone stimulation (Angelin-Duclos et al., 2005). Thyroid hormones are known to be required for terminal erythroid differentiation, however the exact molecular mechanisms underlying thyroid hormone function on erythropoiesis are unknown (Gao et al., 2017). In agreement with our results, thyroid hormones have been previously demonstrated to directly affect proliferation of erythroid progenitors (Golde et al., 1977; Popovic et al., 1977; Dainiak et al., 1978; van Gucht et al., 2017). This may be attributable to higher levels of TH nuclear receptors α (TRα) in early progenitors compared to late stage erythroblasts, as has been previously demonstrated to underlie variable responses to thyroid hormones at different stages of development (Gao et al., 2017).

We also note a significant increase in apoptosis of late-stage erythrocytic precursors in the spleens of treated mice, a previously characterized response to splenomegaly (Ilesanmi, 2010). We suggest that thyroid hormones mediate an apoptotic feedback mechanism that, in response to increased erythrocytic precursor stimulation, controls the number of reticulocytes that are released and is preventative of red blood cell excess. However, the apparent apoptotic mechanism proves insufficient in the short term to overcome the excess production of early precursors; after 1 week of treatment, the experimental mice displayed pronounced polycythemia (**Figure 4**). It appears that over longer periods, in the case of our study a 9-week administration of MP-10, the feedback returns hematological values to normal levels (**Figure 4**). This could be a limitation of the pharmacological model and the result of slight inconsistencies in delivery of the low dosage of MP-10 over the 9 weeks. A knockout model would provide more consistent inhibition of PDE10A and more reliable hematological values. It could, however, be truly representative of what occurs in humans and therefore the reason hyperthyroidism has not yet been consistently linked to polycythemia.

A reduction in RBC count, and a corresponding reduction in EPO has been previously reported in hypothyroid patients, however the reduction in EPO was thought to be associated with reduced oxygen requirement due to diminished BMR (Das et al., 1975). In fact, it has been suggested that thyroid hormones only induce EPO production under hypoxic

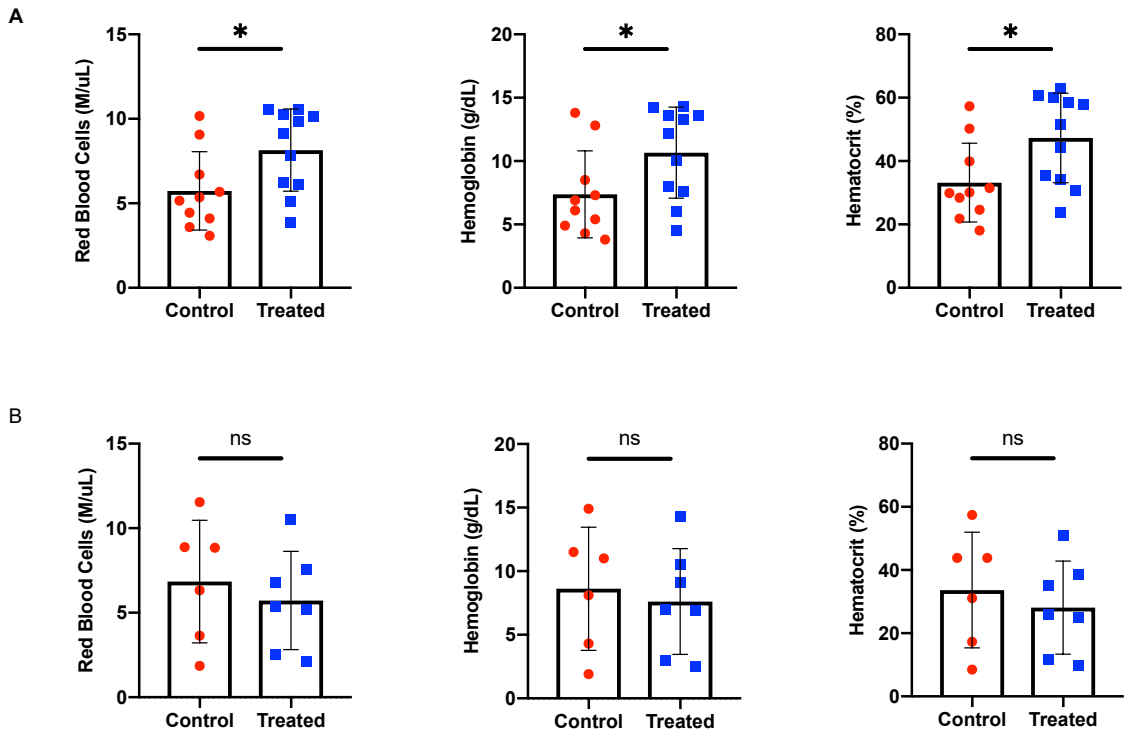

FIGURE 4 | Hematological effects of short- and long-term phosphodiesterase 10A (PDE10A) inhibition. **(A)** After 1 week of treatment, mice receiving MP-10 (10 mg/kg) displayed significantly higher red blood cell (RBC) counts ($p = 0.0317$), an effect also confirmed in humans carrying the Bajau genetic variant (**Table 1**). Treated mice were also found to have significantly higher hematocrit ($p = 0.0259$) and hemoglobin ($p = 0.0458$) than control animals. **(B)** After 9 weeks of treatment with MP-10 (30 mg/kg), there are no apparent hematological differences between treated and control animals. $^{*}p \leq 0.05$.

TABLE 1 | Phosphodiesterase 10A (PDE10A) SNPs are associated with increased red blood cell (RBC) count but not erythropoietin (EPO).

SNP	Chr	Gene	RBC count assoc	EPO assoc
rs3008049	6	PDE10A	2.658×10^{-3}	0.4091598959
rs3008050	6	PDE10A	7.255×10^{-3}	0.226484351
rs3008052	6	PDE10A	– [a]	0.364422382
rs2983527	6	PDE10A	1.128×10^{-2}	0.2426864261

Red blood cell count (from UK Biobank) and EPO (from the PREVEND cohort) association at SNPs of interest. The Bonferroni corrected thresholds for significance are 0.0167 (for RBC), 0.0125 (for EPO).
[a] *This SNP is not available in the UK Biobank data.*

conditions (Fandrey et al., 1994). *In vitro* studies have shown that thyroid hormones can stimulate erythropoiesis directly in systems where hormonal effects on EPO production are eliminated (Malgor et al., 1975; Golde et al., 1977), thus suggesting an EPO independent mechanism, consistent with our findings which showed no difference in *Epo* expression between treated and control animals. It may be that slight differences in expression are obscured by the nature of the measurement. Unfortunately, we were not able to measure TH or EPO in the animals directly. In addition, it is important to note that while *Gapdh* is frequently used for normalization in other TH studies, it is a HIF-dependent gene and as such may change in expression along with our gene of interest.

It is important to note that the adult human spleen is not a site of erythropoiesis as it is in mice. Because of this, observed cell population differences in the mouse spleen should be considered analogous to bone marrow in humans (Brodsky et al., 1966; Bresnick et al., 2018). In addition, erythroblastic islands, which are essential for the maturation of erythroblasts that are fated to enucleate, have been demonstrated *in vivo* in fetal splenic red pulp (Manwani and Bieker, 2008). Therefore, the observations in this study may be extrapolated to human fetal development and the observed changes in spleen size may occur developmentally.

MATERIALS AND METHODS

Mice

For this study, we used wild type C57BL/6J mice. Experiments were performed on weaned animals beginning at 3 and 4 weeks for the short- and long- term studies, respectively. These ages were chosen because most murine spleen development occurs by 3 weeks (Hankir et al., 2016). While the neonatal mouse spleen is a hematopoietic site, by the time of full development the spleen's role is limited to erythropoiesis (Wolber et al., 2002; Angelin-Duclos et al., 2005).

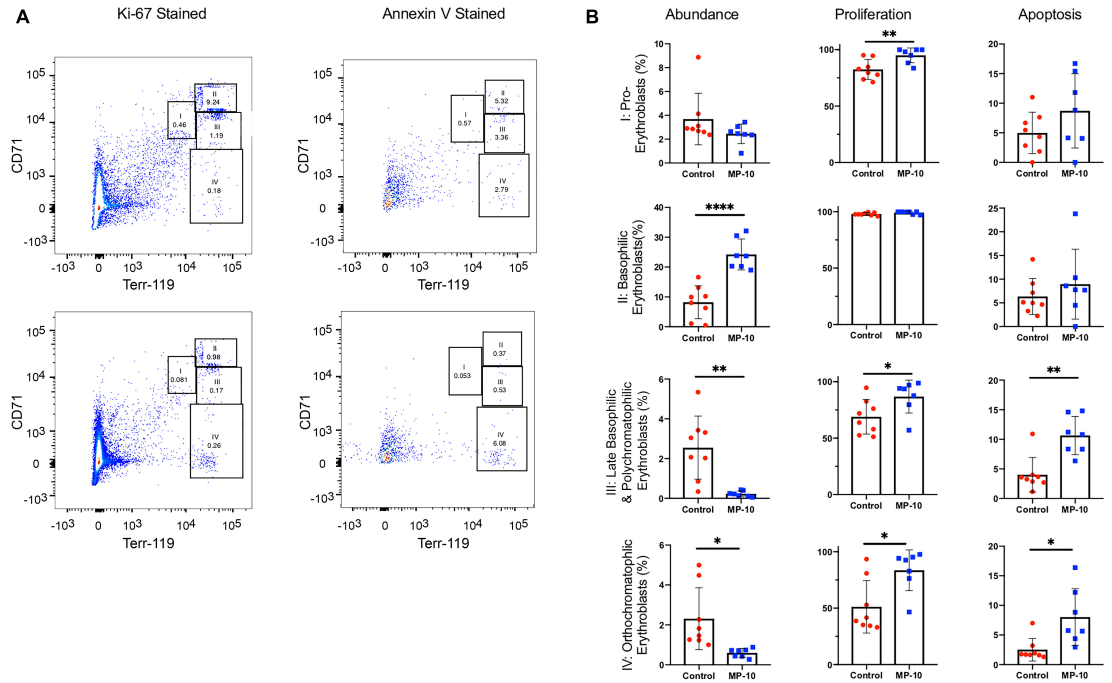

FIGURE 5 | Flow cytometry data show differences in cell precursor populations between control and MP-10 treated mice. **(A)** Representative flow cytometry data of splenic cells from a control mouse and MP-10 (30 mg/kg) treated mouse stained with Ki-67, an indication of proliferation and AnnexinV, an apoptosis marker. The four different erythrocytic populations were defined according to Socolovsky et al. (2001): I: pro-erythroblasts; II: basophilic erythroblasts; III: late basophilic erythroblasts; IV: orthochromatophillic erythroblasts. **(B)** Analyzed flow cytometry data demonstrate MP-10 (30 mg/kg) treated animals have significantly increased proliferation at nearly every stage of development. Comparison of splenic cellular composition between control and MP-10 treated mice demonstrate that treated mice have an increase in proliferation in nearly every precursor population, however proportionally lower abundance of late stage precursors due to increased apoptosis. Results are summarized in **Table 2**. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{****}p \leq 0.0001$.

TABLE 2 | Flow cytometry results summary.

Precursor population/gate	Relative abundance	Proliferation	Apoptosis
Pro-erythroblasts (I)	0.1773	0.0087	0.1718
Basophilic erythroblasts (II)	<0.0001	0.0692	0.3967
Late basophilic & polychromatophilic erythroblasts (III)	0.0020	0.0374	0.0011
Orthochromatophillic erythroblasts (IV)	0.0127	0.0107	0.0102

P-values for flow results, blue or red values indicate the relative proportion of cells under a given designation is significantly higher in treated or control mice, respectively.

TABLE 3 | Thyroid Stimulating Hormone (TSH) association does not correlate with *Epo* association.

SNP	Chr	Gene	TSH assoc.	EPO assoc
rs2046045	5	PDE8B	1.85×10^{-17}	0.7699698027
rs6885099	5	PDE8B	1.95×10^{-56}	0.723791259
rs1382879	5	PDE8B	7.16×10^{-18}	0.5133130162

Thyroid Stimulating Hormone and EPO association values for three significantly thyroid-associated SNPs are found in another phosphodiesterase (PDE8B) known to be associated with thyroid hormones (Panicker et al., 2008; Malinowski et al., 2014).

We only included males because of well of documented, significant differences in free T4 between males and females in various recombinant inbred (RI) strains of mice which create a sex-dependent thyroid hormone signal (McLachlan et al., 2014). To inhibit PDE10A, we used the highly selective PDE10A inhibitor PF-2545920 hydrochloride (MP-10) from Sigma-Aldrich dissolved in DMSO and diluted in 40% 2-Hydroxypropyl-beta-cyclodextrin (HPβCD). In the long-term study, animals were injected daily with either 10 mg/kg of MP-10 or an equivalent volume of vehicle intraperitoneally (*i.p.*) for 9 weeks and were subsequently sacrificed. For the short-term study, animals were injected daily with 30 mg/kg MP-10 or an equivalent volume of vehicle *i.p.* for 1 week, after which they were sacrificed. In both the long- and short-term study, the final injection was performed 2 h before the animals were sacrificed.

Ultrasound Studies

Images were acquired with B-mode, and color Doppler modes using a Vevo 2100 high frequency ultrasound machine (VisualSonics) and a MS550D 22–55 MHz probe. Animals were sedated with isoflurane and scan time was approximately 20 min to identify and image the spleen. Spleen area was calculated from 2-dimensional images using the VisualSonics software.

Flow Cytometry

Whole spleens were collected, washed in RPMI and PBS and passed through 100 and 70 μM mesh to release cell suspensions. Single cell suspensions obtained from spleens were analyzed with the following antibodies: B220-APC, TER119-PE, CD71-FITC and Annexin V PerCP-eFluor710 (eBioscience). Cells were analyzed on a BD LSRFortessa machine (Becton Dickinson) using FlowJo software. The four different erythrocytic populations were defined according to Socolovsky et al. (2001): I: $Ter119^{med}CD71^{high}$ (pro-erythroblasts); II: $Ter119^{high}CD71^{high}$ (basophilic erythroblasts); III: $Ter119^{high}CD71^{med}$ (late basophilic erythroblasts); and IV: $Ter119^{high}CD71^{low}$ (orthochromatophillic erythroblasts). For compensation, single positive labeled cells and OneComp eBeads (Becton Dickinson) were used. Ki-67 PerCP-eFluor710, the intracellular staining to identify proliferating cells was performed according to the Intracellular Fixation & Permeabilization Buffer Set protocol (eBioscience).

Erythropoietin qPCR

For indicated conditions and time-points, tissues were collected and minced followed by RNA extraction using TRIzol Plus RNA Purification Kit (Invitrogen Cat#: 12183555). cDNA synthesis was performed using qScript XLT cDNA SuperMix (QuantaBio Cat# 95048-025). After reverse transcription, qPCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) and analyzed using an Applied Biosystems 7900 HT qPCR instrument. The cycle threshold (Ct) value for each transcript was normalized to *Gapdh*. The comparative Ct method was used to quantify transcript abundance. TaqMan Assays used were; Epo: Mm01202755_m1, Gapdh: Mm99999915_g1 Gapdh.

Histology

Spleens samples were submitted to histology for sectioning and Hematoloxin and Eosin staining. The frozen tissue was sliced at a thickness of 5 microns. Prepared slides were imaged with the Axioslide scanner.

Hematology

Blood was collected from the submandibular vein 24 h before animals were sacrificed. Complete blood counts were conducted using a Drew Scientific Hemavet 950FS with manufacturer mouse settings.

Prevention of REnal and Vascular ENd-Stage Disease Study

Participants from the PREVEND study were used for this study. PREVEND has been used for the association studies with EPO levels, for details on the study protocol see Hillege et al. (2001); Grote Beverborg et al. (2015). In brief, PREVEND was designed to prospectively investigate the natural course of urinary albumin excretion (UAE) and its relationship with renal and cardiovascular disease in a large cohort drawn from the general population. For the current analyses, we used data from the second survey between 2001 and 2003. Serum EPO levels were measured using the IMMULITE EPO assay (DPC, Los Angeles, CA, United States), and a luminometer measured the amount of serum EPO using chemiluminescence. We used 2,691 randomly selected non-anemic individuals, to avoid confounding variables. Analysis was performed on residuals of EPO levels after adjustment for age, gender and the first 10 principal components using an additive genetic model in SNPTEST v2.4.1.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Utah IACUC.

AUTHOR CONTRIBUTIONS

MI devised and performed the experiments and wrote the manuscript. MS assisted with the designing and implementing all the experiments as well as data interpretation. NG, NV, PiV, and PeV generated, analyzed, and interpreted the EPO GWAS data. MR assisted with the experiments. EL assisted in the experimental design. All authors reviewed the manuscript.

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Conflict of Interest: MI was employed by the company Maze Therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Prenatal Hypoxia Induces Cl^- Cotransporters KCC2 and NKCC1 Developmental Abnormality and Disturbs the Influence of $GABA_A$ and Glycine Receptors on Fictive Breathing in a Newborn Rat

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Prenatal hypoxia is a recognised risk factor for neurodevelopmental disorders associated with both membrane proteins involved in neuron homeostasis, e.g., chloride (Cl^-) cotransporters, and alterations in brain neurotransmitter systems, e.g., catecholamines, dopamine, and GABA. Our study aimed to determine whether prenatal hypoxia alters central respiratory drive by disrupting the development of Cl^- cotransporters KCC2 and NKCC1. Cl^- homeostasis seems critical for the strength and efficiency of inhibition mediated by $GABA_A$ and glycine receptors within the respiratory network, and we searched for alterations of GABAergic and glycinergic respiratory influences after prenatal hypoxia. We measured fictive breathing from brainstem in *ex vivo* preparations during pharmacological blockade of KCC2 and NKCC1 Cl^- cotransporters, $GABA_A$, and glycine receptors. We also evaluated the membrane expression of Cl^- cotransporters in the brainstem by Western blot and the expression of Cl^- cotransporter regulators brain-derived neurotrophic factor (BDNF) and calpain. First, pharmacological experiments showed that prenatal hypoxia altered the regulation of fictive breathing by NKCC1 and KCC2 Cl^- cotransporters, GABA/$GABA_A$, and glycin. NKCC1 inhibition decreased fictive breathing at birth in control mice while it decreased at 4 days after birth in pups exposed to prenatal hypoxia. On the other hand, inhibition of KCC2 decreased fictive breathing 4 days after birth in control mice without any change in prenatal hypoxia pups. The GABAergic system appeared to be more effective in prenatal hypoxic pups whereas the glycinergic system increased its effectiveness later. Second, we observed a decrease in the expression of the Cl^- cotransporter KCC2, and a decrease with age in NKCC1, as well as an increase in the expression of BDNF

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and calpain after prenatal hypoxia exposure. Altogether, our data support the idea that prenatal hypoxia alters the functioning of $GABA_A$ and glycinergic systems in the respiratory network by disrupting maturation of Cl^- homeostasis, thereby contributing to long-term effects by disrupting ventilation.

Keywords: prenatal hypoxia, newborn, breathing, NKCC1, KCC2

INTRODUCTION

Development of a mammal neural system is influenced by early life experiences. According to the hypothesis of "developmental programming of health and disease" or "fetal origins of disorder later in life," maternal environment has an impact on foetal development, and even on adult health (Barker et al., 1993; de Boo and Harding, 2006; Li et al., 2012). Foetal growth and development are constrained by oxygen limitations due to maternal environment including high altitude and pathological situations, such as maternal severe pulmonary and cardiac diseases, chronic anaemia, and intrauterine perfusion (Brodsky and Christou, 2004). Prenatal hypoxia has indeed been characterised as a major cause of neurodevelopmental disorders that can lead to chronic neurological disabilities in children (Baud et al., 2004; Golan and Huleihel, 2006). Related to one of the earliest motor behaviours to develop in foetal state, breathing is sensitive to early prenatal hypoxic stress that induces short- and long-term effects in breathing (Kobayashi et al., 1985; Peyronnet et al., 2000; Powell, 2007; Tree et al., 2016).

Breathing depends on a central respiratory drive (CRD) from the brainstem neuronal network in which neural groups interact with several neurotransmitters such as GABA and glycine (Onimaru et al., 1990; Wittmeier et al., 2008; Koizumi et al., 2013; Richter and Smith, 2014; Anderson et al., 2016). GABA and glycine modulate the central respiratory drive by acting on Cl^- channel-receptors, i.e., $GABA_A$ and glycine receptors. In immature neurons, limited expression of the K^+/Cl^- cotransporter (KCC2) that extrudes Cl^- from cells and greater expression of the $Na^+/K^+/Cl^-$ cotransporter (NKCC1), which intrudes Cl^- into cells lead to high intracellular Cl^- concentrations (Blaesse et al., 2009). In such conditions, the activation of $GABA_A$ and glycine receptors induces an increase in Cl^- outward conductance that depolarises neurons and therefore promotes excitation. In mature neurons, the membrane expression of KCC2 increases whereas the NKCC1 expression decreases, thus leading to a weak intracellular Cl^- concentration (Liu and Wong-Riley, 2012). In this condition, the activation of $GABA_A$ and/or glycine receptors induces an increase in Cl^- inward conductance that hyperpolarises neurons that promote inhibition (Rivera et al., 2004; Vinay and Jean-Xavier, 2008; Stil et al., 2009). The expression of KCC2 and NKCC1 has several regulators including brain-derived neurotrophic factor (BDNF), calpain, insulin growth factor, and prenatal hypoxia may up or downregulate the expression or activation of these regulators (Kelsch et al., 2001; Watanabe and Fukuda, 2015). As reported in perinatal periods, CRD appears strongly influenced by the functional status of Cl^- cotransporters. For instance, KCC2-deficent mice die quickly after birth due to a respiratory drive failure, and KCC2 regulates rhythmic respiratory-related activity of hypoglossal nuclei (Hubner and Stein, 2001; Okabe et al., 2015). As Cl^- homeostasis depends on environmental conditions including prenatal stress, we hypothesised that a defect in Cl^- cotransporters disrupts GABA modulation of breathing, as observed after a gestational stress (Delhaes et al., 2014; Pozzi et al., 2020).

In the present study, we examined how changes in the expression of KCC2, NKCC1, GABA/$GABA_A$, and glycine influence CRD at birth by using a model of prenatal hypoxia that also reproduces intrauterine growth restriction (Baud et al., 2004; Pham et al., 2015; Tree et al., 2016). We hypothesise that prenatal hypoxia causes reduced expression of Cl^- cotransporters KCC2 and NKCC1, thus leading to disturbances in respiratory frequency, through abnormal shifts toward depolarisation or hyperpolarisation after activation of $GABA_A$ and glycine receptors. To determine the impact of chronic prenatal hypoxia, pharmacological studies were combined to electrophysiological recordings in *en bloc* preparations from newborn rats. We also quantified the membrane expression of the two Cl^- cotransporters in the brainstem using Western Blot and underlying contributors in their regulation, BDNF by ELISA, and calpain by immunohistochemistry.

MATERIALS AND METHODS

All experimental procedures were approved by the "INT Neurosciences Ethic Committee No. 71 for Animal Research" in Marseille (national number of ethical agreement is C1305518) and were performed in accordance with Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 and French law (2013/118). All efforts were made to minimise the number of animals used and their suffering.

Animals and Chronic Gestational Hypoxia Model

Sprague–Dawley pregnant rats (Charles River, France) were housed with food and water *ad libitum* in a 12-h light/dark cycle. Pregnant rats were exposed to hypoxia (10% O_2/90% N_2) from day 5 to day 20 of gestation, as previously described (Peyronnet et al., 2000). On day 20, they were housed individually and birth occurred in normoxia. Newborn rats grew up in normoxia and constituted the prenatal hypoxia group [prenatal hypoxia (PH); n = 151]. A control group of newborn rats was composed of pups from pregnant rats whose gestation occurred under normoxic conditions [21% O_2; normoxic prenatal group, normoxic prenatal (CONT); n = 162]. This high number of animals is because we have 4 drugs applied on 8 distinct groups: PH and CONT, two ages, medullary-spinal cord (MS)/ponto-medullary-spinal cord

(PMS) preparations for electrophysiological experiments (n = 7–12 per test), Western blot (n = 6–8 per group), and Elisa test quantification (n = 5 per group).

Electrophysiological Recordings in *ex vivo* Central Nervous System Preparations

Experiments were conducted on the medullary–spinal cord (MS) and the ponto–medullary–spinal cord (PMS) preparations isolated under deep cold anesthesia by immersion in ice water from newborn rats from the day of birth (counted as P0) to fourth postnatal day (P4) (Okada et al., 1998; Rousseau and Caravagna, 2015; Tree et al., 2016). Two groups of age were distinguished to assess central breathing network maturation: P0-1 and P3-4. MS and PMS preparations differed only in the rostral cut: at the level of anterior inferior cerebellar arteries, caudal to the VIII cranial nerve exit points for MS preparation, rostral to the fifth cranial nerves at the level of superior cerebellar arteries, and caudal edge of inferior colliculi for PMS preparations. A caudal section was done between the seventh and eighth cervical spinal roots. *Ex vivo* preparations were rapidly dissected out and placed in a recording chamber (5 mL), ventral surface upward. This chamber was continuously superfused (5 mL per min) with artificial cerebro-spinal fluid (a-CSF) maintained at 27°C ± 1°C (pH 7.4) and bubbled with carbogen (95%O_2/5%CO_2). The a-CSF composition was (in mM): 129.0 NaCl, 3.35 KCl, 21.0 $NaHCO_3$, 1.26 $CaCl_2$, 1.15 $MgCl_2$, 0.58 NaH_2PO_4, and 30.0 D-glucose. The fourth cervical ventral root (C4) was sucked into a glass micropipette and its electrical activity was filtered (10–3,000 Hz), amplified (5,000×), integrated (time constant 100 ms), and digitised (sampling frequency, 5,000 Hz) by using Spike 2 data analysis system (Cambridge Electronik design, United Kingdom). The frequency of C4 burst discharges was considered as the fictive respiratory frequency (f_R, expressed in cycles per minute, $c.min^{-1}$). f_R was averaged over 5-min periods regardless of the experimental condition. After the surgical procedure, preparations were superfused for 20 min with a-CSF until stable rhythmic C4 discharges were recorded. During such stable conditions, the collected f_R is considered as "basal condition" or predrug values.

Pharmacological Experiments

After determination of basal f_R, the control a-CSF (i.e., a-CSF without drugs) was then replaced by a-CSF added with one of the following drugs: bumetanide (10 μM; inhibitor of NKCC1); VU0240551 (10 μM, selective KCC2 inhibitor); picrotoxin (20 μM; $GABA_A$ antagonist); strychnin (1 μM; glycine receptor antagonist) for a test period of 20 min. These applications were followed by a recovery period (20 min) under control a-CSF. Drug concentrations have been established according to the literature (Iizuka, 2003; Ren and Greer, 2006), except for VU0240551, which was tested at 1, 5, 10, 25, and 50 μM. Thus, we used 10 μM of VU0240551 to determine the lowest concentration necessary to change fictive breathing in CONT pups. All drugs purchased at Sigma (Sigma-Aldrich, Saint-Quentin-Fallavier, France) were directly dissolved in a-CSF, except for VU0240551, which was previously dissolved in a-CSF that included 0.0025% DMSO.

Western Blot

P0-1 and P3-4 pups were killed by decapitation after deep-cold anesthesia and quickly dissected to search for quantitative expression of NKCC1 and KCC2 at the membrane (Danneman and Mandrell, 1997). The pons was quickly frozen in liquid nitrogen and stored at −80°C. Supernatant protein concentrations were determined with a detergent-compatible (DC) protein assay (Bio-Rad). Briefly, samples were homogenised in cold lysis buffer A and centrifuged at 18,000 *g* for 30 min at 4°C. Samples were then homogenised in cold lysis buffer B without detergent and centrifuged. Pellets were collected in lysis buffer A without DTT. KCC2 or NKCC1 were then immunoprecipitated, separated in 7% SDS-PAGE, and transferred to a polyvinylidene fluoride membrane. After blockade in Tris-buffered saline plus 5% non-fat dry milk, membranes were exposed overnight at 4°C to a polyclonal rabbit KCC2-specific antibody (07-432; MERCK Millipore) diluted 1:500 or a monoclonal NKCC1-specific antibody (clone T4, Developmental Studies Hybridoma bank) diluted 1/500 in the blocking solution. ImmunoPure goat-horseradish-peroxidase–conjugated rabbit or mouse-specific antibody (1:500 in blocking solution, 1 h at 22°C) was used for chemiluminescent detection (Pierce Biotech). Signal intensities were measured with the image analysis software Quantity-One (Bio-Rad).

ELISA Test

P3-4 aged pups were killed by decapitation after freezing with anaesthesia and quickly dissected out. Pons was isolated, quickly frozen in liquid nitrogen, and then conserved at −80°C. Pons were homogenised in cold RIPA lysis buffer (25 mM Tris–HCl pH7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1%SDS; Pierce Biotechnology, Rockford, IL, United States) supplemented with protease and phosphatase inhibitors cocktail (Sigma-Aldrich, France). Samples were then centrifuged at 15,000 *g* for 30 min at 4°C. Total protein concentration of each sample was determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL, United States) and adjusted to 80 μg/mL. BDNF Emax immunoassay system (Promega, France) was performed according to the manufacturer's instructions. BDNF concentrations were expressed as picograms per mL.

Immunohistochemistry

To identify prenatal hypoxia-induced changes in the expression of calpain 1, immunohistochemical detection was performed on PH (n = 5) and CONT (n = 5) newborn rats at P3-P4. Newborn rats were anaesthetised with deep cold anesthesia by immersion in ice water (Danneman and Mandrell, 1997). A block containing pons and medulla oblongata was dissected out from the brain and then fixed by immersion in a fixative solution containing 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 m, pH 7.4) for 48 h and then stored for 24 h in a cryoprotectant solution containing 30% sucrose in phosphate buffer at pH 7.4. Serial coronal sections through the pons and medulla oblongata of all pups were cut on a freezing microtome (Leica CM 1510S) at a thickness of 30 μm. Immunohistochemical detection of calpain

1 was processed on free-floating coronal sections. Sections were first incubated with a mouse monoclonal antibody against calpain 1 (sc-271313; Santa Cruz Biotechnology Inc., CA, United States, 1:2,000 in 1% bovine serum albumin, BSA; 48 h, 4°C) and then with a biotinylated horse anti-mouse antibody (BA-2000, Vector Laboratories, Burlington, ON, Canada; 1/500; 2 h). Calpain 1 was finally identified by using a NovaRed Kit (Vector NovaRED Substrate Kit, Vector Laboratories, Burlington, ON, Canada), which resulted in a red/brown coloration. Antibodies specificity was verified with the omission of primary or secondary antibodies in some sections. No labelling was observed in these sections. All the sections were mounted in sequential caudo-rostral order on silanised slides, air-dried, and coverslipped with EUKITT (Bio Optica, Milan, Italy). Using a Leica microscope (Leica DM 2000; Leica Microsystems, Heidelberg, Germany) with bright-field illumination, representative images were photographed at 10× or 20× magnification with a digital camera (Leica DFC450C, Leica Microsystems, Heidelberg, Germany). Calpain-positive cells were visually counted in each section collected through the respiratory related area of the pons, and therefore throughout the rostrocaudal extent of each nucleus, using a Leica microscope (Leica DM2000; Leica Microsystems, Heidelberg, Germany). We analysed the number of calpain positive cells in locus coeruleus, sub-coeruleus nucleus, lateral and medial parabrachial nuclei, and Kolliker füse nucleus. The mean number of calpain-positive cells per section was calculated for each nucleus in each animal.

Statistics

Data were analysed with GraphPad (GraphPad Prism8, San Diego, CA, United States). Normality of data distribution was assessed using d'Agostino and Pearson normality test. Results were given as mean ± SEM when data complied to normality or as median and interquartile range [Q1; Q3] when normality was not detected. Statistical analyses of electrophysiological data were performed on data averaged over a 5-min time-period. The value of fR was expressed in absolute values ($c.min^{-1}$) in the text. Statistical comparisons of body weight between CONT and PH pups were assessed using a Mann and Whitney test. Statistical comparisons of fR between basal conditions and drug challenges for all groups (MS or PMS, P0-1 or P3-4, CONT or PH) were assessed with one-way repeated measures analysis of variance (ANOVA) followed by the *post hoc* Holm-Sidak's multiple comparison test or with Friedman test followed by the *post hoc* Dunn's multiple comparison. The influence of age and/or prenatal hypoxia on fR was assessed using a two-way analysis of variance (ANOVA) test followed by the *post hoc* two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. To avoid differences related to basal changes in fR caused by the presence or absence of the pons or by differences in age, the comparison of fR between CONT and PH pups during drug challenges was done on normalised fR (expressed in percentage of pre-drug values of fR). Comparisons of the effect on fR between PH and CONT at two different age groups within the last 5 min of each pharmacological test were done using two-way analysis of variance (ANOVA) test followed by the *post hoc* two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli to decipher an age and/or prenatal hypoxia effect on fR. For Western blot analysis, comparisons of KCC2 and NKCC1 expression and the ratio NKCC1/KCC2 between PH and CONT and P0 and P4, respectively, were done using a Kruskal–Wallis test followed by the *post hoc* two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. For ELISA test and immunohistochemistry, CONT group was compared to PH group for each age set using Mann and Whitney test. Level of statistical significance was set at $p < 0.05$.

RESULTS

Prenatal hypoxia leads to a decrease in body weight. At P0-1, we noted a lower weight ($p = 0.006$) in the PH group (6.18 ± 0.09 g; $n = 75$) in comparison with CONT group (6.56 ± 0.07 g; $n = 85$). At P3-4, this lower body weight persisted ($p = 0.027$) with mean weights of 8.96 ± 0.17 and 9.56 ± 0.17 in PH ($n = 76$) and CONT ($n = 77$) groups, respectively. Age influenced fR in *ex vivo* preparations. Indeed, in MS preparations, fR increasesd with age in CONT pups ($p = 0.0002$) without there being any change in PH pups, whereas in PMS preparations, fR increased in PH pups ($p = 0.0002$) without there being any change in CONT groups. Prenatal hypoxia influenced fR in *ex vivo* preparations. Indeed, at P0-1, fR was higher in PH pups than in CONT pups in MS preparations ($p = 0.036$), whereas in presence of the pons, fR was lower in PH pups than in the CONT group ($p = 0.025$).

Abnormalities in Fictive Breathing Modulation by NKCC1 Induced by Prenatal Hypoxia

P0-1

In the CONT group, 10 μM bumetanide–aCSF perfusion decreased fR during the last 5 min of exposure in MS preparations ($p = 0.020$; $n = 12$; **Figures 1A,B**) and from the sixth minute in PMS preparations ($F_{4,32} = 7.176$, $p = 0.0003$; $n = 9$; **Figures 1C,D**). In contrast, in the PH group, fR was not influenced by blockade of NKCC1 cotransporter in MS ($n = 9$, $p = 0.61$; **Figures 1A,B**) and PMS ($n = 8$, $p = 0.12$; **Figures 1C,D**) preparations. Although intragroup effects were different, normalised fR (in % of pre-bumetanide values) was similar in CONT and PH in both MS and PMS preparations (**Figures 1E,F**).

P3-4

In the CONT group, fR was not modified by blockade of NKCC1 cotransporter regardless of the preparation: MS ($p = 0.21$; $n = 8$; **Figures 1A,B**) and PMS ($p = 0.053$; $n = 9$; **Figures 1C,D**). In the PH group, exposure to bumetanide–aCSF did not change fR in the MS preparations ($p = 0.46$; $n = 8$; **Figures 1A,B**), but decreased fR in the PMS preparations ($F_{4,28} = 3.068$, $p = 0.032$; $n = 8$; **Figures 1C,D**) from the sixth minute of bumetanide–aCSF exposure. Normalised fR (in % of pre-bumetanide values) appeared to be lower in PH group than in CONT group in P3-4 PMS preparations ($p = 0.012$), but similar between CONT and PH in P3–4 MS preparations (**Figures 1E,F**).

P0-1 vs. P3-4

In the CONT group, the effect of age was present in PMS but not in MS preparations, fR was lower in P3-4 pups PMS preparations

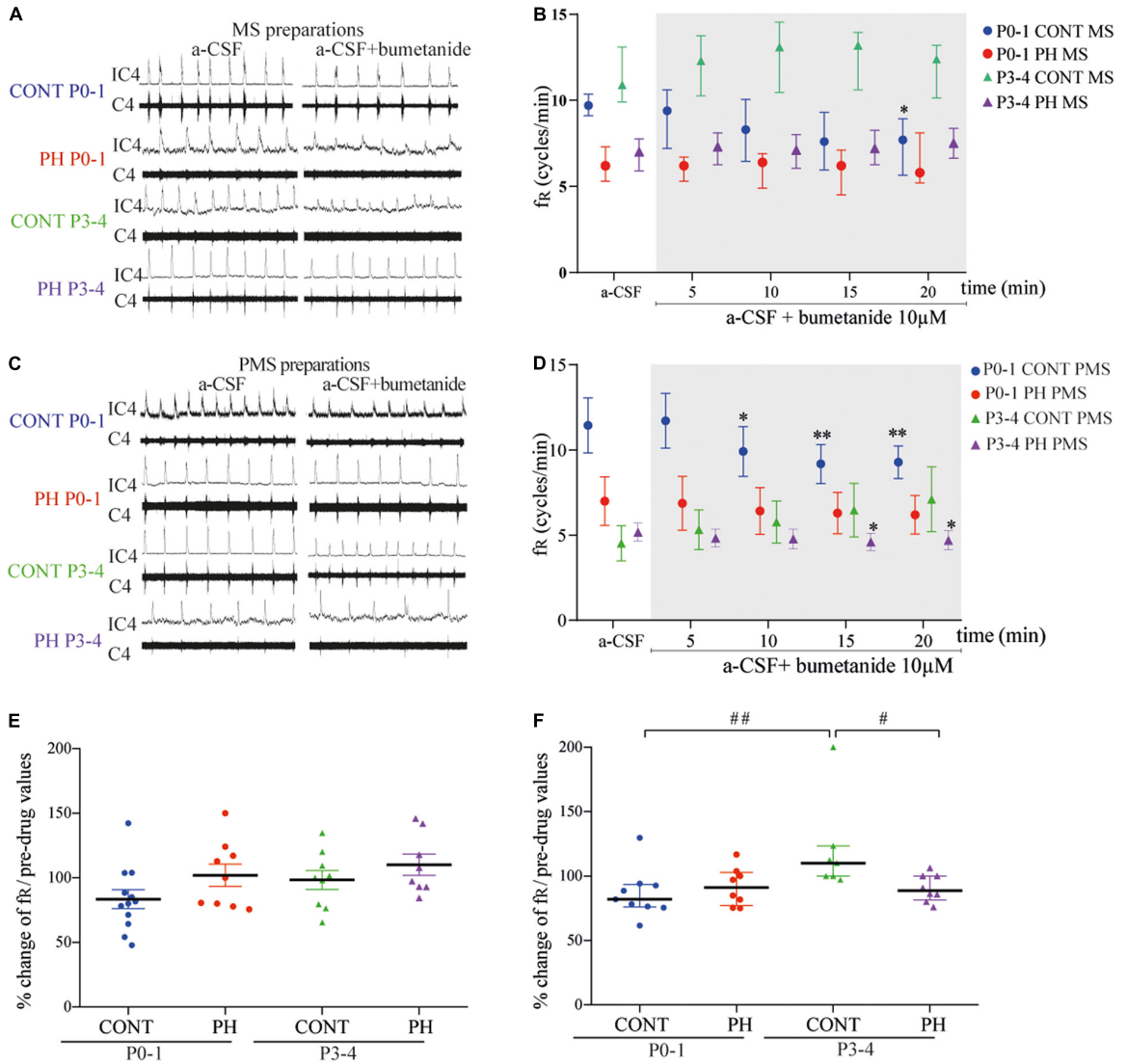

FIGURE 1 | NKCC1 inhibition in the pons altered fictive breathing. **(A,C)** Representative recordings in the medulla **(A)** and ponto-medullary **(C)** preparations before (a-CSF) and during (a-CSF + bumetanide) bumetanide application, in controls (CONT) and prenatal hypoxic (PH) pups, at P0-1 and P3-4. **(B,D)** Frequency of fictive breathing before (a-CSF) and every 5 min during (a-CSF + bumetanide 10 µM) bumetanide application, in CONT and PH pups, at P0-1 and P3-4. The fR is expressed as median [Q1; Q3] in medulla (MS) and as mean ± SEM in ponto-medullary (PMS) preparations. **(E,F)** Scatter plots with surimposed mean ± SEM in medulla **(E)** and median [Q1; Q3] in ponto-medullary **(F)** preparations, illustrating changes in fR during the last 5 min of bumetanide exposure, expressed as percentages of pre-drug values. * indicates significant intragroup differences in fR between a-CSF condition and bumetanide application. # indicates significant intergroup differences in fR between PH and CONT or P0-1 and P3-4. *$p < 0.05$, **$p < 0.01$, #$p < 0.05$,##$p < 0.01$. C4 electrical activity of the 4th cervical ventral nerve root; IC4: integrated activity of the C4 ventral nerve root.

during the last 5 min under aCSF-bumetanide ($p = 0.004$; **Figure 1F**), than in P0-1 preparations. In PH pups, normalised fR was similar in both ages regardless of the preparation (**Figure 1F**).

Abnormalities in Fictive Breathing Modulation by KCC2 Induced by Prenatal Hypoxia

P0-1

In the CONT group, 10 µM VU0240551 application had no influence on fR in MS ($p = 0.71$; $n = 8$; **Figures 2A,B**) and PMS preparations ($p = 0.62$; $n = 10$; **Figures 2C,D**). Same results were found in PH group, in which fR was unaffected in MS ($p = 0.29$; $n = 8$) and PMS preparations ($p = 0.44$; $n = 8$). There was therefore no difference in normalised fR (in % of pre-VU0240551 values) between MS and PMS in PH and CONT preparations during aCSF–VU0240551 exposure (**Figures 2E,F**).

P3-4

In the CONT group, 10 µM VU0240551 application had no influence on MS preparations ($p = 0.83$; $n = 8$; **Figures 2A,B**), but fR decreased from the sixth minute of KCC2 blockade in PMS

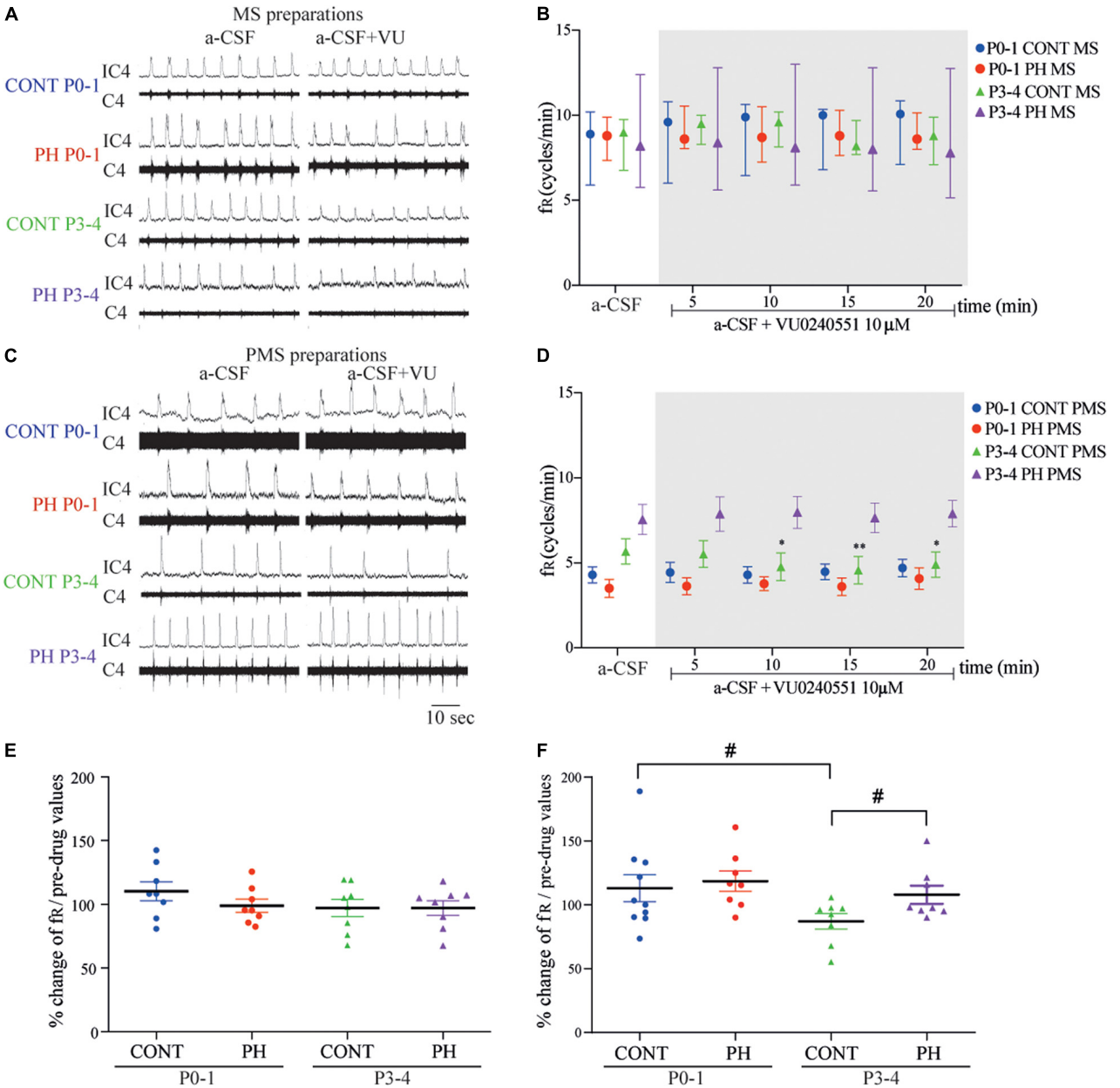

FIGURE 2 | Impaired breathing related to KCC2 blockade was suppressed in prenatal hypoxic pup preparations. **(A,C)** Representative recordings of medulla **(A)** and ponto-medullary **(C)** preparations before (a-CSF) and during (a-CSF+VU) VU0240551 application, in controls (CONT) and prenatal hypoxic (PH) pups, at P0-1 and P3-4. **(B,D)** Frequency of fictive breathing before (a-csf) and every 5 min during (a-CSF + VU0240551 10 µM) VU0240551 application, in CONT and PH pups at P0-1 and P3-4. The fR is expressed as median [Q1; Q3] in medulla (MS) and as mean ± SEM in ponto-medullary- (PMS) preparations. **(E,F)** Scatter plots with surimposed mean ± SEM, illustrating changes in fR during the last 5 min of VU0240551 exposure, expressed as percentages of pre-drug values, in medulla **(E)** and ponto-medullary **(F)** preparations. *Indicates significant intragroup differences in fR between a-CSF condition and VU0240551 application. # Indicates significant intergroup differences in fR between PH and CONT or P0-1 and P3-4. *$p < 0.05$, **$p < 0.01$, #$p < 0.05$. C4 electrical activity of the fourth cervical ventral nerve root; IC4: integrated activity of the C4 ventral nerve root.

preparations ($F_{4,28} = 4.482$, $p = 0.0063$; $n = 7$; **Figures 2C,D**). In contrast, in PH group, under VU0240551 exposure, fR was not modified in neither MS ($n = 8$, $p = 0.46$) nor PMS ($p = 0.58$; $n = 8$) preparations. Normalised fR (in % of pre-VU024055 values) was therefore lower in CONT than in PH ($p = 0.043$) in PMS preparations (**Figure 2F**).

P0-1 vs. P3-4

In MS preparations from both groups of animals, fR was similar with age during VU0240551 exposure, whereas in PMS preparations, a significant decrease in f_R occurred at P3-4 compared to P0-1 in CONT pups ($p = 0.035$; **Figures 2E,F**). This age change in KCC2 blockade was not observed in PH pups.

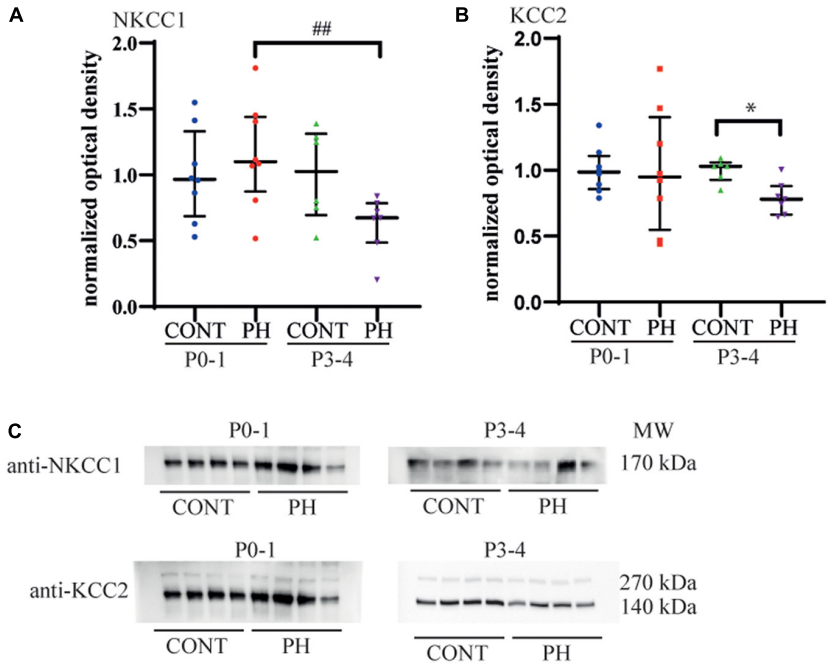

FIGURE 3 | KCC2 expression decreased in the pons of prenatal hypoxic pups at P3-4, compared to age matched controls. **(A)** Expression of NKCC1 and **(B)** KCC2 in the pons at P0-1 and P3-4 in CONT and PH groups, expressed as normalised optical density. **(C)** Images showing Western blots of NKCC1 and KCC2 at P0-1 and P3-4 in CONT and PH pups. In PH pups, NKCC1 expression was lower at P3-4 than at P0-1. At P3-4, KCC2 expression was lower in PH than in CONT group. Scatter plots with surimposed median [Q1; Q3]. *$p < 0.05$ indicates significant differences between CONT and PH groups and $^{\#\#}$ $p < 0.01$ indicate significant effect of age.

Membrane Expression of Cl^- Cotransporters

Since prenatal hypoxia disturbed both NKCC1 and KCC2 regulation of fictive breathing dependently from the pons, membrane expression of Cl^- cotransporters was analysed at this level. At P0-1, NKCC1 and KCC2 membrane expressions were similar in both groups, CONT and PH ($p = 0.48$ and $p = 0.7$, respectively; **Figures 3A–C**). By P3-4, although NKCC1 expression tended to be lower in PH pups than in CONT, there was no significant difference ($p = 0.07$; **Figures 3A,C**). At P3-4, KCC2 membrane expression was significantly decreased in the PH group in comparison with CONT pups at P3-4 ($p = 0.019$; **Figures 3B,C**). In PH pups, a significantly higher NKCC1 membrane expression was presented in P0-1 group compared to P3-4 group ($p = 0.006$; **Figures 3A,C**), and this effect of age was absent in CONT. The ratio NKCC1/KCC2 was similar in PH and CONT at both ages.

Prenatal Hypoxia Increased Brain-Derived Neurotrophic Factor Levels in Brainstem

Since prenatal hypoxia induced a decrease in the membrane expression Cl^- cotransporters in the pons at P3-4, we quantified BDNF, a contributor of their regulation, in this region at that age. We noted a significant increase in BDNF content ($U = 3.5$; $p = 0.031$; **Figure 4**) in PH pups in comparison with CONT pups.

Prenatal Hypoxia Increased the Number of Calpain-Positif Neurons in Brainstem Respiratory Structures

As prenatal hypoxia both supressed the decrease in fR induced by VU024055 and decreased the membrane expression of KCC2, we analysed distribution of calpain-positive neurons in pons aeras related to breathing. Locus Coeruleus and subcoeruleus nucleus had significantly more calpain-positive cells in PH pups ($p = 0.0079$ for each nucleus). The increase in calpain-positive cells was slight but significant in the Kolliker fuse ($p = 0.031$). We did not observe any difference in calpain positive cells in lateral and medial parabrachial nuclei ($p = 0.31$; **Table 1** and **Figure 5**).

Prenatal Hypoxia Disturbed the Modulation of Fictive Breathing Modulation by $GABA_A$ or/and Glycine Receptors Throughout Differential Effects on Medulla Oblongata and Pons

Picrotoxin application on MS or PMS preparations induced tonic discharges, regardless of age and the presence of the pons, as previously shown (Shao and Feldman, 1997; Iizuka, 2003).

P0-1

In CONT group, fR was not modified by picrotoxin exposure in neither MS ($p = 0.58$; $n = 10$; **Figures 6A,B**) nor PMS ($p = 0.54$; $n = 9$; **Figures 6C,D**) preparations, compared to that of the

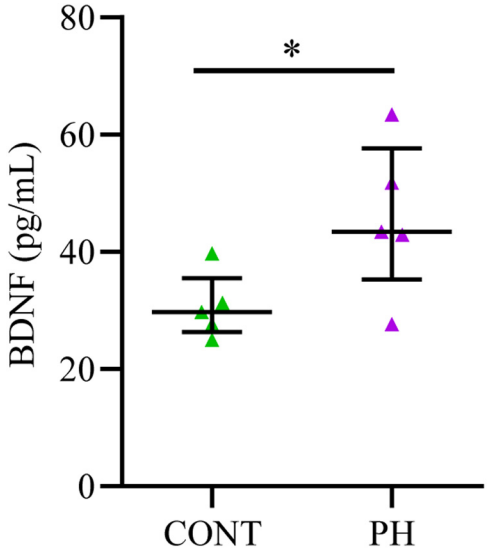

FIGURE 4 | BDNF concentration increased in the pons of prenatal hypoxic pups, compared to control pups. Concentrations of BDNF evaluated by ELISA in the pons of P3-4 control and hypoxic pups expressed in pg/mL after tissue processing. Values are shown as median [Q1; Q3]. *$p < 0.05$ indicates significant differences between CONT and PH groups.

TABLE 1 | Calpain expression in respiratory related areas of the pons in CONT an PH pups.

	CONT	PH
LC	0.3 [0.15,0.7]	4.6 [2.45,5.45] **
SubC	1.6 [0.5,2.95]	16.7 [10.8,21.35] **
lPB mPB	6.8 [1.35,10.3]	7.7 [4.85,18.95]
KF	1.7 [0.2,2.1]	4.4 [2.55,13.1] *

*Values represent the number of calpain positive cells per section in PH and CONT pups at P4 (median with interquartile). LC, locus coeruleus; SubC, sub coeruleus; lPB, lateral parabrachial; mPB, median parabrachial; KF, Kölliker-fuse nucleus. Asterisks indicate significant differences between PH and CONT group, *$p < 0.05$ and **$p < 0.01$.*

basal condition. In PH, fR of MS preparations also remained unchanged ($p = 0.91$; $n = 8$; **Figures 6A,B**) during exposure to picrotoxin. By contrast, fR was increased in PMS preparations of the PH group ($F_{4,32} = 12.93$, $p < 0.0001$; $n = 8$; **Figures 6C,D**) from 5 min during exposure to picrotoxin, compared to the basal condition. As a consequence, normalised fR (in % of pre-picrotoxin values) was higher in PMS preparations of PH pups than in CONT pups during the last 5 min of a-CSF–picrotoxin exposure ($p = 0.0003$; **Figure 6F**).

P3-4

In CONT group, picrotoxin–a-CSF perfusion enhanced the fR from the fifth minute of exposure in MS ($p = 0.0043$; $n = 7$; **Figures 6A,B**) and in PMS ($F_{4,28} = 9.845$, $p < 0.0001$; $n = 8$; **Figures 6C,D**) preparations, compared to basal conditions. In the PH group, blockade of $GABA_A$ also induced an increase in fR in MS ($p = 0.016$; $n = 7$; **Figures 6A,B**) and PMS ($F_{4,28} = 65.84$, $p < 0.0001$; $n = 8$; **Figures 6C,D**) preparations. In MS preparations from PH pups, normalised fR (in % of pre-picrotoxin values) was lower than fR in CONT pups during the last 5 min of a-CSF–picrotoxin exposure ($p = 0.041$; **Figure 6E**).

In CONT pups, normalised fR under picrotoxin was higher at P3-4 than P0-1 in MS ($p = 0.0107$; **Figure 6E**) preparations. In PH pups, normalised fR (in % of pre-picrotoxin values) was similar at P0-1 and P3-4 group in MS ($p = 0.92$; **Figure 6E**) and in PMS ($p = 0.23$; **Figure 6F**) preparations during a-CSF-picrotoxin exposure.

Prenatal Hypoxia and Glycine Receptors Blockade

P0-1

In CONT group, strychnine had no effect on fR regardless of the preparations MS ($p = 0.84$, $n = 10$; **Figures 7A,B**) or PMS ($p = 0.16$, $n = 9$; **Figures 7C,D**). In the PH group, strychnine exposure increased fR slightly and temporarily during strychnine exposure between 11 and 15 min of the test in MS preparations ($F_{4,32} = 2.943$, $n = 9$, $p = 0.035$; **Figures 7A,B**), whereas no change was observed in the PMS preparations ($p = 0.19$; $n = 8$; **Figures 7C,D**). Normalised fR (in % of pre-strychnine values) was not different between the PH and CONT pups at this age.

P3-4

In the CONT group, glycine receptors blockade had an opposite effect on MS and PMS preparations. In MS preparations, fR was enhanced from the tenth min of exposure until the end of the test ($F_{4,28} = 3.447$, $p = 0.02$; $n = 8$; **Figures 7A,B**). In contrast, in PMS preparation, fR was decreased from the tenth minute until the end of strychnine–aCSF exposure ($F_{4,28} = 4.598$, $p = 0.005$; $n = 8$; **Figures 7C,D**). In the PH group, strychnine–aCSF application had no impact on fR in MS preparations ($p = 0.11$; $n = 9$; **Figures 7A,B**). In PMS preparations, the ANOVA repeated measure test followed with a *post hoc* test indicated a significant increase in fR in the last 5 min of the strychnine exposure ($F_{4,28} = 2.887$, $p = 0.040$; **Figures 7C,D**). Normalised fR (in % of prestrychnine values) was significantly higher in the PH group than in the CONT group during the last 5 min of strychnine exposure in PMS preparations ($p = 0.011$; **Figure 7F**).

During blockade of glycine receptors, normalised fR (in % of pre-strychnine values) remains similar in MS preparations in P0-1 and P3-4 pups in CONT ($p = 0.27$) and in PH pups ($p = 0.76$; **Figure 7E**). Unlike in PH group, a significant increase in normalised fR (in % of pre-strychnine values) was observed in PMS preparations in P3-4 pups in comparison with P0-1 pups ($p = 0.026$; **Figure 7F**), whereas age had no effect in CONT pups in PMS preparations.

DISCUSSION

Our study focused on the impact of prenatal hypoxia on Cl^--dependent regulation of central respiratory drive. Interests surrounding this question arose from the following observations: (1) alterations of chloride homeostasis related to a change in membrane expression in NKCC1 and KCC2, which can result from environmental insults, including prenatal stress,

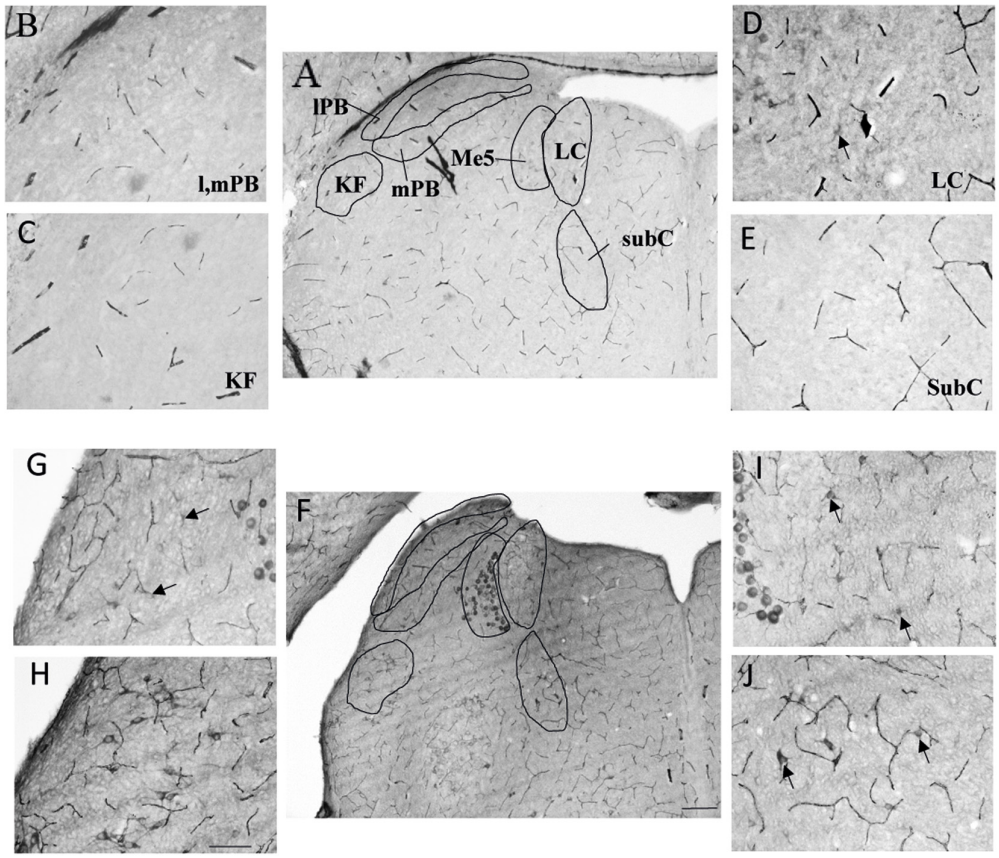

FIGURE 5 | Expression of calpain was stronger in the pons of prenatal hypoxic pups than in controls. Photomicrography of labelling of calpain in breathing-related nuclei in the pons of PH **(A–E)** and CONT **(F–J)** pups. **(B–E)** Higher magnification of A (CONT). **(G–J)** Higher magnification of F (PH). lPB, lateral parabrachial nucleus; mPB, median parabrachial nucleus; KF, kolliker fuse nucleus; LC, locus coeruleus; subC, subcoeruleus. **(A,F)** Scale bar = 200 μm; **(B–E,G–J)** Scale bar = 100 μm. Black arrows point at calpain-expressing cells.

(2) prenatal hypoxia is known to induce neurodevelopmental abnormalities, and (3) central respiratory drive, one of the earliest functions to develop in foetus, which is sensitive to prenatal hypoxic stress. Through analysing fictive breathing on *ex vivo en bloc* preparations under pharmacological applications, we showed that prenatal hypoxia disrupts NKCC1 and KCC2 Cl^--dependent regulation of central respiratory drive and influences GABA/$GABA_A$ and glycine systems whose cell effects depend on intracellular Cl^- concentration. Using western blot, ELISA test, and immunohistochemistry, we demonstrated that these functional data were closely linked to a pontin decrease in membrane expression of Cl^- cotransporters, an increase in BDNF in the pons, and an enhanced number of calpain-positive neurons in nuclei related to breathing.

Age-Dependant Effect of Central Respiratory Drive Modulation by NKCC1/KCC2 in the First Days of Postnatal Life

Our pharmacological results revealed changes in chloride homeostasis in the first few days after birth which led to changes in CRD modulation. For instance, blockade of NKCC1 decreased fR at P0-1 but produced no effect at P3-4. By contrast, KCC2 did not seem to modulate CRD at birth, but its blockade at P3-4 decreased fR when the pons was included in the preparation. Thus, chloride homeostasis strongly depends on NKCC1, but not on KCC2, in the respiratory network cells at P0-1, whereas opposite effects were found at P3-4. Such observations are consistent with data from the literature showing an age-dependent change in the effects of chloride-mediated conductances on fR. In rat brainstem–spinal cord preparations taken between E17 embryonic day and P2, a pretreatment with bumetanide was made in which the NKCC1 inhibitor, used in the present study, abolished the chloride-dependant $GABA_A$ respiratory effect, whereas the inhibition of KCC2 by furosemide had no significant effect before E18 but decreased fR in neonatal preparations (Ren and Greer, 2006). Thus, in addition to the impact on CRD of chloride cotransporters in the transition from foetus to newborn, our data showed that the CRD was strongly influenced by the maturation of chloride homeostasis during the first few days of postnatal life. Concerning the disappearance of respiratory modulation by NKCC1, membrane expression of NKCC1 in the medullary respiratory neurons did not vary within

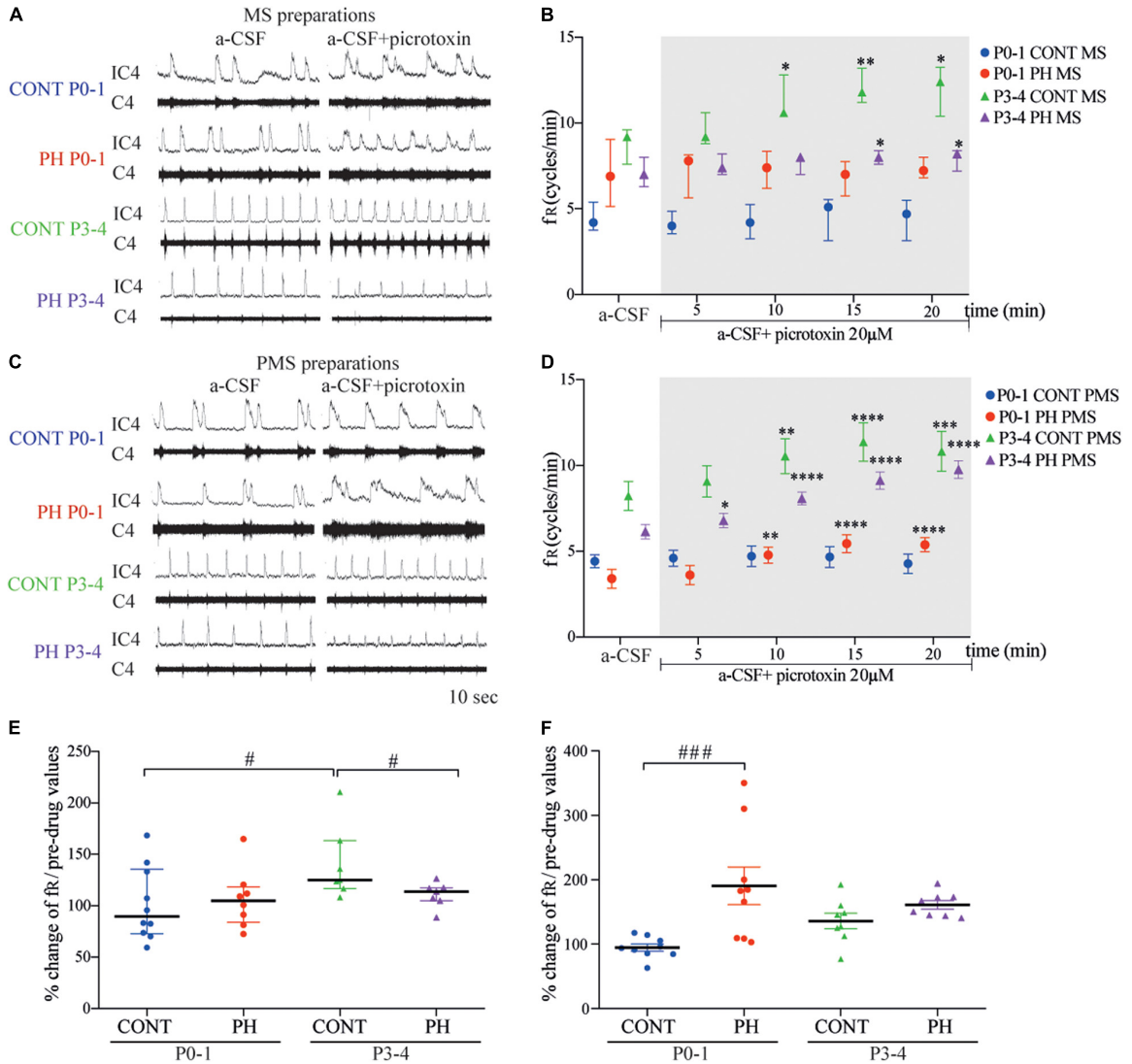

FIGURE 6 | $GABA_A$ receptor blockade increased fictive breathing frequency at P3-4 in all conditions. **(A,C)** Representative recordings of medulla **(A)** and ponto-medullary **(C)** preparations before (a-CSF) and during (a-CSF + picrotoxin) picrotoxin application, in control (CONT) and prenatal hypoxic (PH) pups, at P0-1 and P3-4. **(B,D)** Frequency of fictive breathing before (a-CSF) and every 5 min during (a-CSF + picrotoxin 20 µM) picrotoxin application, in CONT and PH pups, at P0-1 and P3-4. The fR is expressed as median [Q1; Q3] in medulla (MS) and as mean ± SEM in ponto-medullary (PMS) preparations. **(E,F)** Scatter plots with surimposed median [Q1; Q3] in medulla **(E)** and mean ± SEM in ponto-medullary **(F)** preparations, illustrating changes in fR during the last 5 min of picrotoxin exposure, expressed as percentages of pre-drug values. * indicates significant intragroup differences in fR between a-CSF condition and picrotoxin application. # indicates significant intergroup differences in fR between PH and CONT or P0-1 and P3-4. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, $^{\#}p < 0.05$, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$. C4 electrical activity of the 4th cervical ventral nerve root; IC4: integrated activity of the C4 ventral nerve root.

the first week of postnatal life (Liu and Wong-Riley, 2012; Wong-Riley et al., 2019). Maturation of its respiratory effect must, therefore, not depend on a generalised decrease in expression in respiratory cells but rather on its functionality. In such a context, we did not observe any significant decrease in NKCC1 membrane expression between P0-1 and P3-4. It is likely that maturation of respiratory modulation by NKCC1 within the first few days of life depends on the regulation of its phosphorylated form as already shown (Watanabe and Fukuda, 2015). On the implementation of CRD modulation by KCC2, our data suggest that maturation of KCC2 takes place heterogeneously within the respiratory network since we observed the appearance of a modulation of CRD at P3-4 only in presence of the pons. To our knowledge, our study is the first to discriminate medulla oblongata- or pons-dependent influences on the respiratory modulation by KCC2 in the first few days of life. While histological data have shown a gradual increase in KCC2 expression in cells of different medullary respiratory neuronal groups continuous from P0 to P10 (Wong-Riley et al., 2019), there are no data at the pontine level. In the medulla oblongata, overall KCC2 expression in respiratory nuclei reached maximum at P10 with, for example, an increase in preBötzinger complex and parafacial respiratory group by around 50% of P0

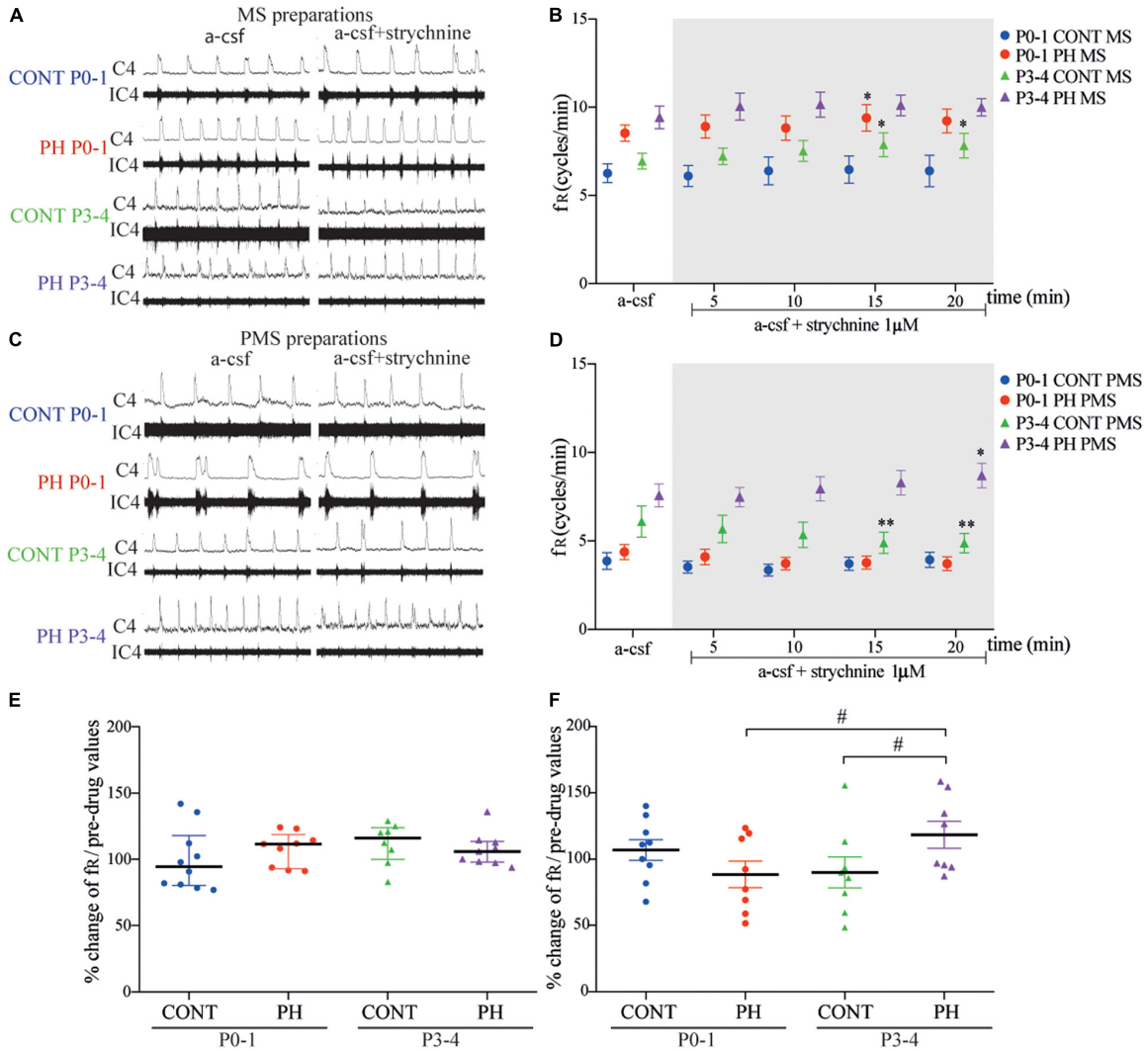

FIGURE 7 | Glycine receptor channels blockade in prenatal hypoxic pup medullo-spinal preparation increased fictive breathing frequency but decreased in pontine-medullo-spinal preparation. **(A,C)** Representative recordings of medulla **(A)** and ponto-medullary **(C)** preparations before (a-CSF) and during (a-CSF +strychnine) strychnine application, in controls (CONT) and hypoxic (PH) pups, at P0-1 and P3-4. **(B,D)** Mean ± SEM of fictive breathing frequency before (a-CSF) and every 5 min during (a-CSF + strychnine 1 µM) strychnine application, in CONT and PH pups, at P0-1 and P3-4, in medulla (MS) and ponto-medullary (PMS) preparations. **(E,F)** Scatter plots with surimposed median [Q1; Q3] in medulla **(E)** and mean ± SEM in ponto-medullary **(F)** preparations illustrating change of fR during the last 5 min of strychnine exposure, expressed as percentage of pre-drug values. * indicates significant intragroup differences in fR between a-CSF condition and strychnine application. # indicates significant intergroup differences in fR between PH and CONT or P0-1 and P3-4. *$p < 0.05$, **$p < 0.01$, #$p < 0.05$. C4 electrical activity of the fourth cervical ventral nerve root; IC4: integrated activity of the C4 ventral nerve root.

expression at P4 and 100% at P10 (Wong-Riley et al., 2019). Since we observed no effect of KCC2 blockade on CRD at P3-4 in medullary-spinal cord preparations, it is likely that KCC2 were either not addressed to the membrane or not functional. As we observed a respiratory impact in presence of the pons, we hypothesised that KCC2 maturation is more advanced in the pons at P3-4 than in the medulla oblongata in regard to respiratory regulation. Since our data suggest that there is no difference in the membrane addressing of KCC2 between P0-1 and P3-4, we hypothesised that the CRD modulation by KCC2 involving pons is linked to protein regulation, as we suggested for NKCC1 (Kelsch et al., 2001; Watanabe and Fukuda, 2015).

Prenatal Hypoxia Led to a Delayed Pons-Dependent Maturation of NKCC1/KCC2 Cl⁻ Cotransporters in the Modulation of Central Respiratory Drive

Pharmacological blockade of Cl⁻ cotransporters in *ex vivo* preparations of PH newborn rats suggests that prenatal hypoxia disturbs the pons-dependent maturation of chloride CRD modulation which takes place within the first days of postnatal life. First of all, at P0-1, NKCC1 did not regulate fR in PH preparations in contrast to the regulation observed in CONT pups and at P3-4. NKCC1, despite a drop in his expression with

age, still regulated the CRD in PH preparations with the pons, which was not the case at this stage in CONT. Second of all, at P3-4, although normal maturation led to a CRD regulation by KCC2 that involves pontine areas, this regulation was not observed in PH preparations. All together, these observations suggest a pivotal role of the pons in the effect of prenatal hypoxia on chloride CRD modulation within the first few days of life. Our data regarding the membrane expression of Cl^- cotransporters suggest a different effect of prenatal hypoxia on the two types of chloride transporters; although the pontine NKCC1 membrane expression was similar in CONT and PH, the membrane expression of KCC2 decreased in the pons of PH pups at P3-4. Unlike what was discussed concerning normal development, regulation of membrane expression of NKCC1 might be responsible for the effect of prenatal hypoxia on CRD regulation as well as regulations of the functional state of NKCC1 through dynamic regulation, such as phosphorylation variations (Watanabe and Fukuda, 2015). Concerning KCC2, membrane expression regulation seems to be involved. In fact, prenatal hypoxia ischemia induced a decrease in the expression of KCC2 related to an excess of calpain activation. This protein mediated the cleavage of KCC2 (Jantzie et al., 2016; Plantier et al., 2019). Higher levels of BDNF and an increase in calpain content in pontin respiratory nuclei observed in PH pups suggest the involvement of these underlying contributors. BDNF *via* activation of its high affinity receptor, TrkB, has indeed decreased KCC2 transcript and protein expression as shown in other physiological contexts (Rivera et al., 2002). Calpains, cystein intracellular proteases activated by intracellular Ca^{2+} or by BDNF and its receptor TrkB, lead to cleavage of KCC2 (Zadran et al., 2010; Puskarjov et al., 2012; Plantier et al., 2019). Among the pontin respiratory structures impacted by delayed maturation of KCC2, we hypothesised that Kolliker–Fuse nucleus that displayed an increase in calpain-positive neurons played a pivotal role in the alterations of CRD Cl^- regulation because of their recognised role in the CRD as the "pontin respiratory group" (Ikeda et al., 2017). It should also be noted that our histological data suggest an impact of prenatal hypoxia on two pontine structures that participate in the adaptation of CRD during hypoxic or hypercapnic challenges: the locus coeruleus and the subcoeruleus nucleus (Coates et al., 1985; Breen et al., 1997; Oyamada et al., 1998; Nitsos and Walker, 1999; Walker et al., 2000; Joubert et al., 2016). Such an observation may suggest that the disregulation of KCC2 induced by prenatal hypoxic in locus coeruleus and subcoeruleus could alter the ability to regulate CRD in a context of gas challenges.

Prenatal Hypoxia Altered the Regulation of Central Respiratory Drive by GABA/$GABA_A$ and Glycine

Developmental Aspect of the GABA/$GABA_A$ and Glycine Impact on Central Respiratory Drive

Based on our results, GABA and glycine do not seem essential to newborn rhythmogenesis, but seem crucial for the shaping and modulating of the overall respiratory network output. In P0-1 group of normoxic pups there was no change in fictive breathing frequency in medulla or ponto-medullary preparations during $GABA_A$ or glycine receptors blockade. Nevertheless, in P3-P4 group, medulla or ponto-medullary preparations exhibited an increase in fictive breathing when picrotoxin was applied, compared to the baseline condition. This observation supports the hypothesis that GABA became "inhibitory" between P0-1 and P3-4. It has been shown that bicuculline application, a $GABA_A$ antagonist, increased C4 burst frequency in medulla preparation from P0-4 rat pups (Kato et al., 2000), but no exact detail was given regarding a possible variation between ages. Moreover, in a study using mouse medulla slice, blockade of $GABA_A$ receptors did not alter rhythmic bursts in the XIIn between P0-P2, but increased this activity in slices from P4-P6 pups (Ritter and Zhang, 2000), thus confirming the postnatal set-up of the GABA inhibitory role. We found similar results when glycinergic receptors were blocked, strychnine had no effect at P0-P1 but rather at P3-P4. One could enhance or depress fictive breathing differentially according to the absence or the presence of the pons in preparations which supports the idea that glycinergic systems, such as the GABAergic system, undergo drastic changes after birth. It also highlights the role of the pons in the central respiratory command, and how it changes after birth. We then focused on the comparison between preparations with and without the pons in order to better understand its function and how GABA and glycine are involved.

Abnormal Development of GABA/$GABA_A$ and Glycine Inhibition of Central Respiratory Drive After Prenatal Hypoxia

In P0-1 group of normoxic pups, we did not observe any difference in fictive breathing frequency in medulla or ponto-medullary preparations during $GABA_A$ or glycine receptors blockade. However, P0-1 preparations with or without pons coming from PH pups exhibited significant differences. The fictive breathing frequency was increased during $GABA_A$ blockade in ponto-medullary preparations and during glycine receptors blockade in medullary preparations. Since the f_R in the PMS preparation was lower in PH pups than the f_R in CONT groups, and since $GABA_A$ receptor blockade increased the f_R in PH but not in CONT, it is possible that prenatal hypoxia leads to increased efficiency of the GABAergic system on CRD.

The stimulating effects of glycine have already been described in studies where prenatal medulla preparations were exposed to nicotine. Direct glycine exposure was made and it showed fictive breathing depression in rat medulla preparations, compared to control preparations. Depression was accentuated by exposure to glycine baths and was abolished using strychnine (Luo et al., 2004, 2007). Interestingly, from P0-1 we obtained an effect of strychnine in PH preparations which was not detectable in CONT pup preparations, which suggests that a glycinergic modulation of the respiratory network in the medulla was altered by the prenatal hypoxia. In fact, strychnine exposure can lead to a difference in loss between PH and CONT preparations. Concerning the effect

of $GABA_A$ blockade, picrotoxin induced an increase in fictive breathing frequency in ponto-medullary PH pup preparations, but not in the CONT group. This result suggests a change in the GABAergic pontine influence of medullary respiratory network after prenatal hypoxia. Muscimol, an agonist of $GABA_A$ receptor, has been shown to enhance the depression of fictive breathing frequency or apnea duration in medulla-spinal cord preparations (Luo et al., 2004, 2007). Our results indicate that prenatal hypoxia had no impact on GABAergic modulation of breathing at the medulla level, but modified GABAergic pontine modulation of medullary respiratory network.

The influences of activation by $GABA_A$ or glycine receptors on breathing network change with maturation (Paton and Richter, 1995; Ritter and Zhang, 2000; Zhang et al., 2002; Ren and Greer, 2006; Okabe et al., 2015). In P3-4 aged pups, the $GABA_A$ antagonist increased fictive breathing frequency in the two groups regardless of the preparations. While glycine receptor blockade increased f_R in medulla preparation and decreased it in ponto-medullary preparation of CONT pups, prenatal hypoxia led to different results as follows: in medulla preparations, strychnine had no influence but it slightly enhanced fictive breathing frequency in the ponto-medullary preparations at the end of the test. The changes in the influence of the blockade of $GABA_A$ or glycine between normoxic and PH pups on fictive breathing frequency might be related to the differential maturation of NKCC1 and KCC2 cotransporters in the pons or medulla. In normoxic pups, bumetanide had no effect on both preparations; whereas it decreased f_R in ponto-medullary preparations of the PH pups, and had no influence on the medullary preparations. The KCC2 cotransporters inhibition with VU0240551 had no effect in PH pups in the two preparations, but it decreased fictive breathing frequency in ponto-medullary preparations with no effect on medullary preparations in CONT pups. Results in CONT pups are concordant with literature. Before P1, pretreatment with bumetanide abolished the influence of muscimol on breathing, while the blockade of KCC2 cotransporters was ineffective. However, it became effective after P1 and either attenuated or abolished the effect of muscimol (Ren and Greer, 2006) or decreased the XII burst frequency in mouse (Okabe et al., 2015). Furthermore, additional experiments could investigate whether prenatal hypoxia modulates the number of GABA and glycine receptors. If this were the case, such modulations could contribute to the alterations highlighted in this study.

CONCLUSION

In conclusion, the present study highlights impact of Cl^- cotransporter disruption caused by prenatal hypoxia on the respiratory drive by pointing out the involvement of pontin neural groups and $GABA/GABA_A$ and glycin neurotransmission systems. Our data also suggest that pharmacological paradigms aimed to reduce BDNF, or that calpain levels could represent promising strategies to normalise KCC2 levels in the context of prenatal hypoxia, or high risks for the emergence of neurodevelopmental disorders, a medical field where there are little pharmacological preventive approaches.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the INT Neurosciences Ethic Committee No. 71 for Animal Research.

AUTHOR CONTRIBUTIONS

CC contributed substantially to acquisition and analysis of electrophysiological and pharmacological data and manuscript preparation. AC contributed substantially to acquisition and analysis of immunohistochemical data, statistical analysis of electrophysiological data, and manuscript preparation. J-OC review of manuscript. SL and CB contributed substantially to acquisition of Western Blots. LB contributed substantially to data interpretation, literature search, discussion of results and implications, and writing of the manuscript. JP contributed substantially to study design and manuscript preparation. FC contributed substantially to study design, data interpretation, literature search, analysis of data, and writing of the manuscript. All authors are accountable for all the aspect of the work.

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The Cardiovascular and Metabolic Effects of Chronic Hypoxia in Animal Models: A Mini-Review

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Animal models are useful to understand the myriad physiological effects of hypoxia. Such models attempt to recapitulate the hypoxemia of human disease in various ways. In this mini-review, we consider the various animal models which have been deployed to understand the effects of chronic hypoxia on pulmonary and systemic blood pressure, glucose and lipid metabolism, atherosclerosis, and stroke. Chronic sustained hypoxia (CSH)—a model of chronic lung or heart diseases in which hypoxemia may be longstanding and persistent, or of high altitude, in which effective atmospheric oxygen concentration is low—reliably induces pulmonary hypertension in rodents, and appears to have protective effects on glucose metabolism. Chronic intermittent hypoxia (CIH) has long been used as a model of obstructive sleep apnea (OSA), in which recurrent airway occlusion results in intermittent reductions in oxyhemoglobin saturations throughout the night. CIH was first shown to increase systemic blood pressure, but has also been associated with other maladaptive physiological changes, including glucose dysregulation, atherosclerosis, progression of nonalcoholic fatty liver disease, and endothelial dysfunction. However, models of CIH have generally been implemented so as to mimic severe human OSA, with comparatively less focus on milder hypoxic regimens. Here we discuss CSH and CIH conceptually, the effects of these stimuli, and limitations of the available data.

Keywords: animal modeling, chronic hypoxia, cardiovascular disease, high altitude, sleep, metabolism

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INTRODUCTION

Animal models have been useful for demonstrating various physiological effects of hypoxia, thus providing deeper understanding of the impact of hypoxemia in human disease. Chronic sustained hypoxia (CSH) and chronic intermittent hypoxia (CIH) are each associated with cardiovascular and metabolic changes, which can be adaptive or maladaptive. In this mini-review we consider the outcomes associated with both CSH and CIH as they pertain to cardio-metabolic disease. Specifically, we will address cardiovascular and metabolic outcomes of CSH and CIH models in animals which aim to mimic human disease states. We will not focus on models of acute sustained or intermittent hypoxia (lasting minutes to hours), which may have variable consequences. Moreover, in this mini-review, we consider only CSH and CIH models which might resemble chronic hypoxic conditions in humans. Intriguing reports of the effects of intermittent hypoxia on neuroplasticity with low-frequency hypoxic episodes lasting several minutes (Gonzalez-Rothi et al., 2015; Navarrete-Opazo

et al., 2015) are subjects of other expert reviews (Randelman et al., 2021). Finally, we note that our intention is to cover significant breadth of understanding of the topic of cardio-metabolic consequences of chronic hypoxia in animal models, sacrificing some depth of specific models and outcomes. We invite the reader to explore specific citations for important study details.

HYPOXIA AS A MODEL OF HUMAN DISEASE

Both CSH and CIH in animal models have been used to simulate various disease states. CSH has been applied to rodents at varying fraction of inspired oxygen (FiO_2), generally ranging from 0.10 to 0.15 (Hislop and Reid, 1976; Cowburn et al., 2017; Ioja et al., 2018; Prieto-Lloret et al., 2021), either been normobaric or hypobaric relative to sea level. Though resulting peripheral saturations are not always considered, the severity of CSH is a critical variable: For instance, CSH of FiO_2 0.10 as a model of high altitude exposure might recapitulate the effective oxygen content at an altitude of 5800 m (Mt. Kilimanjaro), whereas an FiO_2 of 0.15 might be representative of a lower altitude (2400 m, Aspen, CO). In considering analogues of human disease, an FiO_2 of 0.10 would be expected to model only very hypoxemic diseases like cyanotic heart disease, whereas an FiO_2 of 0.15 might model chronic obstructive pulmonary disease (COPD), or other chronic lung diseases which are far more common.

Similarly, CIH has been applied to animal models in a variety of ways, although most studies roughly reproduce conditions used by Fletcher *et al.*, who first studied CIH in rodents as a model of OSA (Fletcher et al., 1992b). In CIH, multiple variables of desaturation and resaturation are important to define. In Fletcher's experiments, rats were exposed to rapid reductions of FiO_2 from 0.21 to 0.05 over 12 s, then quickly returned to 0.21. This process was repeated every 90 s (corresponding to an oxyhemoglobin desaturation index [ODI] of 40 events/h), for 7 h per day, for up to 5 weeks. Each of these variables—rate of deoxygenation, depth of deoxygenation, rate of reoxygenation, ODI, duration of daily exposure, and overall experiment duration—may be manipulated in different animal experiments (Farré et al., 2018). At least one study has demonstrated tissue-specific effects of various hypoxic profiles of CIH in rodents (Reinke et al., 2011). Thus, there are several considerations when designing animal experiments seeking to elucidate the physiological effects of either CSH or CIH.

CARDIO-METABOLIC EFFECTS OF CHRONIC SUSTAINED HYPOXIA

CSH and Pulmonary Hypertension

In humans and in animal models, acute alveolar hypoxia has been shown to cause pulmonary vasoconstriction, leading to acute pulmonary hypertension (PH) (Fishman, 1976; Wagenvoort, 1977; Rabinovitch et al., 1979; Perkin and Anas, 1984; Voelkel, 1986). Both hypoxic pulmonary vasoconstriction and PH may revert after cessation of hypoxic exposure. By contrast, exposure to CSH results in chronic PH which may be irreversible (Meyrick and Reid, 1978; Stenmark et al., 2009). Vascular remodeling due to CSH consists of muscularization of the small arteries of the alveolar wall and proliferation of cells expressing α-smooth muscle actin, followed by thickening of the precapillary pulmonary arteries, inflammation, and fibrosis of the large proximal pulmonary arteries (Stenmark et al., 2009). CSH causes PH so reliably in rodents that it has been widely adopted as a model for studying mechanisms and downstream effects of PH. However, the response to CSH is variable between species (Stenmark et al., 2009). Although CSH leads to PH both in mice and rats, the degree of vascular remodeling is typically less in mice (Hislop and Reid, 1976; Frank et al., 2008; Cahill et al., 2012).

Sustained Hypoxia and Systemic Blood Pressure

While CSH causes PH in rodent models, the effect of CSH on systemic blood pressure is less clear. Acute ascent to high altitude, an inherently hypoxic environment, can reversibly increase systemic blood pressure (Bender et al., 1988; Wolfel et al., 1991, Wolfel et al., 1994). Epidemiological studies have shown that humans living at high altitude have *lower* systemic blood pressure than those living at sea level (Rotta, 1947; Ruiz and Peñaloza, 1977), highlighting the difference between acute exposure and those acclimatized to such an environment. In rodents exposed to normobaric or hypobaric CSH, results have been mixed. Vilar *et al.* demonstrated a reduction in blood pressure in spontaneously hypertensive rats after exposure to normobaric CSH (FiO_2 of 0.10 for 8 weeks) (Vilar et al., 2008), induction of pro-angiogenic pathways; and they showed that neutralizing antibodies targeting vascular endothelial growth factor-A (VEGF-A) both abrogated the effects of hypoxia on angiogenesis, and increased blood pressure. Other studies also showed that CSH decreased systemic blood pressure in young spontaneously hypertensive rats (Henley and Tucker, 1987), and that hypoxia mitigated blood pressure elevation in the renal hypertensive rat (Fregly, 1963, Fregly, 1970). However, one study demonstrated that CSH (FiO_2 of 0.10) did change blood pressure in male rats at durations of anywhere from 1 to 30 days, despite an increase in carotid body catecholaminergic signaling (Hui et al., 2003). Our group has also not observed changes in systemic blood pressure in young mice exposed to 40 days of CSH of similar severity (Zhen et al., 2021). Vaziri *et al.* demonstrated *increased* blood pressure in rats exposed to hypobaric CSH (effective FiO_2 of 0.10–0.11) that persisted even after the restoration of normoxia (Vaziri and Wang, 1996). Thus, the effects of CSH on systemic blood pressure are complex, and perhaps dependent on the specific conditions and animals.

Effects of CSH on Atherosclerosis and Stroke

Atherosclerosis is the major underlying etiology of cardiovascular disease, which is the leading cause of death worldwide (Mendis et al., 2011). Evidence for the contribution of hypoxia to the

progression of atherosclerosis is largely circumstantial. Hypoxia inducible factor-1α (HIF-1α), a subunit of HIF-1, the major regulator of the cellular response to hypoxia, is normally quickly hydroxylated and degraded in normoxia. In hypoxia, however, HIF-1α is stabilized and can dimerize with HIF-1β, allowing binding to hypoxia responsive elements in the promoter regions of target genes of interest (Iyer et al., 1998). HIF-1α is stabilized in macrophages and smooth muscle cells near the necrotic core of atherosclerotic vascular lesions in humans and in animal models (Sluimer et al., 2008; Lim et al., 2013; Ferns and Heikal, 2017), and HIF-1 has been implicated in atherosclerosis progression (Kasivisvanathan et al., 2011). Moreover, hyperbaric oxygen (FiO_2 1.0, 2.4–2.5 atm) improves atherosclerosis in both rabbits and mice (Kudchodkar et al., 2000, Kudchodkar et al., 2007, Kudchodkar et al., 2008). It is therefore conceivable that hypoxia could contribute to the development of atherosclerosis, but to our knowledge, CSH has never been shown to directly impact atherosclerosis in animal models.

Atherosclerosis, among other factors, may lead to acute ischemic stroke, which causes over 130,000 deaths in the United States yearly. Patients with pre-existing atherosclerotic lesions who then become hypoxemic (e.g., respiratory failure in the ICU setting) may develop sufficient brain ischemia to manifest as a stroke. However, recent data suggest that acute hypoxic exposure in animal models of ischemic stroke may be protective. Mice with stroke induced by middle cerebral artery occlusion and then exposed to variably severe hypoxia (FiO_2 of 0.07–0.12) for two to 8 weeks (Zhang et al., 2020) showed improved collateral blood flow in a "dose-dependent" manner, with more severe and longer duration of hypoxia generating more robust collateral circulation. These effects were durable even after cessation of hypoxia. These data suggest that while some effects of CSH may be maladaptive, some might be beneficial, and that adaptive responses to hypoxia may present in unique ways.

Metabolic Effects of CSH

Despite our ability to implement CSH as a stimulus with relative ease in animal studies, the metabolic effects of CSH are less well explored than the cardiovascular effects. Gamboa *et al.* were the first to recognize the potentially beneficial effects of CSH on glucose metabolism (Gamboa et al., 2011), finding that CSH with an FiO_2 of 0.10 reduced plasma fasting glucose and insulin, increased insulin sensitivity, and improved insulin-dependent glucose uptake by skeletal muscle. Since that time, similar findings have been replicated by us (Zhen et al., 2021) and others (Lee et al., 2013; Ioja et al., 2018), with additional data demonstrating hypoxia-dependent effects on the liver transcriptome (Zhen et al., 2021) and changes in liver and skeletal muscle mitochondrial function (Ioja et al., 2018). Lipid metabolism also appears to be altered in CSH, with elevated serum triglyceride and low-density lipoprotein levels resulting from CSH with an FiO_2 of 0.10 (Zhen et al., 2021). We and others have noted that CSH causes weight loss in rodents. In our studies, however, we found a complex interaction between hypoxia and weight, and that beneficial metabolic effects of CSH cannot solely be explained by weight reduction (Zhen et al., 2021).

CARDIO-METABOLIC EFFECTS OF CHRONIC INTERMITTENT HYPOXIA

CIH has been used to model OSA, the most common respiratory disease in the world (Benjafield et al., 2019). Epidemiologic associations have been made between OSA and a wide variety of adverse health outcomes, including cardiovascular disease, diabetes, cognitive and mood disorders, and others. However, OSA has several significant manifestations aside from intermittent hypoxemia, including hypercapnia, intrathoracic pressure swings, and fragmented sleep. CIH models attempt to understand the mechanisms by which the hypoxemia of OSA may uniquely contribute to these outcomes of interest.

CIH and Pulmonary Hypertension

OSA in humans is associated with PH, although the effect is typically mild (Sajkov and McEvoy, 2009) and the impact of OSA on PH independent of other comorbidities has been debated (Chaouat et al., 1996; Sajkov et al., 1999). In OSA, the duration of hypoxemia resulting from respiratory events (apneas or hypopneas), rather than the frequency of respiratory events as gauged by the apnea-hypopnea index *per se*, is linked with more severe pulmonary hypertension (Samhouri et al., 2020). In early animal models involving dogs, repetitive airway occlusion was induced by tracheal obstruction of variable duration. These studies showed that pulmonary arterial (PA) pressure increased as a function of the severity of desaturation (Iwase et al., 1992). Further, the authors showed that airway occlusion in animals allowed to breathe 100% oxygen (which prevented significant desaturations), did not increase PA pressure. Likewise, when another set of dogs were allowed to breathe hypoxic gas without airway occlusion, PA pressures increased. These observations suggested that hypoxemia is likely the most critical of the several physiological manifestations of OSA to cause PH. There are several studies examining the impact of CIH on pulmonary hypertension in rodent models (Fagan, 2001; McGuire and Bradford, 2001; Campen et al., 2005; Nisbet et al., 2009; Nara et al., 2015; Snow et al., 2020; Zhen et al., 2021). Some of these studies appear to show increases in right ventricular systolic pressure, right ventricular mass, and/or pulmonary vascular remodeling in response to CIH, although we did not observe these effects in young C57BL/6J mice (Zhen et al., 2021). CIH also does not increase right ventricular pressures to the same degree as CSH (Fagan, 2001; Zhen et al., 2021). Any putative effect of CIH to worsen pulmonary hypertension may be due to changes in nitric oxide bioavailability (Nisbet et al., 2009) and/or increases in endothelin-1 expression and endothelial damage (Wang et al., 2013), leading to pulmonary vasoconstriction.

CIH and Systemic Blood Pressure

As mentioned above, the first demonstrated physiological effects of CIH were to increase systemic blood pressure in rats (Fletcher et al., 1992b). Since that time, this finding has been demonstrated by others (Fletcher, 2001; Prabhakar and Kumar, 2010). The major mechanism by which CIH is thought to induce hypertension is by activation of the sympathetic nervous

system. Fletcher *et al.* showed that surgical denervation of peripheral chemoreceptors in the carotid body prevented CIH-induced elevations in arterial blood pressure in rats (Fletcher et al., 1992a). CIH also impairs endothelium-dependent vasodilation of skeletal muscle resistance arteries (Tahawi et al., 2001; Phillips et al., 2004) and causes vascular remodeling (Fletcher et al., 1992b). CIH increases the responsiveness of the carotid body to hypoxia, causing upregulation of pro-inflammatory cytokines, and activation of the sympathetic nervous system (Lesske et al., 1997; Braga et al., 2006; Dick et al., 2007; Lam et al., 2012, Lam et al., 2014; Zoccal et al., 2019). HIF-1 has also been implicated in the development of hypertension in animal models, via downstream effects on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Yuan et al., 2011; Schulz et al., 2014; Semenza and Prabhakar, 2015). The CIH-mediated increase in blood pressure persists even after cessation of CIH, and CIH may increase blood pressure in male rats in as little as 2 days of exposure (Hinojosa-Laborde and Mifflin, 2005). Our group has noted that there may be acclimatization to CIH causing normalization of blood pressure over prolonged periods (4–5 weeks) (Zhen et al., 2021), but more work is needed to define this effect further.

Effects of CIH on Atherosclerosis and Stroke

While exposing wild-type mice to CIH may induce vascular inflammation and remodeling (Gileles-Hillel et al., 2014), it does not appear to result in overt atherosclerosis (Savransky et al., 2007b; Drager et al., 2013), even after prolonged exposure (e.g., 20 weeks) (Song et al., 2012). However, atherosclerosis is observed in wild-type mice fed a high cholesterol diet in conjunction with exposure to CIH (Savransky et al., 2007b). Additionally, in studies using ApoE-deficient mice, which are more susceptible to atherogenesis, CIH exposure induces atherosclerosis (Jun et al., 2010; Arnaud et al., 2011; Fang et al., 2012; Gautier-Veyret et al., 2013). The major mechanism for the development of atherosclerosis in CIH appears to be the excess expression of pro-inflammatory pathways. Nuclear factor kappa B (NF-κB) is important for the development of atherosclerosis in rodents exposed to CIH (Fang et al., 2012; Song et al., 2018), and HIF-1 also may play a role in the development of CIH-induced atherosclerosis (Drager et al., 2013; Zhou et al., 2014).

Compared to the outcomes mentioned above, few animal studies have examined the relationship between CIH and stroke, even though human epidemiological studies have strongly linked OSA to stroke risk (Dyken et al., 1996; Yaggi et al., 2005; Das and Khan, 2012). CIH increases the brain's susceptibility to hypoxia by altering cerebral blood flow (Phillips et al., 2004; Capone et al., 2012). Mechanisms for this include increased endotheliln-1 and increased oxidative stress via NADPH oxidase (Capone et al., 2012). Canzani *et al.* demonstrated that intermittent airway obstruction increased reperfusion injury in a mouse model of ischemia-reperfusion injury (Cananzi et al., 2020). Another intriguing study showed that CIH with a nadir FiO_2 of 0.10 may be neuroprotective, whereas a nadir FiO_2 of 0.06 may exacerbate neurological damage (Jackman et al., 2014), suggesting that the specific model of CIH, mimicking a specific severity of OSA, is fundamentally important.

Metabolic Effects of CIH

CIH also reliably impacts glucose and lipid metabolism. CIH induces insulin resistance and glucose intolerance in obese mice, whether due to diet or genetic modification (leptin-deficient Ob^-/Ob^- mice) (Polotsky et al., 2003; Drager et al., 2011). We and others have noted similar effects of CIH in lean mice (Iiyori et al., 2007; Zhen et al., 2021). Although some groups have noted either sex-specific effects of CIH on glucose metabolism (Marcouiller et al., 2021), or improvement in glucose tolerance with CIH (Polotsky et al., 2003; Carreras et al., 2012; Thomas et al., 2017), this is usually accompanied by an increase in whole-body insulin resistance, suggesting the complexity of the response to CIH on glycemia, which may at best be mixed, and in some scenarios deleterious. Additionally, CIH worsens nonalcoholic fatty liver disease and other types of liver injury in mice with diet-induced obesity (Savransky et al., 2007a, Savransky et al., 2007c; Mesarwi et al., 2021), and alters lipid metabolism (Drager et al., 2012; Jun et al., 2012; Yao et al., 2013; Zhen et al., 2021). It is important to note that the CIH model used in these studies is frequently designed to simulate severe OSA—that is, with severe reductions in nadir FiO_2 (0.04–0.07), and a high ODI. The effects of less severe CIH on glucose and lipid metabolism are not well described.

FUTURE DIRECTIONS

Though much has been accomplished regarding our understanding of the diverse cardio-metabolic consequences of CSH and CIH, there are clearly areas which merit further investigation. First, there are gaps in our understanding of the physiological effects of milder CIH and CSH. CSH has been investigated mostly with an FiO_2 of 0.10, which likely represents a level of hypoxemia more severe than commonly observed in chronic heart/lung diseases in humans. It has been suggested that one might expect adaptive, rather than maladaptive, physiological responses to milder CIH (Navarrete-Opazo and Mitchell, 2014). Second, some of the outcomes presented in this mini-review have only a minimal amount of accompanying mechanistic data; there is undoubtedly room to devote more complete exploration of these concepts. Third, in particular when considering effects of CSH, one must consider whether normobaric hypoxia differs from hypobaric hypoxia, which has relevance for studies involving physiological outcomes of CSH models intended to mimic exposure to high altitude. Although there has been debate about this topic for years (Millet et al., 2012; Mounier and Brugniaux, 2012), animal studies examining the effects of CSH are typically not performed in both conditions, creating uncertainty about the impact of atmospheric pressure on the outcome being measured. Indeed, the uncertainty on this point extends to human-based research as well (Coppel et al., 2015). Finally, in our group, we have noted unique cardio-metabolic consequences of combined sustained and intermittent hypoxia,

or "overlap hypoxia", which may be used to model the COPD/OSA overlap syndrome, or periodic breathing at high altitude (Zhen et al., 2021). A systematic approach to understanding the hypoxemia of this unique condition is needed.

CONCLUSION

Both CSH and CIH are associated with unique, and sometimes maladaptive, physiological responses, though there are considerable differences between these types of hypoxic exposures. CSH and CIH are intended to mimic hypoxemia in human disease states, but the heterogeneity of hypoxemia severity in cardiovascular and pulmonary disease mandates that attention be given to novel and more nuanced models. Future work can be directed toward these goals, as well as toward better understanding of the mechanisms by which hypoxia alters cardio-metabolic physiology in animals.

AUTHOR CONTRIBUTIONS

All authors agree to be accountable for the content of the work in this manuscript. OM, AS, and LB all contributed equally to the writing of the manuscript. LB and OM made final edits.

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REVIEW
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Time Domains of Hypoxia Responses and -Omics Insights

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The ability to respond rapidly to changes in oxygen tension is critical for many forms of life. Challenges to oxygen homeostasis, specifically in the contexts of evolutionary biology and biomedicine, provide important insights into mechanisms of hypoxia adaptation and tolerance. Here we synthesize findings across varying time domains of hypoxia in terms of oxygen delivery, ranging from early animal to modern human evolution and examine the potential impacts of environmental and clinical challenges through emerging multi-omics approaches. We discuss how diverse animal species have adapted to hypoxic environments, how humans vary in their responses to hypoxia (i.e., in the context of high-altitude exposure, cardiopulmonary disease, and sleep apnea), and how findings from each of these fields inform the other and lead to promising new directions in basic and clinical hypoxia research.

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1 INTRODUCTION

In parallel with the evolution of aerobic metabolism, various organisms evolved mechanisms for adapting to decreased oxygen (O_2) availability. These alterations can be categorized by time domains ranging from gradual changes in atmospheric O_2 concentrations over millions of years to immediate physiological responses upon exposure to hypoxic stress. In this review, we discuss adaptations and responses to hypoxia that span 1) millions of years of non-human animal evolution, 2) hundreds of generations since modern humans have occupied high altitudes, 3) physiological and epigenetic changes within a lifespan, and 4) physiologic and pathophysiologic hypoxic challenges. We highlight how different species have adapted to reduced O_2 availability and how humans exhibit variable individual capacities to alter hypoxia pathway responses. We approach these topics from multiple perspectives and emphasize the importance of integrating physiological and -omics effects of hypoxia for future progress in biological and medical applications.

2 TIME DOMAIN 1: ADAPTATION TO HYPOXIA ACROSS NON-HUMAN SPECIES

2.1 Oxygen Over Geologic Time and its Impact on Early Animal Pathway Evolution

Since the emergence of eukaryotes and multicellular life, Earth's atmospheric and oceanic O_2 levels have been in constant flux. It is estimated that atmospheric O_2 ranged between 15 and 30% over the last 550 million years (Berner, 1999). During this time, most of the animal phyla we know today emerged and evolved, adapting to changes in O_2 availability. Many major events in the evolutionary history of life coincided with rising atmospheric O_2 levels (Falkowski et al., 2005; Berner et al., 2007; Mills et al., 2014).

Key hypoxia sensing and response pathways evolved to ensure prompt physiological responses when O_2 demand exceeded supply. For example, the hypoxia-inducible factor (HIF) pathway coordinates expression of thousands of genes in response to hypoxia (Semenza, 2020) and is highly conserved with a majority of metazoans expressing HIF homologues (Hampton-Smith and Peet, 2009). The HIF pathway evolved in the common ancestor of Bilateria, Cnidaria, and Placozoa, as modern representatives of earlier-branching metazoan lineages (Ctenophores and Poriferans) lack the ability to respond to changes in O_2 *via* transcriptional regulation (Mills et al., 2018). Once the HIF pathway was established, it underwent changes in complexity as O_2 requirements became more demanding in larger animals and tissue metabolic requirements became specialized (Taylor and McElwain, 2010).

2.2 Physiological Adaptations to Hypoxia in Tetrapods

Comparative studies provide empirical generalizations about the nature of hypoxia adaptations and yield unique insight into adaptive mechanisms that would otherwise remain unknown. Air-breathing animals adapt to high-altitude hypoxia through physiological adjustments that sustain O_2 flux to tissue mitochondria and thereby support aerobic ATP production. The first physiological response to hypoxemia is to increase ventilation to minimize the decline in the arterial partial pressure of O_2 (P_{O2}), which occurs within the first minutes of hypoxia exposure through the hypoxic ventilatory response (Teppema and Dahan, 2010; Ivy and Scott, 2015) (further discussed in **Section 4**). Studies of deer mice reveal that evolutionary adaptation to high altitude changes the hypoxic ventilatory chemoreflex to further increase breathing and pulmonary O_2 uptake while attenuating sympathoadrenal activation (Scott et al., 2019; Storz and Scott, 2019). Highland deer mice maintain higher rates of alveolar ventilation and preserve ventilatory sensitivity to CO_2 relative to lowland conspecifics (Ivy and Scott, 2017, 2018). Known exceptions to this common increase in ventilation are the naked mole rat and guinea pig (Gonzalez-Obeso et al., 2017). The naked mole rat is an extremely hypoxia-tolerant mammal that lives underground and has been shown to decrease ventilation upon hypoxia (Pamenter et al., 2015).

Changes in lung structure and function also represent important adaptations to chronic hypoxia at high altitude. Hypoxia during early life can impair septation (the partitioning of saccules into alveoli) and thus impede lung development (Blanco et al., 1991; Ambalavanan et al., 2008). These effects are overcome in high-altitude deer mice (West C. M. et al., 2021), whereby they and many other high-altitude animals exhibit larger lungs and/or higher alveolar surface density than low-altitude counterparts (Pearson and Pearson, 1976; Lechner, 1977; Maina et al., 2017). High-altitude deer mice are also more effective at ventilation-perfusion matching in chronic hypoxia than low-altitude mice (West CM. et al., 2021). These changes help increase the morphological and physiological capacities for O_2 diffusion of the lungs, and thus help augment arterial P_{O2} and O_2 saturation in chronic hypoxia.

Another adaptive mechanism to improve arterial O_2 saturation is achieved through genetically based changes in hemoglobin (Hb), the protein responsible for circulatory O_2 transport (Storz, 2016, 2019). Many vertebrates native to high altitude have evolved increased Hb-O_2 affinity (Storz, 2016) and, in some cases, the adaptive protein modification helps safeguard arterial O_2 saturation under severe hypoxia (Tate et al., 2017, 2020; Storz and Bautista, 2021). These changes are attributed to amino acid replacements at numerous sites in the *a*- and *ß*-chain subunits of the $\alpha_2\beta_2$ Hb tetramer (Storz et al., 2010; Natarajan et al., 2015a, 2015b, 2016, 2018; Tufts et al., 2015; Zhu et al., 2018; Signore et al., 2019). A regulatory mechanism of adaptation was

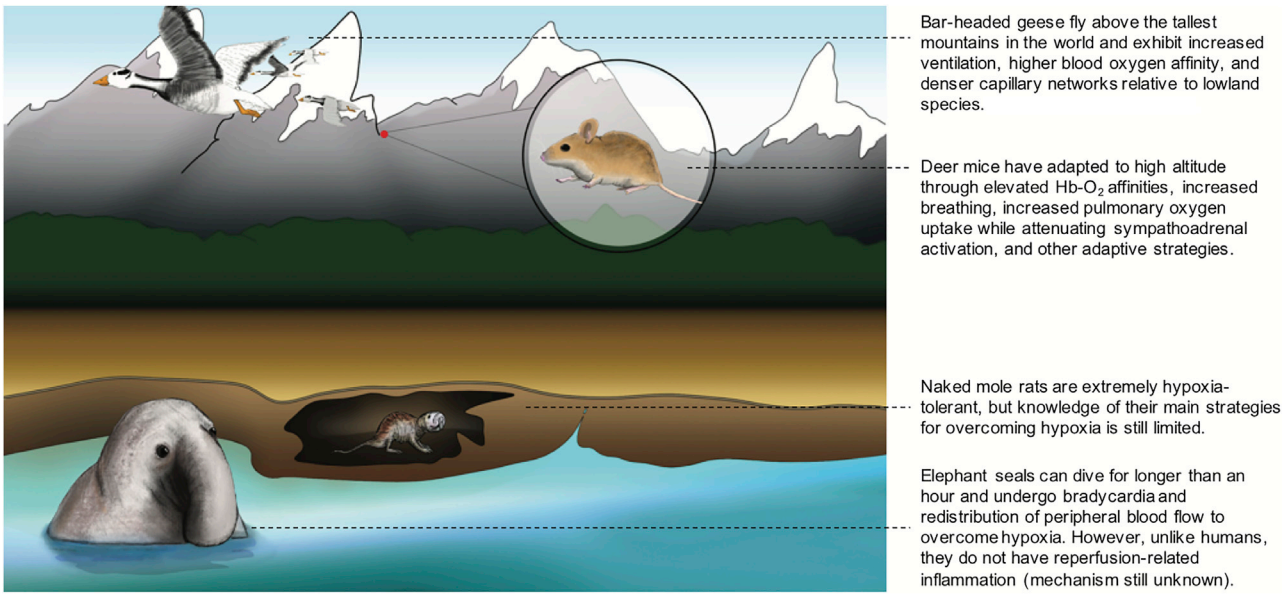

FIGURE 1 | Different strategies that animals utilize to overcome hypoxia. Bar-headed geese fly above the tallest mountains in the world and exhibit increased ventilation, higher blood oxygen affinity, and denser capillary networks relative to lowland species among other adaptive traits. Deer mice have adapted to high altitude through elevated Hb-O_2 affinities, increased breathing, increased pulmonary oxygen uptake while attenuating sympathoadrenal activation, and other adaptive strategies. Naked mole rats are extremely hypoxia-tolerant, but knowledge of their main strategies for overcoming hypoxia is still limite. Elephant seals can dive for longer than an hour and undergo bradycardia and redistribution of peripheral blood flow to overcome hypoxia. However, unlike humans, they do not have reperfusion-related inflammation (mechanism still unknown).

also documented in the Tibetan antelope, whereby an increased Hb-O_2 affinity is achieved *via* an Hb isoform switch (Signore and Storz, 2020). The convergent evolution of increased Hb-O_2 affinity in high-altitude taxa highlights the importance of this phenotype in hypoxia adaptation (Storz, 2016). However, numerous exceptions have also been documented where high-altitude specialists have not evolved elevated Hb-O_2 affinities in comparison with lowland sister taxa (Revsbech et al., 2013; Janecka et al., 2015).

Although an elevated Hb-O_2 affinity can improve arterial O_2 saturation under conditions of severe hypoxia, the resultant increase in arterial O_2 content only translates into an increased capacity for O_2 transport to tissues if it is associated with a sufficiently high tissue O_2 diffusion capacity (Wearing et al., 2021). Accordingly, bar-headed geese and high-altitude deer mice have evolved elevated Hb-O_2 affinities (Storz et al., 2010; Jendroszek et al., 2018; Natarajan et al., 2018) in conjunction with derived muscle phenotypes that improve O_2 diffusion capacity and O_2 utilization, as characterized by increased capillary density, oxidative fiber density, mitochondrial volume density, and mitochondrial oxidative capacity (Scott GR. et al., 2015, Scott et al., 2015 G. R.; Lui et al., 2015; Mahalingam et al., 2017, 2020; Tate et al., 2017, 2020; Nikel et al., 2018; Storz et al., 2019). High-altitude deer mice can also achieve higher cardiac output in hypoxia than low-altitude mice (Tate et al., 2020; Wearing et al., 2022). The combined effects of increases in arterial O_2 saturation, cardiac output, and tissue O_2 extraction lead to pronounced increases in aerobic capacity in hypoxia (Tate et al., 2020). Storz and Scott (2019) provide an overview of O_2-transport pathway mechanisms.

The distinct responses to hypoxia across high-altitude birds shows how attempts to interpret how species match O_2 supply to O_2 demand requires consideration of the integrated function of all the steps in the O_2 transport cascade. Thus, while bar-headed geese demonstrate surprisingly large increases in ventilation accompanied by a fall in pulmonary O_2 extraction in hypoxia (Scott and Milsom, 2007; Lague et al., 2016), this produces a respiratory alkalosis that should enhance Hb-O_2 binding, increasing arterial O_2 content (CaO_2) and reducing the need to increase cardiac output (Lague et al., 2017). On the other hand, the high mass-specific cardiac output seen in high-altitude speckled teal and ruddy ducks despite a large O_2 carrying capacity of the blood appears to be essential to support the high mass-specific metabolic rates of these smaller species (Ivy et al., 2019; Laguë et al., 2020; Milsom et al., 2021). In short, there is tremendous diversity in how different high-altitude avian species match O_2 supply to demand. Different strategies animals utilize to mitigate impacts of hypoxia are illustrated in **Figure 1**.

2.3 Physiological Adaptations to Hypoxia in Marine Mammals

Some of the most dramatic mammalian adaptations to hypoxia have evolved in diving mammals that recolonized marine environments 50–30 million years ago (Berta, 2015). Unlike high-altitude or burrowing animals, marine mammals do not

face issues with pulmonary-O_2 loading since O_2 uptake occurs at sea level. Instead, hypoxemia is induced by breath-hold diving and results from depletion of blood and muscle O_2 stores (Thewissen et al., 2009). In deep-diving mammals, the lungs contribute very little to O_2 storage capacity, as the animals routinely experience alveolar collapse at depth, forcing reliance on blood and muscle O_2 stores during dives (McDonald and Ponganis, 2012). For this reason, deep-diving mammals evolved to have extremely high concentrations of Hb in blood and myoglobin in muscle as a mechanism to increase O_2 storage capacity (Ponganis, 2015). To maximize the use of on-board O_2 stores, marine mammals exhibit dramatic cardiovascular adjustments while diving and surfacing, known as the dive response. At the onset of a breath-hold dive, marine mammals experience dramatic reductions in heart rate, which is matched by a high degree of peripheral vasoconstriction to maintain a steady mean arterial pressure (Davis and Williams, 2012). This bradycardia and tissue ischemia ensures that hypoxia-sensitive organs, such as the brain and heart, have consistent supplies of blood arriving throughout the dive. Other organs (e.g., kidney, liver, spleen), which experience severely reduced blood delivery during dives, must utilize alternative O_2 stores (e.g., myoglobin), or rely on anaerobic metabolism to meet energetic demands (Kooyman et al., 2021). When an animal surfaces from a dive, ischemic tissues undergo reperfusion and heart rate increases well above resting levels to maintain mean arterial pressure. Despite a lifestyle of chronic ischemia-reperfusion events to many of their tissues, these mammals appear to have no detrimental side effects and, therefore, represent excellent mammalian models to study potential cytoprotective mechanisms.

Hypoxia tolerance in marine mammals has been studied extensively in the northern elephant seal, an elite diver that spends up to eight months continuously foraging at sea and conducting dives that may be in excess of one and a half hours (Hassrick et al., 2010). Elephant seals undergo routine apneas even when on land when asleep, exhibiting physiological adjustments similar to those during dives mentioned previously (Ponganis et al., 2006; Stockard et al., 2007; Tift et al., 2013). During these terrestrial apneas, elephant seals experience a reduced muscle blood flow of only 46% of the average muscle blood flow during normal breathing (Ponganis et al., 2008). The animals routinely experience hypoxemia during these terrestrial apneas (PaO_2 ~ 30 mmHg), and the degree of blood O_2 depletion during dives is even more dramatic (PaO_2 ~ 10 mmHg) (Meir et al., 2009). Considering these animals spend over 80% of their lives in a breath-hold, it is remarkable that their tissues can tolerate these degrees of hypoxemia and repeated ischemia/reperfusion events.

In fact, unlike humans and most other mammals, marine mammals do not show evidence of tissue damage in response to repeated bouts of severe hypoxia or ischemia-reperfusion events (Vázquez-Medina et al., 2011; Vázquez-Medina et al., 2012). Potential strategies to avoid injury include mechanisms to cope with ischemic inflammation and reperfusion-derived oxidant generation. In particular, the glutathione system likely plays a key role in antioxidant defense in marine mammals. Glutathione levels in tissues and circulation are significantly higher in diving than in non-diving mammals (Vázquez-Medina et al., 2007; García-Castañeda et al., 2017). Similarly, several genes involved in the glutathione system are under positive selection or expanded in marine mammals (Yim et al., 2014; Zhou et al., 2018).

Limiting ischemic inflammation is crucial to avoid reperfusion injury. Evidence shows that deep-diving Weddell and elephant seals possess a yet-to-be-identified anti-inflammatory component in their plasma (Bagchi et al., 2018). These same two species also have the highest levels of endogenous carbon monoxide (CO) ever measured, with levels in the blood approaching those seen in chronic cigarette smokers (Pugh, 1959; Tift et al., 2014; Tift and Ponganis, 2019). Interestingly, exposure to low or moderate levels of CO has been shown to have potent cytoprotective effects (Motterlini and Otterbein, 2010). The primary source of endogenous CO production in mammals is the degradation of heme by heme oxygenase enzymes (Tenhunen et al., 1968). Therefore, elevated Hb and myoglobin stores result in more heme that could be degraded to produce CO endogenously. The mechanism of cytoprotection is also not completely understood, but there are several studies that have now shown CO can provide tissue-specific protection against injuries associated with hypoxia and ischemia-reperfusion events (Tift et al., 2020). These unanswered questions may be best examined in the elephant seal due to its propensity for voluntary breath-holds and the existence of established endocrine, biochemical, and molecular techniques to work with the animals (Khudyakov et al., 2015; Crocker et al., 2016). Ongoing investigations using *ex vivo* systems that are amenable to physiological manipulation and molecular perturbation can also complement *in vivo* studies while providing insights into mechanisms that confer natural tolerance to hypoxemia and ischemia/reperfusion in diving mammals as described by Allen and Vazquez-Medina (2019) and Lam et al. (2020).

2.4 Genomic Analyses Across Multiple Species Reveal Converging Patterns of Hypoxia Adaptation

For species that have adapted to hypoxic environments, key traits show consistent genetic evidence for convergent evolution. The HIF pathway is reported as a major genetic target of selection in multiple species native to the Tibetan Plateau. For example, *Endothelial PAS Domain Protein 1* (*EPAS1*), the gene that encodes the HIF-2α subunit, is reported to be important for high-altitude adaptation in Tibetan yak and antelope (Wang et al., 2015), snakes (Li et al., 2018), dogs (Wang et al., 2014), and wolves (vonHoldt et al., 2017) (reviewed in Pamenter et al., 2020). These key adaptations in HIF-pathway genes have been well summarized recently across many hypoxia-adapted species (Storz, 2021; Storz and Cheviron, 2021), including domesticated animals (Witt and Huerta-Sánchez, 2019). While the phenotypic effects of many of these naturally occurring *EPAS1* variants are not well understood, recent studies of highland deer mice have shown that allelic variation is associated with altered cardiovascular function and transcriptomic responses to hypoxia (Schweizer et al., 2019). Specifically, the allele that predominates at high altitude is associated with an elevated heart rate under hypoxia and

reduced expression of genes involved in catecholamine biosynthesis and secretion. Many of these effects seem to be attributable to a single non-synonymous substitution that disrupts the interaction between HIF-2 alpha and its transcriptional co-activator, CREB-binding protein (Song et al., 2021). Further evidence for convergent adaptation is reported at the Hb gene region across various species and impacts Hb-O_2 affinity, as mentioned in **Section 2.2** (Storz 2019).

Experiments in the fruit fly, *Drosophila melanogaster*, have also yielded insights into key genetic pathways for hypoxia adaptation, primarily involving the Notch pathway. The Notch signaling pathway is highly conserved across animal species and regulates many aspects of development, cell-cell signaling, and tissue renewal (Kopan and Ilagan, 2009). In hypoxia, Notch-responsive promoters are activated, and the HIF-1 transcription factor is recruited to these promoter sites (Gustafsson et al., 2005). Notch pathway genes were shown to underlie hypoxia adaptation and increased Notch activity conferred improved hypoxia tolerance in *Drosophila* after hypoxia exposure for 200 generations (Zhou et al., 2011, 2021) and have been reported as putatively adaptive genes in Andean populations at high altitude (Bigham et al., 2009). Research aimed at analyzing the cross-talk between HIF and NOTCH pathways in highland populations will provide greater insight into long-term hypoxia adaptations in humans (O'Brien et al., 2022).

Information from extinct species provide additional insight into the genetic factors underlying long-term hypoxia adaptation. The growing accessibility of ancient DNA sequencing coupled with *in vitro* expression systems permits the resurrection of phenotypes once lost to evolutionary time (Campbell et al., 2010; Mirceta et al., 2013; Huerta-Sánchez et al., 2014; Campbell and Hofreiter, 2015; Signore, 2016). For example, the extinct great auk (*Pinguinus impennis*) is an ideal candidate to study phenotypic changes associated with breath-hold hypoxia, as this alcid very recently diverged from its aerial relatives (razorbills and murres) and became a flightless diving specialist. While some morphological changes associated with the great auk's air-to-sea transition are present in the fossil record, the critical physiological processes that accompanied this transition have been lost. However, ancient DNA sequencing and *in vitro* expression of the great auk's Hb proteins suggest this species also evolved an adaptive increase in Hb-O_2 binding affinity (Berenbrink et al., 2017). Information on ancient adaptive trends in DNA can also be gleaned from existing animals' genomes. For example, an adaptive gene region with linked genetic makers (i.e., a haplotype) containing *EPAS1* and *Protein Kinase C Epsilon* (*PRKCE*) is thought to have originated in high-altitude Tibetan wolves and mixed into highland dogs' genomes, a process called introgression, more than 10,000 years ago (vonHoldt et al., 2017). This introgression mirrors the finding of Denisovan (archaic human) DNA in modern Tibetan human genomes, also in the form of a haplotype containing *EPAS1* (Huerta-Sánchez et al., 2014; Hu et al., 2017). Understanding adaptive introgression in other populations and at other genomic locations could provide important insight into evolutionary processes in hypoxia-adapted species.

3 TIME DOMAIN 2: ADAPTATION TO HYPOXIA IN HUMANS

3.1 Physiological Adaptations to Hypoxia in Humans

Compared to other high-altitude species, humans have persisted at high altitude for a relatively short period of time. Humans first inhabited high altitudes hundreds of generations ago, with reports suggesting as long as 30,000 to 40,000 years on the Tibetan Plateau (Rademaker et al., 2014; Capriles et al., 2016; Zhang et al., 2018). Although human occupation of high altitude is significantly shorter than other species mentioned above, humans display distinct physiological adaptations to hypoxia that have developed over thousands of years, and these patterns of adaptation vary by continental group (Beall, 2007; Bigham et al., 2009; Simonson, 2015; Luks et al., 2021).

While there is considerable variation within populations, studies of high-altitude residents indicate many individuals of Tibetan ancestry exhibit larger lung volumes, elevated resting ventilation, and elevated hypoxic ventilatory responses compared to both Han Chinese high-altitude residents and Andean high-altitude groups (Hackett et al., 1980; Sun et al., 1990; Droma et al., 1991; Zhuang et al., 1993; Beall et al., 1997; Curran et al., 1997; Moore, 2000; Wu and Kayser, 2006; Beall, 2007). Contrary to Tibetans, many Andean groups exhibit blunted ventilatory responses to hypoxia (Beall, 2007; Heinrich et al., 2020) with associations noted between hematocrit and daytime and sleep oxygen saturation in Andean men and women as well as blunted heart rate response to hypoxia in men (Heinrich et al., 2020).

Variation in hemoglobin concentration ([Hb]) has been well characterized and replicated in many studies of high-altitude populations. Tibetan highlanders generally maintain [Hb] with ranges typically observed in populations living at or near sea-level, while Andean highlanders have, on average, much higher [Hb] (Beall, 2007). Native Amhara high-altitude residents of the Simien Plateau of Ethiopia demonstrate similar [Hb] and erythropoietin concentrations to Tibetans and are also able to maintain higher O_2 saturation levels than either Andeans or Tibetans (Beall et al., 2002). In contrast, Native Oromo high-altitude residents of the Bale Plateau of Ethiopia have elevated hemoglobin concentration and low O_2 saturation (Lundgrin et al., 2013).

The precise mechanisms underlying differences in [Hb] phenotypes among high-altitude human populations remains an active area of research. Studies suggest plasma volume as the key adaptive phenotype that underlies lower [Hb] in Sherpa relative to Andean males with comparable blood volumes (Stembridge et al., 2019). In Tibetan males, relatively lower [Hb] was associated with higher peak VO_2, which was further associated with heart and muscle diffusion components of O_2 transport (Simonson et al., 2015). It is further plausible that variation in red cell lifespan/destruction underlie within and across population variation (Tift et al., 2020). Additional genetic studies, as discussed in **Section 3.2**, and further physiological and functional assessments will provide much needed insight into these mechanisms.

Differences are also apparent in traits associated with blood flow and O_2 diffusing capacity, particularly relating to the

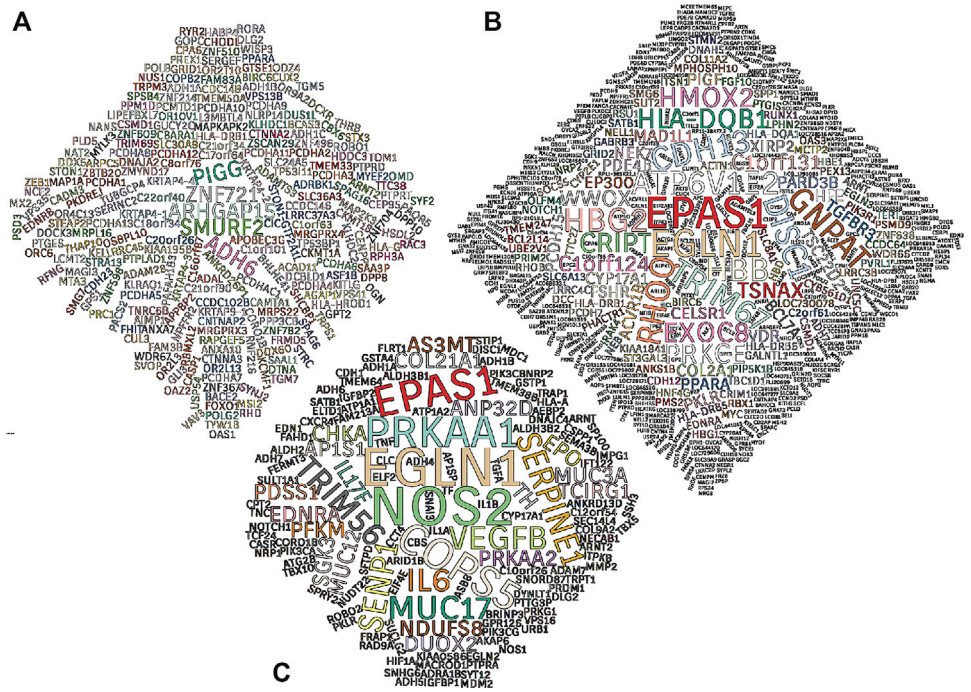

FIGURE 2 | Word clouds of genes under positive selection. Genes reported as top targets of positive selection in high-altitude human populations illustrated in word clouds. A total of 31 publications were used to establish word clouds based on four, fifteen, and twelve original studies from Ethiopian **(A)**, Tibetan **(B)**, and Andean **(C)** populations, respectively. All genes included are mentioned by name in the main text of at least one study and/or mentioned more than once in the supplementary materials section of the publication. Text size is indicative of the number of times a top selection candidate gene is mentioned. Gene symbols are validated using the Ensembl genome database. Major statistical methods considered in this analysis are FST, PBS, iHS and XP-EHH. Gene lists of this analysis can be found in the Supplementary Table.

signaling molecule and vasodilator nitric oxide (NO). Tibetans display higher exhaled NO than Andeans (Beall et al., 2001), and this elevated NO is associated with enhanced pulmonary blood flow (Hoit et al., 2005) and may account for the lower pulmonary artery pressures observed in Tibetans (Groves et al., 1993; Hoit et al., 2005; Beall, 2007). Tibetans also demonstrate higher circulating NO and bioactive NO products compared to sea-level residents, with the former being associated with variants in the regulator gene of nitric oxide synthase, *GCH1* (Guo et al., 2017), and the latter with higher forearm blood flow (Erzurum et al., 2007). Some Himalayan Sherpa also demonstrate higher sublingual capillary densities and microcirculatory blood flow at high altitude (5,300 m) compared to sea-level residents (Gilbert-Kawai et al., 2017). As discussed in **Section 5.3**, individual variation in NO could further underlie susceptibility to high-altitude illnesses, such as high altitude pulmonary hypertension.

While distinct adaptive phenotypes are observed across high-altitude human populations, it is important to recognize that substantial within-population variation exists among each group. Furthermore, the extent to which observed trait differences reflect genetically based adaptive changes, environmentally induced effects, and/or plasticity in terms of gene-environment interactions requires additional functional investigation (Hall et al., 2020). The likely importance of gene-environment interactions in shaping phenotypes in high-altitude natives is emphasized by recent work in deer mice, which shows that high-altitude populations have evolved altered responses to chronic hypoxia for several cardiorespiratory traits (Storz and Scott, 2019).

3.2 Genomic Evidence for Positive Selection in Humans

All studies reporting genetic adaptation to high altitude employ statistical tests that derive from observations that a) only two of the four forces driving genetic evolution–gene flow and natural selection–have directional effects, and b) of these, gene flow affects all loci (particular positions in the genome) in the same way, whereas natural selection acts on specific loci (Lewontin and Krakauer, 1973). While such tests have their limitations (Jensen et al., 2016), the wide range of tests employed and consistent results across multiple studies provide substantial evidence that natural selection has operated on specific genes in the multigenerational residents of Ethiopian, Himalayan, and Andean high-altitude populations. The genes reported in many of these studies are contained within regions of the genome that exhibit a distinct pattern (i.e., a "selective sweep," summarized in Simonson (2015)). In most cases, the specific genetic change in the DNA underlying the adaptation is unknown but is likely within or near the gene reported. A compilation of genes reported in human high-altitude adaptation studies are visually summarized in **Figure 2**, highlighting top adaptive genes reported among Ethiopians (**Figure 2A**), Tibetans (**Figure 2B**), and Andeans (**Figure 2C**).

While many HIF pathway genes are reported as targets of selection in highland populations, only some are associated with known, putatively adaptive phenotypes and these associations vary across populations. In genomic studies of Tibetans, allelic variants at HIF genes such as *EPAS1* and *Egl-9 Family Hypoxia Inducible Factor 1* (*EGLN1*) exhibit significant associations with [Hb] (Beall et al., 2002; Simonson et al., 2010; Yi et al., 2010) but do not provide information about the direct modulation of this phenotype (reviewed in Simonson (2015)). Lower [Hb] in Tibetan males was also associated with regulatory variants in the *Heme oxygenase 2* (*HMOX2*) gene, reported as a top selection candidate gene in Tibetans (Simonson et al., 2010), and these regulatory variants were associated with higher *HMOX2* expression in cell culture analyses (Yang et al., 2016). These findings suggest upregulation of the heme oxygenase/carbon monoxide (HO/CO) pathway that is involved in heme degradation–the same HO/CO pathway relevant to elephant seal adaptation (discussed in **Section 2.3**). Elevated [Hb] in Andeans is associated with increased endogenous CO, suggesting potential convergence of elevated CO through different rates of erythrocyte production versus lifespan/destruction in each population (Tift et al., 2020).Variants within the HIF genes and other adaptive genetic factors may affect various steps in the O_2 transport pathways and/or metabolic O_2 utilization that prevent cellular O_2 levels from declining as much as they would otherwise thereby attenuating the hypoxic stimulus that induces erythropoiesis or plasma volume contraction (Storz, 2021; Storz and Cheviron, 2021). The relatively low [Hb] observed in many Tibetan highlanders may potentially be an indirect consequence of how allelic variants of genes like *EPAS1* modulate components of O_2-sensing/transport.

From an evolutionary perspective, identifying functional links between genotypes showing evidence of positive selection and reproductive success is of particular importance. One such strategy has focused on determinants of birth weight since a) birth weight is the most important determinant of neonatal or infant mortality, b) mortality risk during pregnancy and through the first year of life is greater than at any other time prior to the end of the reproductive period (i.e., the period during which natural selection is acting), and c) the profound effect of high altitude to reduce birth weight (Jensen and Moore, 1997). Tibetan and Andean ancestry confer protection against hypoxia-associated reductions in birth weight compared to newcomer groups residing at the same altitude, reviewed in Moore (2021). In Andeans, this effect appears to stem, in part, from greater uteroplacental blood flow and O_2 delivery during pregnancy at high altitude in women of Andean versus European ancestry (Julian et al., 2009). Such effects appear to be genetic, not developmental, in origin (Julian et al., 2011). One genomic region near adaptive protein kinase, AMP-activated, alpha 1 (*PRKAA1*), the gene encoding the α1 catalytic subunit of adenosine monophosphate kinase (AMPK), not only shows evidence of positive selection in Andeans but has also been associated with greater birth weight and uterine artery diameter at high altitude, a major determinant of uteroplacental blood flow (Bigham et al., 2014). Moreover, AMPK activation *in vitro* in pregnant human uterine vessels or *in vivo* in murine models have potent vasodilator effects that are associated with maintenance of fetal growth during high-altitude exposure (Lane et al., 2020; Lorca et al., 2020). While much remains to be learned, given that drugs such as metformin activate AMPK and have been safely used in pregnancy, such studies offer the possibility of yielding new therapies for treating or preventing pregnancy disorders characterized by uteroplacental ischemia and hypoxia.

3.3 Evolutionary Significance in Medicine

In the fields of precision and personalized medicine, it is necessary to consider individual genetic factors that may underlie variation in particular phenotypes or pathologies. In present-day highland populations, unique evolutionary histories help contextualize such variation, i.e., adaptations and maladaptations. Each population's genetic landscape has been shaped by standing (existing) genetic variation, admixture (mixture of genetic material from different populations), and/or *de novo* (novel) mutations that have occurred throughout hundreds of generations at high altitude. An example pertinent to the history of Tibetan adaptation is the introgression of the previously mentioned *EPAS1* gene region as mentioned in **Section 2.4**. The genetic sequence in this region has proven crucial for Tibetan adaptation and is more similar to an archaic human population that no longer exists, the Denisovans. The sequence of DNA at the *EPAS1* region is more similar to Denisovan DNA than other available human genome sequences, reflecting adaptive introgression from this population tens of thousands of years ago (Huerta-Sánchez et al., 2014; Hu et al., 2017).

Furthermore, while a vast majority of genome-based association studies have focused on populations of European ancestry (Sirugo et al., 2019), an understanding of physiological and genomic variation in other historically understudied populations are sorely needed to provide a more complete understanding of human variation relevant to hypoxia tolerance and genomic medicine. It is further important that as raw genomic data continue to increase in value as a global commodity, researchers ensure genome donors have a say in how their information is utilized and that part of the benefits received are returned to the original participants (Fox, 2020; Hall et al., 2020).

4 TIME DOMAIN 3: PHYSIOLOGICAL AND EPIGENETIC CHANGES IN RESPONSE TO HYPOXIA WITHIN A LIFETIME

In addition to long-term generational population-level adaptation, responses to acute (minutes) or chronic hypoxic stress (days) may occur in order to maintain oxygen homeostasis. These responses may be under genetic/epigenetic control and reflect ranges of species, population, and individual variation. Such differences could prove adaptive to a point or lead to maladaptive phenotypes, e.g., in cases of overcompensation such as excessive erythrocytosis (Hancco et al., 2020) as discussed in **Section 5.2**. The first physiological response to an acute hypoxic stimulus is the hypoxic ventilatory response (HVR), which involves an increase in minute ventilation due to the activation of the peripheral chemoreceptor mainly located in the carotid bodies (Powell et al., 1998; Teppema and Dahan, 2010;

Ivy and Scott, 2015; Pamenter and Powell, 2016). This response shapes how one acclimatizes to hypoxia and has the potential to impact the extent of hypoxia experienced, thereby impacting molecular changes that may lead to a cascade of physiological changes. Epigenetic modifications, in interaction with the genome, the environment, and other regulatory factors, provide a mechanism for environmental stresses such as hypoxia to modify gene expression early in development and throughout the life course. While various steps of O_2 transport may be altered over shorter time domains within the lifetime, this Section focuses primarily on the initial hypoxia responses involving the first steps of O_2 transport.

4.1 Hypoxia Responses in Early Life Stages

The magnitude of hypoxia responses vary across life stages. The initial increase of ventilation in neonates is moderate compared to the HVR observed in adults (Teppema and Dahan, 2010; Dzal et al., 2020), and the HVR is usually accompanied by a decrease in metabolic rate not observed in adulthood (Mortola, 2004; Mortola and Maskrey, 2011). The HVR in newborns is mediated by the peripheral chemoreceptors and progressively increases with age as the result of chemosensory reflex maturation (Teppema and Dahan, 2010; Dzal et al., 2020) and a decrease of HVD with maturation (Bissonnette, 2000; Renolleau et al., 2001). The newborn phase is critical for the development of respiratory control (Carroll, 2003), as changes in O_2 levels, such as hypoxia or hyperoxia, may cause alterations in respiratory control with long-lasting effects (Carroll, 2003; Bavis, 2005, 2020; Lofaso et al., 2007; Teppema and Dahan, 2010). Given the long-lasting repercussions of O_2–related challenges, it is plausible that hypoxic events, *via* epigenetic modifications, may result in phenotypic plasticity. Such modifications may lead to notable physiological changes and/or distinct gene expression, proteomic, and metabolomic profiles as discussed further in this Section and the final Section of this review. Indeed, recent reports describe epigenetic changes with hypoxia exposure in neonates (Bustelo et al., 2020; Tong et al., 2021), and epigenetic modifications in peripheral chemoreceptors have been induced with hypoxia exposure (Nanduri et al., 2012, 2017a, 2017b; Prabhakar, 2013). These findings highlight the crucial impact of O_2 levels in early stages.

4.2 Ventilatory Responses to Acute and Sustained Hypoxia

Ventilatory responses vary based on the duration of hypoxia exposure. The HVR is a reflex response initiated when glomus or type 1 cells in the carotid body detect a decrease in the arterial levels of oxygen, producing an increase in intracellular Ca^{2+} and release of one (or more) neurotransmitters to terminals of the carotid sinus nerve (Teppema and Dahan, 2010; Ortega-Sáenz et al., 2013; Prabhakar and Semenza, 2015; Iturriaga et al., 2021). These neurotransmitters produce an increase in the frequency of action potentials through the glossopharyngeal nerve to respiratory centers of the brainstem, resulting in activation of the phrenic nerve, activation of the diaphragm, and increased respiratory frequency and tidal volume to produce increased ventilation (Teppema and Dahan, 2010; Pamenter and Powell, 2016; Ortega-Sáenz and López-Barneo, 2020). The specific nature of the O_2 sensor in the glomus cells is currently debated in terms of: a) metabolism-related mechanisms (i.e., processes occurring in the mitochondria), b) membrane-linked O_2 sensor mechanisms (e.g., potassium and/or calcium channels, or olfactory receptors), and c) mechanisms involving gasotransmitters determining or modulating the chemoreceptor activity (e.g., carbon monoxide, hydrogen sulfide, nitric oxide) (Prabhakar and Semenza, 2015; López-Barneo et al., 2016; Iturriaga et al., 2021), which are all plausible targets for different adaptations. For example, how the HVR is beneficial under specific durations and patterns of hypoxia, the extent of genetic/epigenetic control versus other mechanisms of physiological plasticity, and links to reproductive outcomes at altitude are active areas of research.

4.3 Acclimatization

Whereas acute hypoxia induces HVR, longer exposure to a continuous hypoxic stimulus for days, weeks, or years (or chronic hypoxia) produces ventilatory acclimatization to hypoxia, which is a further increase of ventilation during hypoxic stimulation and when breathing normoxic air (Powell et al., 1998; Dempsey et al., 2014). For example, upon sojourn to high altitude, the HVR increases over 2–14 days and remains elevated, along with resting ventilation and arterial O_2 saturation, for at least eight weeks as a manifestation of ventilatory acclimatization to hypoxia (Sato et al., 1994; Hupperets et al., 2004). While the timescale varies, and it has been shown to take over 10 days for ventilation to stabilize in some individuals (Powell et al., 1998). It has been suggested that a blunting of the HVR (hypoxic desensitization) reported among Andean populations (Chiodi, 1957; Severinghaus et al., 1966; Weil et al., 1971; Beall et al., 1997; Léon-Velarde et al., 2003) suggests an elevated ventilatory response cannot be maintained over longer time periods and will eventually decline (Zhuang et al., 1993). This idea was supported by another long-term acclimatization study showing HVR declined in some individuals of European ancestry after 45 days (Forster et al., 1971). If blunting of chemoreflex responses is typical in some sea-level residents after long-term altitude exposure and in Andean highlanders, it is intriguing that high HVR is maintained in some Tibetan highlanders. Selective pressure acting on standing and/or adaptive genetic variation or epigenetic differences in Tibetan populations may have contributed to the maintenance of this trait (Simonson et al., 2010; Simonson, 2015). Given that HVR magnitude also varies both within and across human populations, it is key genetic factors likely contribute, at least in part, to differences in ventilatory control (Collins et al., 1978; Brutsaert et al., 2005). The extent of variability in short- and long-term acclimatization responses across individuals and within various populations remains to be fully explored and would benefit from longitudinal analyses.

While the genetic underpinnings of the magnitude of HVR are not well understood, HIF regulators may play a key role in ventilatory acclimatization to hypoxia. For example, heterozygous *Hif-1α* knockout mice exposed to 72 h of hypoxia have reduced ventilation during normoxia and acute hypoxia compared to homozygous *Hif-1α* mice (Kline et al.,

2002). In addition, site-specific deletion of *Hif-1α* in the brainstem nucleus tractus solitarius of adult mice did not affect the acute HVR in normoxia but blunted the acute HVR in mice exposed to chronic hypoxia for 7 days (Moya et al., 2020). Hodson et al. (2016) showed that inducible inactivation of the HIF regulator prolyl hydroxylase 2 (Phd2) in mice resulted in an increased HVR, and that deletion of *Epas1*, which encodes for Hif-2α, but not Hif-1α, prevented this increase of HVR. Additional work also suggests that *Epas1* and potentially other genetic components may play a key role in ventilation, primarily through O_2–sensing in glomus cells of the carotid body (Bishop and Ratcliffe, 2020; Moreno-Domínguez et al., 2020). Moreno-Domínguez et al. (2020) ablated *Epas1* in mice, which resulted in a reduced HVR. They achieved a similar reduction in HVR when they genetically deleted *Cox4i2*. Studies regarding the genetics of the HVR should be a prolific area for future experimental studies.

While increases in ventilation and also heart rate are immediate responses to hypoxia, other physiological changes can occur within days and weeks to improve tissue O_2 delivery in chronic hypoxia (Imray et al., 2011). Chronic mild hypoxia, between 8 and 12% O_2, triggers profound vascular remodeling in the central nervous system (CNS) of adult mice, resulting in a greater than 50% increase in blood vessel density throughout the CNS over a period of 2–3 weeks (Boroujerdi and Milner, 2015). This process encompasses an angiogenic response that includes endothelial proliferation (Li et al., 2010), arteriogenic remodeling (Boroujerdi et al., 2012), enhanced transient expression of remodeling proteins such as fibronectin (Boroujerdi and Milner, 2015), and sustained elevated expression of proteins involved in blood-brain barrier integrity (e.g., tight junction proteins and vascular basement extracellular matrix proteins such as laminin) (Li et al., 2010). Such changes may provide protection in stroke and multiple sclerosis as discussed in **Section 5.4**.

4.4 Hypoxia and the Epigenome Across the Lifespan

Hypoxia in early life environments may have profound and lasting effects on the development of adult physiology through many different molecular, physiological, and morphological changes. Epigenetics is most broadly defined as the study of mitotically heritable changes to DNA that do not alter the nucleotide sequence (Holliday, 1987). The most commonly studied epigenetic mechanisms include DNA methylation (methyl groups modifying cytosines preceding guanines, or CpG sites), histone modifications (methyl, acetyl, and other chemical tags modifying the proteins around which DNA is wrapped), and non-coding RNAs (Allis and Jenuwein, 2016). Because the epigenome sits at the nexus of the genome and the environment, it is a layer of regulation that is of particular interest in the study of early hypoxia exposures.

Some regions of the epigenome are characterized by increased plasticity during the critical window in human development from preconception to early childhood (Hochberg, 2011). During this time, the epigenome undergoes active and passive reprogramming and thus is very susceptible to environmental influences (Faulk and Dolinoy, 2011; Buganim et al., 2013). Upon gamete formation, and again after fertilization, DNA methylation is erased and re-established *de novo* in a cell- and tissue-specific manner (Reik and Dean, 2001; Seisenberger et al., 2012). Moreover, mounting evidence suggests that early life events such as mother's psychosocial stress levels, nutrition, and exposure to heavy metals and endocrine disrupters such as bisphenol-A (BPA), can affect DNA methylation, predisposing a developing child to adverse health outcomes later in life (Dolinoy et al., 2007; Senut et al., 2012; Klengel et al., 2014; Thayer and Non, 2015; Non, 2021).

The epigenetic processes involved in adaptation to high-altitude hypoxia are just beginning to be explored, as summarized by Julian (2017, 2019). Recently developed animal models have revealed how epigenetic changes contribute to negative outcomes in prenatal hypoxia exposure. For example, in rats, epigenetic changes induced by hypoxia in critical stages of prenatal development can lead to increased cardiac vulnerability to hypoxia in adults (Xiong et al., 2016) and epigenetic reprogramming in response to maternal hypoxia that manifests in adult offspring (Lv et al., 2019). Additionally, intermittent hypoxia exposure in late gestation of mice has shown to induce DNA methylation changes across nearly 700 genes, many associated with metabolic regulation and inflammation, in adult male offspring (Khalyfa et al., 2017).

While early life is hypothesized to be a sensitive window for epigenetic changes, exposure to hypoxia across the lifespan can also result in epigenetic changes. Alkorta-Aranburu et al. (2012) analyzed epigenetic differences across adult human populations living at different altitudes in Ethiopia and identified four CpG sites with significantly different methylation levels in saliva samples from high- versus low-altitude Oromo Ethiopians. However, these sites were not found in genes known to be relevant to hypoxia response. A large study of epigenetic variation in hundreds of Quechua individuals in Peru living at high versus low altitude identified associations with DNA methylation at two different loci (Childebayeva et al., 2019b). Specifically, time spent at high altitude was associated with higher levels of DNA methylation at the repetitive element known as the *Long Interspersed Nuclear Element 1* (*LINE-1*) throughout the genome and lower methylation levels at the promoter region of *EPAS1*. Moreover, a few epigenome-wide studies of lifetime and early developmental altitude exposures in the same population of Andeans found differentially methylated loci in various genes, including *Superoxide Dismutase 3* (*SOD3*), a gene that plays a role in antioxidant defense against oxidative stress, as well as accelerated epigenetic aging in those living at high relative to low altitude (Childebayeva et al., 2021a). The authors also identified associations between DNA methylation and the altitude adaptive phenotype of fraction of exhaled nitric oxide (Childebayeva et al., 2021a). Surprisingly, even short-term hypoxia, such as that experienced by Europeans ascending Everest, elicited distinct epigenetic changes at key HIF loci, including *EPAS1* (Childebayeva et al., 2019a), along with other genes in HIF and RAS pathways (Childebayeva et al., 2021b). These findings speak to plasticity not just in development, but potentially throughout one's lifetime, suggesting epigenetic factors may play a role in acclimitization to high altitude.

Other mechanisms of developmental physiology at high altitude are discussed elsewhere (Jochmans-Lemonie and Joseph 2018), including changes to the cardiorespiratory system, as well as thermoregulatory processes during post-natal development.

Experimental studies in animal models also yield important insights into epigenetic modulation in the context of hypoxia. Adult rats exposed to long-term (30 days) intermittent hypoxia had higher levels of DNA methylation and down-regulation of genes related to antioxidant enzymes, such as superoxide dismutase genes *Sod1*, *Sod2*, *catalse* (*Cat)*, *Thioredoxin reductase 2* (*Txnrd2)*, *Peroxiredoxin 4* (*Prdx4*), and *Glutathione peroxidase 2* (*Gpx2)*, measured in the carotid body, the adrenal medulla, and brainstem regions associated with the carotid body reflex (Nanduri et al., 2017a). The same methylation differences were not seen in rats exposed to short-term (10 day) intermittent hypoxia, suggesting sustained or chronic exposure is necessary to produce these epigenetic effects. Of note, the carotid body has a distinct response to intermittent versus chronic hypoxia, which may explain these differences (Nanduri et al., 2017a). Furthermore, treatment with a DNA methylation inhibitor during hypoxia exposure or recovery blocked epigenetic changes and led to less reactive O_2 species and stabilized blood pressure and breathing, suggesting that epigenetics play a role in the pathology of long-term intermittent hypoxia exposure through regulation of antioxidant enzymes (Nanduri et al., 2017a).

5 TIME DOMAIN 4: HYPOXIA AND DISEASE THROUGHOUT THE LIFE COURSE

5.1 Hypoxia and Sleep

Sleep is a distinct state from wakefulness and a uniquely susceptible window for hypoxic exposures. After sleep onset, compensatory ventilatory responses to gas exchange abnormalities and upper airway obstruction can influence oxygenation patterns (Khoo et al., 1996; Wellman et al., 2011). If ventilation remains steady and sleep remains continuous, sustained hypoxia ensues. In contrast, vigorous ventilatory response and arousals from sleep result in transient increases in ventilation and oxygenation, resulting in intermittent hypoxia. Thus, individual differences in ventilatory reflexes and co-morbid cardiopulmonary disease give rise to unique patterns of hypoxia, which vary in frequency and severity.

Hypoxia is a cardinal feature of sleep-disordered breathing and has been implicated in the pathogenesis of cardiovascular and metabolic comorbidities (Drager et al., 2015). Obstructive sleep apnea (OSA) is the most common form of sleep disordered breathing and affects up to one billion people worldwide (Benjafield et al., 2019). OSA is characterized by repetitive pharyngeal collapse during sleep, leading to intermittent hypoxemia and hypercapnia with associated catecholamine surges and arousals from sleep (Dempsey et al., 2010). OSA has been associated with neurocognitive, cardiovascular, and metabolic sequelae including pulmonary hypertension (Sajkov et al., 1999). Therapy with continuous positive airway pressure (CPAP) alleviates hypoxemia, reduces systemic blood pressure, improves neurocognitive performance (Muñoz et al., 2000; Dempsey et al., 2010), and normalizes pulmonary artery pressures (Sajkov et al., 2002). CPAP may also reduce cardiovascular risk (Sassani et al., 2004; Buchner et al., 2007; Kohler et al., 2008), although data are still evolving (McEvoy et al., 2016). The causal pathway describing the role of OSA-induced cardiovascular comorbidities remain under investigation. Some of those mechanisms include carotid body activation, epigenetic changes, hypercapnia, autonomic function, inflammatory pathways, and oxidative stress (Dempsey et al., 2010; Mesarwi et al., 2015; Iturriaga, 2018; Benjafield et al., 2019).

Animal models have long been used to investigate the role of hypoxia in the pathogenesis of sleep-disordered breathing-related cardiometabolic disease. In seminal work, Fletcher et al. (1992) illustrated that chronic intermittent hypoxia (CIH) mimicking severe OSA caused an increase in systemic blood pressure. Since that time, several other investigators have extended these findings and showed that IH, during daylight hours when rodents are normally asleep, leads to dysglycemia and insulin resistance (Polotsky et al., 2003; Iiyori et al., 2007), dyslipidemia (Li et al., 2005), atherosclerosis (Savransky et al., 2007), and pulmonary hypertension (Fagan, 2001; Campen et al., 2005), supporting concurrent findings in human subjects. Studies in mice exposed to overlap (sustained and intermittent) hypoxia revealed this combined stress leads to systemic and pulmonary hypertension without protective effects typically associated with sustained hypoxia (Zhen et al., 2021). We have derived considerable mechanistic insight about the role of hypoxia in sleep and pulmonary illness from these and other such studies.

In addition to OSA, central sleep apnea (CSA) is common in high-altitude sojourners. During acute high-altitude exposure, the increase in ventilatory chemosensitivity, particularly to hypoxia, leads to unstable breathing patterns (Burgess and Ainslie, 2016). Desaturation events cause periods of hyperventilation which result in hypocapnia-induced apneas or hypopneas, which lead to subsequent desaturation events and arousals. The severity of this periodic breathing pattern appears to increase at higher elevation and may worsen or improve with acclimatization depending on the elevation and degree of increase in the hypoxic ventilatory response (Andrews et al., 2012; Burgess et al., 2013; Orr et al., 2018; Frost et al., 2021). In long term high-altitude residents, sleep disordered breathing remains common and may be linked to the development of excessive erythrocytosis or chronic mountain sickness as discussed in the next Section. Several studies report more frequent central and/or obstructive apnea events in individuals with chronic mountain sickness compared to individuals without chronic mountain sickness at the same elevation (Sun et al., 1996; Julian et al., 2013; Rattner et al., 2014; Pham et al., 2017; Heinrich et al., 2020). Continuous desaturation events on top of existing chronic hypoxemia may lead to significant oxidative stress and inflammatory events which could play key roles in this pathogenesis.

5.2 Chronic Mountain Sickness

Chronic Mountain Sickness (CMS) is a manifestation of maladaptation to life at high altitude and affects a large number of people living above 2500 m (Villafuerte and

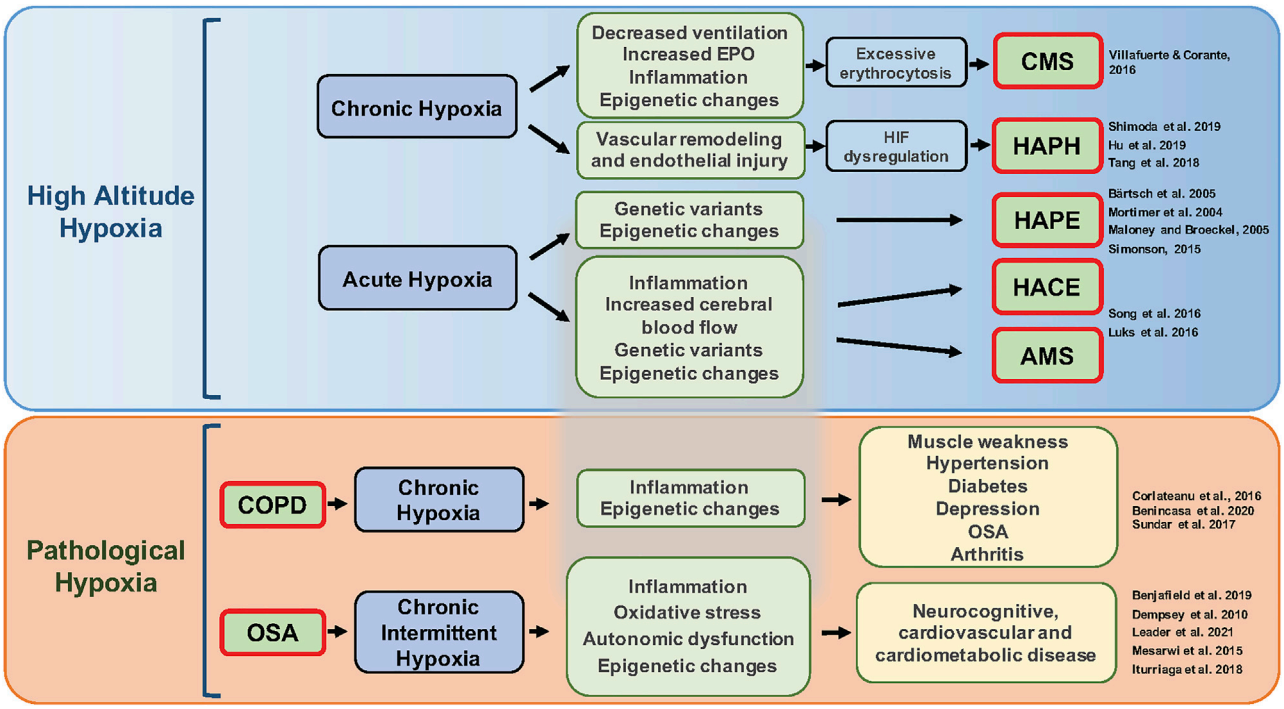

FIGURE 3 | Pathological conditions associated with different patterns of hypoxia. Acute or chronic exposure to high-altitude hypoxia (upper panel) can lead to several diseases including chronic mountain sickness (CMS), pulmonary hypertension, high-altitude pulmonary edema (HAPE), high-altitude cerebral edema (HACE), and acute mountain sickness (AMS). Mechanisms underlying this variation include genetic factors, plasticity and/or lack of plasticity in ventilatory responses to hypoxia, excessive erythrocytosis, hypoxia-inducible factor (HIF) dysregulation, epigenetics, and inflammation. Additionally, clinical diseases such as chronic obstructive pulmonary disease (COPD) and obstructive sleep apnea (OSA) lead to chronic (sustained) hypoxia and chronic intermittent hypoxia, respectively (lower panel) and can trigger subsequent inflammation, oxidative stress, changes in gene expression, autonomic dysfunction, and hypercapnia that further contribute to various comorbidities.

Corante, 2016). The excessive production of red blood cells (excessive erythrocytosis) characterizes the condition and is associated with signs and symptoms that affect an individual's well-being, social life, and frequently employment (Villafuerte and Corante, 2016). While increased erythrocytosis improves oxygen content, excessive levels are maladaptive. At present, it is well established that chronic hypoxemia resulting from life at high altitude is the main underlying factor; however, the main pathophysiological mechanism remains elusive. As mentioned in **Section 4.3**, loss of ventilatory acclimatization to altitude hypoxia leading to central hypoventilation has been proposed as the principal mechanism explaining accentuated hypoxemia and the subsequent excessive erythropoietic response (León-Velarde and Richalet, 2006). However, the significant variability in the apparent causes of excessive erythrocytosis suggests that the origin of the condition involves multiple levels (Hancco et al., 2020). Some individuals with CMS develop severe hypoxemia, possibly due to depressed ventilation during day or night that triggers excessive erythrocyte production while others have moderate hypoxemia but increased plasma erythropoietin and erythropoietin availability (as determined by the decreased soluble form of the erythropoietin receptor), or increased local erythropoietin production or sensitivity at the bone marrow level (Hancco et al., 2020). This variability suggests genetic adaptation and lack of adaptation at various levels in highlanders from the same population with and without CMS (Zhou et al., 2013).

5.3 High-Altitude Pulmonary Hypertension and Pulmonary Edema

Hypoxic pulmonary vasoconstriction (HPV) refers to the contractile response of pulmonary blood vessels in response to reduced alveolar O_2 tension (Sylvester et al., 2012; Swenson, 2013). Contrary to what happens in systemic circulation, the local hypoxic stimulus produces HPV and reduces the blood flow to areas of the lung that are poorly ventilated. Chronic hypoxia can produce arterial remodeling and changes in vascular reactivity inducing a sustained increase in pulmonary arterial pressure leading to high-altitude pulmonary hypertension (HAPH) (Xu and Jing, 2009). These diseases and other pathological conditions associated with hypoxia are illustrated in **Figure 3**.

HAPH is a clinical condition characterized by high pulmonary arterial pressure or high systolic pulmonary arterial pressure, as well as right ventricular hypertrophy, heart failure, and absence of excessive erythrocytosis (León-Velarde et al., 2005). Changes in the levels and function of HIFs are involved in the mechanisms producing HAPH (Tang et al., 2018; Hu et al., 2019; Shimoda et al., 2019) but, more specifically, modulation of HPV through

changes in the activity of K^+ or Ca^{2+} channels, or vasoactive molecules such as nitric oxide or endothelin-1 (ET-1), could contribute to the development of HAPH (Sylvester et al., 2012; Mirrakhimov and Strohl, 2016). However, the fact that HAPH is not normalized by O_2 inhalation, or agents that inhibit HPV, suggests other factors could also contribute to HAPH (Sylvester et al., 2012). HAPH is more prevalent in men than women who haven't reached menopause (Aldashev et al., 2002, 2005) and varies among different populations living at high altitude (Fagan and Weil, 2001), which suggests a genetic component, as reviewed by Eichstaedt, Benjamin, and Grünig (2020) with potential involvement of various candidate genes (Eichstaedt et al., 2020)Aldashev et al. (2002). In support of this idea, HAPH is more common in Andean than Tibetan populations, with pulmonary arterial pressures in Tibetans living at 3,658 m comparable to sea level values (Groves et al., 1993).

Additional candidate genes that may play a role in HAPH include those involved in the nitric oxide-associated pathways due to their vasoactive properties. As mentioned in **Section 3.1**, exhaled nitric oxide is substantially elevated in Tibetans compared to Andeans highlanders and lowlanders at sea level (Beall, 2007). León-Velarde and Mejía (2008) suggest that variants in *ENOS* (the gene encoding endothelial nitric oxide synthase) are associated with endogenous NO production and may contribute to HAPH susceptibility (Sofowora et al., 2001), but these observations have yet to be experimentally validated. Other genes linked to nitric oxide-associated pathways are reviewed in Eichstaedt, Benjamin, and Grünig (2020). It remains difficult to dissect the direct physiopathology of HAPH from mechanisms present in common comorbidities such as high-altitude pulmonary edema and CMS.

In severe cases, HAPH can lead to high-altitude pulmonary edema (HAPE). HAPE occurs when excessive HPV leads to increased alveolar capillary permeability and fluid leak into the lung tissue. While HAPE pathophysiology is well characterized, there are limited predictors of individual susceptibility (Bärtsch et al., 2005). Individual genetic variants and epigenetic markers have been correlated with aspects of HAPE pathophysiology and susceptibility but validation of these isolated 'hits' is lacking (Mortimer et al., 2004; Maloney and Broeckel, 2005; Simonson, 2015). Our understanding of how genomic influences relate to short- and long-term hypoxic and hypobaric physiology is limited in part due to isolated candidate genes analyses (Simonson and Malhotra, 2020), limited sample sizes, and lack of stratification for genotype-phenotype analyses. Recently, chronic, as opposed to acute, altitude exposure has been identified as a potential cause of a type of "mountain residence" HAPE (Ebert-Santos, 2017), challenging the paradigm that all HAPE is triggered by acute altitude exposure. Thorough characterization of this chronic-induction population, in rigorous comparison with those susceptible to classic acute-induction HAPE, would be a logical first step in learning about long-term altitude exposure.

The mechanisms underlying HPV involve processes that carry over from the transition from placental-fetal oxygenation to pulmonary O_2 supply at birth. However, these responses may not prove beneficial in severe lung disease and do not provide an advantage at high altitude. Therefore, the mechanisms essential at a crucial time point of birth has a cost under different environmental or pathological conditions later in life. While HPV contributes to complications in HAPE and OSA, it also occurs in patients with acute lung injury and pneumonia (Naeije and Brimioulle, 2001). HPV is an evolutionarily conserved response, observed in a variety of species including humans, with analogs in the gill circulation of fish and skin circulation of amphibians (Moudgil et al., 2005). HPV was first described as an adaptation by Euler and Liljestrand (1946) who recognized that HPV improves O_2 uptake by diverting pulmonary blood flow to better aerated parts of the lungs. This physiologic effect is put to use during lung surgery when one lung is purposefully not ventilated in order to minimize intraoperative bleeding (Dunham-Snary et al., 2017). HPV compensates for regional alveolar hypoxia, as in bronchopneumonia, but not hypoxia affecting the whole lung, as in OSA and HAPE. Oral and intravenous pulmonary vasodilators, e.g., calcium channel blockers, interfere with HPV and can worsen oxygenation and mortality in critically ill lung patients (Naeije and Brimioulle, 2001; Karam et al., 2017). However, vasodilators are recommended for patients with HAPE. This difference reflects the potential beneficial role of HPV in lung injury/pneumonia and its pathological activation in HAPE. Hypobaric hypoxia is a novel condition for which lowland dwelling humans have not had time to evolve an optimal response. By contrast, some groups with a long history of high-altitude residence have a blunted HPV which might protect them against pulmonary hypertension and HAPE (Dunham-Snary et al., 2017). Other high-altitude taxa exhibit reduced HPV, such as yaks (Anand et al., 1986), llamas (Reyes et al., 2020), the Tibetan pika (Ge et al., 1998), and high-altitude deer mice (West CM. et al., 2021), providing a likely example of convergent evolution.

5.3 Hypoxia and Inflammatory Responses

Hypoxia may also play a key role in initiating inflammation or exacerbating preexisting inflammatory states (Eltzschig and Carmeliet, 2011; Pham et al., 2021). One example is chronic obstructive pulmonary disease (COPD), whereby chronic low-grade systemic inflammation contributes to development of comorbidities including weight loss and muscle wasting, hypertension, diabetes, depression, obstructive sleep apnea, and arthritis (Corlateanu et al., 2016). It is possible that epigenetic changes are part of the mechanisms underlying such phenotypes in COPD (Corlateanu et al., 2016; Sundar et al., 2017; Benincasa et al., 2021). The role of hypoxia and inflammatory in more acute conditions, e.g., COVID-19, remains an active area of research (Simonson et al., 2021).

Another example of hypoxia-induced inflammation is observed in some high-altitude research studies, since inflammation has been implicated as a contributing factor to acute mountain sickness (AMS), development of HAPE (Duplain et al., 2000; Hartmann et al., 2000; Grocott et al., 2007; Lemos et al., 2013; Boos et al., 2016), and high-altitude cerebral edema (HACE) (Song et al., 2016; Luks et al., 2017). Additionally, genes encoding inflammatory cytokines are found to be under evolutionary selection in Andean and Tibetan populations (Foll et al., 2014), which may afford protection instead of exacerbating hypoxemia.

Animal models indicate that inflammation also contributes to ventilatory acclimatization to hypoxia. For example, rats treated with ibuprofen during exposure to sustained hypoxia showed a blocked response to acute hypoxia without affecting the persistent hyperventilation in normoxia. Ibuprofen treatment also prevented the increase of interleukin 1β (IL-1β) and interleukin-6 (IL-6) in the nucleus tractus solitarius when compared to rats exposed to sustained hypoxia but administrated with saline (Popa et al., 2011). Stokes et al. (2017) showed that inhibition of microglia in rats using minocycline prevented the complete development of ventilatory acclimatization to hypoxia with blunted responses to acute hypoxia. In addition, their results indicate minocycline prevented the increase of IL-6 observed in the nucleus tractus solitarius of rats exposed to hypoxia per 24 h (Hocker et al., 2017).

After tissue trauma and injury, HIF-1 appears to protect the host by preventing infection (Bogdanovski et al., 2017) *via* regulating inflammation and increasing bactericidal capacity of phagocytes (Peyssonnaux et al., 2005) and further stimulates angiogenesis and promotes tissue repair (Umschweif et al., 2013). Attenuated HIF-1α signaling and impairment of downstream immune responses are theoretical concerns when supplemental O_2 is given for patients with infection. Although impaired O_2 delivery in sepsis and septic shock was thought to cause multiple organ dysfunction and mortality (Tuchschmidt et al., 1992), multiple trials aimed at increasing O_2 delivery in sepsis have failed to show a benefit for this approach (Hayes et al., 1994; PRISM Investigators et al., 2017). Some studies suggest that excess O_2 can harm sepsis patients (Demiselle et al., 2018; Perner et al., 2020). The appropriate O_2 target in critical illness is a controversial topic that is the focus of ongoing trials (Perner et al., 2020).

5.4 Therapeutic Applications

Hypoxia is generally considered to impair physiological function and limit performance. However, there is mixed evidence that chronic (hypobaric) hypoxia may be beneficial in certain diseases. Some studies suggest high-altitude residents have decreased rates of cancer, e.g., lung cancer rates are lower in Peru compared to the United States after controlling for known confounding factors such as cigarette smoking (Thiersch et al., 2017; Thiersch and Swenson, 2018). Further studies are necessary to show specific effects of hypoxia, such as determining if cancer risks differ between those who reside at high altitude and those who have moved to sea level.

Chronic intermittent hypoxia (CIH), which can occur with obstructive sleep apnea, is usually considered harmful as a cause of inflammation and oxidative stress (Labarca et al., 2020), resulting in well-known pathological effects on cardiovascular physiology and metabolism (Marin et al., 2005; Barnes et al., 2022). However, CIH has also been reported to produce beneficial effects. For example, 15 cycles in inspired O_2 administered daily to produce cycles of arterial P_{O2} similar to those occurring with OSA can improve motor neuron function in both preclinical and clinical studies (Vose et al., 2022). This line of research is based on the observation that CIH can result in long term facilitation (LTF) of phrenic nerve activity, and increased ventilation that persists in normoxia following CIH (Gonzalez-Rothi et al., 2015). Hence, it is possible that there may be some beneficial effects of hypoxia that interact with harmful effects and their complex interaction might influence evolutionary responses to hypoxia. There are several examples of such balancing selection, such as the well-known effect of sickle cell disease to provide an evolutionary advantage by protecting against malaria despite causing cardiovascular disease (Piel et al., 2010).

Hypoxic pre-conditioning at this level of hypoxia confers marked protection in several animal models of neurological disease including ischemic stroke and multiple sclerosis (Miller et al., 2001; Dore-Duffy et al., 2011; Dunn et al., 2012), raising the question of therapeutic application of hypoxic pre-conditioning as mentioned in **Section 4.3**. However, the optimal dose, duration and frequency of hypoxic treatment that confers maximal protection from neurological disease, and the cerebrovascular impact of "naturally occurring" hypoxia, e.g., high altitude residents, aircrew, and patients with cardiopulmonary disease, sleep apnea, and other respiratory pathologies, remains unknown.

Pharmacological interventions to increase Hb-O_2 affinity, observed in may highland species as discussed in **Section 2.2**, have shown to improve hypoxia tolerance in mice, indicating this as a potential therapeutic avenue for hypoxia-induced pathologies (Dufu et al., 2017). A drug called Voxeletor (previously GBT440) increases Hb-O_2 affinity through its interactions with hemoglobin S to inhibit polymerization and is currently being tested in sickle cell disease patients (Estepp, 2018). Several other therapies revolve around promoting increased persistence of expression of fetal hemoglobin (HbF), an isoform of Hb which is produced around 6 weeks of pregnancy (Linch, 1998) and usually persists for 2–4 months after birth (Schechter, 2008). HbF has a higher affinity to O_2 than adult Hb, allowing for the fetus to more efficiently scavenge O_2 *in utero* and as a neonate (Wang and Zhao, 2010). HbF is found in approximately 3–7% of adult red blood cells; however, these levels are increased in individuals with beta-thalassemia, sickle cell disease, or acute erythropoietic stress (Italia et al., 2007; Kim et al., 2015) and the extent of individual variation on a global level has yet to be established. Some individuals with sickle cell disease and HbF persistence lack the symptoms and phenotypes associated with disease, offering promising directions for therapeutic development (Fathallah and Atweh, 2006). Natural variation in a number of genes have been associated with fetal Hb persistence, with several mechanisms described (Thein et al., 2009), along with studies to develop therapies that chemically modulate HbF levels. One mechanism involves reducing the amount of adult Hb in diseases like alpha- or beta-thalassemia, resulting in observed upregulation of HbF to compensate for the lack of adult Hb genes (Wahed and Dasgupta, 2015). Other mechanisms have been attributed to mutations within the promotor regions of the genes *HBG1* and *HBG2* (Karakaş et al., 2015). These promotor variants can result in new transcription factors binding to the promotor or disruption of the binding on major repressors, such as *BCL11A* and *ZBTB7A*, allowing for continued expression of HbF (Thein et al., 2009; Martyn et al., 2018). The use of compounds such as hydroxyurea have also been shown to

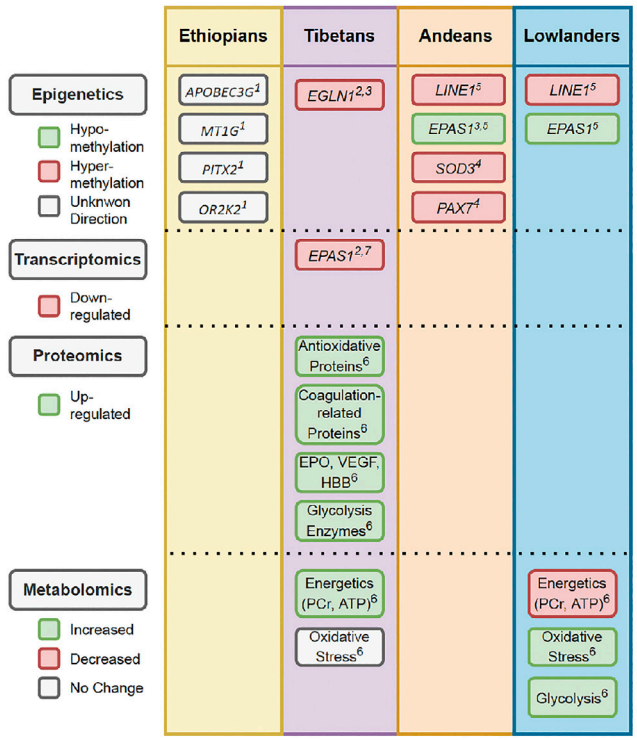

Figure 4. Omics Adaptation to Altitude in Ethiopians, Tibetans, Andeans, and lowlanders who visit high-altitude environments (>3600 m).
Genes with hypo- (green) and hyper- (red) methylated regions, differential transcriptomic regulation and/or up- (green) and down- (red) regulated families of proteins are listed by highland population. Metabolites and metabolic factors with increased levels and those that are activated in the corresponding population at high altitude are shown in green (red indicates downregulation).

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FIGURE 4 | Omics Adaptation to Altitude in Ethiopians, Tibetans, Andeans, and lowlanders who visit high-altitude environments (>3600 m). Genes with hypo- (green) and hyper- (red) methylated regions, differential transcriptomic regulation and/or up- (green) and down- (red) regulated families of proteins are listed by highland population. Metabolites and metabolic factors with increased levels and those that are activated in the corresponding population at high altitude are shown in green (red indicates downregulation).

promote HbF expression in sickle cell disease patients (Cokic et al., 2003). Studies have tied this effect to increases in nitric oxide radical and cyclic-GMP levels (Cokic et al., 2003) while alternative factors have been proposed such as selective vasodilation or decreased platelet activation (Glover et al., 1999).

6 APPLICATION OF MULTI -OMIC TOOLS TO ADVANCE THE FIELD

Adaptation and acclimatization to hypoxia involve coordinated efforts across many biological systems within the human body. These systems can be studied in parallel through multiple different "-omics" approaches, highlighted in **Figure 4**, which summarizes the various epigenetic, transcriptomic, proteomic, and metabolomic studies of high-altitude adaptation to date. As proximal phenotypes, these data may provide insight into the prioritization of precise genetic variants, which remain largely unknown, and ways to test their functional significance in highlanders and other human populations (e.g., lowland groups with shared genetic variation). With more comprehensive collections of -omics data, it is possible to generate hypotheses about mechanisms underlying hypoxia adaptation that can be tested through gene-editing and functional investigations (Hall et al., 2020).

6.1 High-Altitude Hypoxia Transcriptomics and Proteomics

With the advent of high-throughput sequencing and improved pipelines for sequencing technologies, information about global gene expression and regulation can now be obtained *via* RNAseq, which determines RNA quantity in a given sample (Wang et al., 2019). In a multi-omics data analysis in Tibetans that included transcriptomics analyses, Xin et al. (2020) developed a statistical model that correlated regulatory elements with gene expression, ultimately indicating down-regulation of *EPAS1* as potentially adaptive in Tibetans, corroborating previous experimental evidence from studies such as Peng et al. (2017). Findings of Xin et al. (2020), among others, demonstrate the depth and power behind the use of multiple -omics to identify potential adaptive mechanisms outside protein-coding variants in the genome. In addition to these studies in Tibetans, recent research has focused on -omics profiles in individuals acclimatizing to altitude as discussed in **Section 6.2**. Additional studies focused specifically on high-altitude acclimatization provide greater insight into short-term hypoxia responses that, when coupled with -omics data, yield important insight into potential mechanisms of individual hypoxia responses as well (Subudhi et al., 2014).

Proteomics is the global analysis of proteins and, in conjunction with mass spectrometry, can provide additional insight into high-altitude adaptation (Altelaar et al., 2013; Gao

et al., 2017). As reviewed by Gao, Luo and Ni (2017), the majority of proteomic studies in relation to high altitude have focused on either acclimatization of lowlanders or high-altitude related illnesses. Proteomic studies on highlanders are rare, but studies such as Ahmad et al. (2013) and Du et al. (2019) have reported differentially expressed proteins, mainly part of the inflammatory pathway, involved in coagulation cascades, antioxidative stress, and glycolysis. Both studies compared plasma of native highlanders to non-acclimatized lowlanders.

In addition to studying highlanders, other proteomic studies have attempted to identify novel biomarkers for AMS (discussed in **Section 5.4**). For example, Julian et al. (2014) measured plasma protein levels of individuals residing in the Denver, Colorado, United States, metropolitan area (1,650 m) after acute hypobaric hypoxia exposure, comparing the protein levels of those susceptible to AMS and those resistant to AMS. They found an increase in the abundance of proteins with antioxidant properties in individuals susceptible to AMS but not in those resistant to AMS. Lu et al. (2018) conducted a similar study in individuals of Chinese ancestry and found reduction of proteins related to tricarboxylic acid cycle, glycolysis, ribosome, and proteasome in the AMS resistant (AMS−) group, but not in AMS susceptible (AMS+) group. While all the aforementioned studies analyzed plasma, Jain et al. (2018) analyzed saliva for potential proteomic biomarkers. They also found increases in levels of antioxidant enzymes in addition to several other proteins, including apoptosis inducing factor-2. Their results indicate that proteomic analysis of saliva may be a feasible, non-invasive method to measure acclimatization, or more importantly, failure to acclimatize in disease states such as HAPE or HACE.

6.2 High-Altitude Hypoxia Metabolomics Across Time Domains

Most adaptive traits identified in highland populations to date involve adjustments in O_2 delivery, yet tolerance to hypoxic environments also includes adjustments in cellular O_2 utilization and particularly to mitochondrial oxidative metabolism (Murray, 2016). One functional tool employed to investigate the complex metabolic interactions occurring in response to an environmental perturbation is metabolomics (O'Brien et al., 2015). Application of this approach in high-altitude studies has revealed metabolic signals of adaptation and acclimatization and evidence for significant remodeling of metabolism at a tissue-specific and system-wide level.

In the Himalayan Sherpa, metabolomics was employed alongside measures of mitochondrial respiratory capacity in skeletal muscle, thus combining *ex vivo* functional measures with metabolite levels *in vivo*, to assess metabolic alterations with ascent to 5300 m (Horscroft et al., 2017). Despite the fall in O_2 delivery with ascent, Sherpas demonstrated increased skeletal muscle ATP and phosphocreatine (PCr) concentrations, suggesting improvement in energetic reserve, alongside no change in oxidative stress markers. This remarkable preservation of muscle energetics and protection against oxidative stress was accompanied by a shift away from fatty acid oxidation (FAO), with suppression of both FAO capacity and expression of transcriptional regulator of fatty acid metabolism PPARα (Horscroft et al., 2017). The O_2 requirement of ATP synthesis is greater during FAO than glucose oxidation. This shift away from FAO therefore suggests an adaptive hypometabolic state and reduction in cellular O_2 requirements in the Sherpa (Murray et al., 2018), as supported by enhanced mitochondrial coupling efficiency (Horscroft et al., 2017). These metabolic adaptations were associated with the putatively advantageous allele of *PPARA* (Horscroft et al., 2017), previously reported as an adaptive target in Tibetans (Simonson et al., 2010). In stark contrast to Sherpas, lowlanders demonstrated depletion of skeletal muscle PCr and ATP alongside a sharp rise in oxidative stress markers with ascent (Horscroft et al., 2017). While there was evidence of a shift away from FAO through suppression of a PPARα target (carnitine palmitoyl transferase 1 B) and enhanced oxidative coupling efficiency, lowlanders also displayed evidence of incomplete FAO through an increase in long-chain acylcarnitines: total carnitine ratio (Horscroft et al., 2017), potentially resulting in production of harmful lipid intermediates (Koves et al., 2008).

Insight into metabolic acclimatization to high altitude has also been gleaned from metabolomics analysis of placenta collected at 3100 m following C-section or vaginal delivery from women of sea-level ancestry living at high altitude (Tissot van Patot et al., 2010). In contrast to sea level, labor at altitude generated greater ATP, ATP/ADP ratios and higher PCr in the absence of large changes in glucose, lactate, or free amino acids. This shift in metabolites occurred alongside evidence of decreased oxidative stress, lower lipid peroxidation and increased antioxidant capacity. Together, these changes implied that metabolic adaptation had occurred in response to maternal hypoxia at high altitude during pregnancy. This led to a blunted response to the hypoxia-induced metabolic stress of labor, with less reliance upon anaerobic glycolysis or protein catabolism to maintain energetic homeostasis (Tissot van Patot et al., 2010).

Metabolic signals have also been identified at the systemic level in response to high-altitude exposure. In the largest of these studies to date, metabolomic and lipidomic analyses were conducted on plasma obtained from 198 subjects at baseline and across four locations on the ascent to Everest Base Camp (5,300 m). Metabolites undergoing progressive changes with ascent were identified. Increasing lactate and decreasing glucose pointed towards increased reliance upon anaerobic glycolysis. This shift occurred alongside evidence of fat store mobilization, with decreasing triglycerides associated with *de novo* lipogenesis and increasing levels of free fatty acids such as palmitic, linoleic, and oleic acids (O'Brien et al., 2019).

Increased reliance upon glycolysis with high-altitude acclimatization is supported by other metabolomics studies, which demonstrate raised skeletal muscle glycolytic intermediates in lowlanders (Horscroft et al., 2017), raised circulating lactate (Tissot van Patot et al., 2009) and induction of erythrocyte glycolysis (Liu et al., 2016; Sun et al., 2016). Erythrocyte glycolytic pathways are tightly linked to O_2 delivery, with raised sphingosine 1-phosphate (S1P) and phosphorylation of AMP-activated protein kinase both contributing towards hypoxia-induced 2,3-bisphosphoglycerate (2,3-BPG) (Liu et al., 2016; Sun et al., 2016), a negative allosteric regulator of Hb-O_2 binding affinity, thus facilitating O_2 release (Chanutin and Curnish, 1967). The induction of erythrocyte 2,3-BPG was shown to persist throughout prolonged (16 day) stay at

altitude (D'Alessandro et al., 2016; Liu et al., 2016) and one week after descent (D'Alessandro et al., 2016).

Metabolomics has also been applied in the context of high-altitude pathology, as discussed in **Section 5**, with the aim to identify circulating biomarkers. In comparison to control subjects, those suffering from HAPE (10 per group) demonstrated distinct metabolic profiles, including increases in a number of amino acids (such as valine, lysine, and isoleucine), decreased glucose, and low density lipoproteins (Luo et al., 2012). Next research steps should aim to determine what physiology and biomarkers are common and perform research to understand the underlying mechanism.

While relatively few high-altitude studies have employed metabolomics techniques, current evidence suggests it is an effective approach for identifying metabolic signals of adaptation and acclimatization to high altitude. It provides the most insight when combined with functional measures to elucidate mechanisms associated with these signals, as demonstrated by Horscroft et al. (2017), Sun et al. (2016), and Liu et al. (2016).

6.3 Multi -Omics in Other Extreme Environments

While high altitude is the main environment in which humans encounter hypoxia, multi -omic tools can and have been applied to other extreme environments where humans may face the physiological challenge of low O_2. One such instance is in diving populations, as exemplified by the Bajau people of the Philippines who are known for their underwater breath-holding abilities. The Bajau people spend an average of 60% of their work day underwater, and undergo hypoxic states during breath holds (Schagatay et al., 2011; Ilardo et al., 2018). Ilardo et al. (2018) identified genetic variants under positive selection within the Bajau that corresponded with larger spleen size, potentially conferring a physiological advantage during dives by providing a larger reservoir of red blood cells.

Further multi -omic work is being conducted in the final human frontier, where humans may encounter hypobaric and potentially hypoxic conditions during space travel. Currently, on-board the International Space Station (ISS), great care is taken to maintain a steady partial pressure of O_2 near to that at the Earth's sea-level, but explorers conducting extravehicular activity may be exposed to hypobaric hypoxic conditions (Norcross et al., 2015). Despite the normal O_2 levels on-board the ISS, an integrated -omics study of an astronaut during a one-year mission on-board the ISS revealed changes in the expression pattern of genes that have been implicated in hypoxia in rodent models (Garrett-Bakelman et al., 2019). Furthermore, the ambient carbon dioxide (CO_2) concentration in space is in excess of normal atmospheric conditions (0.7 versus 0.03%) (Matty, 2010; Cronyn et al., 2012). Studies are being conducted to understand the health impact of elevated CO_2 on astronauts and the role of genetics in individual variability in response to elevated CO_2 in space flight (Laurie et al., 2017). While the space travel environment may differ from high altitude, hypoxia and hypercapnia remain key concerns in maintaining astronaut health, and multi-omics analyses may provide insight into how to counteract environmental stressors (Schmidt and Goodwin, 2013; Beheshti et al., 2018).

7 CONCLUSION

Humans and other animals have experienced hypoxia across various time scales. This synthesis of existing knowledge of hypoxia responses across time domains integrates information from comparative animal and human studies and explores disease consequences for modern humans. Findings and perspectives across each of these domains contribute to unique and promising future directions for evolutionary and clinical hypoxia research. As tools and techniques become more sophisticated, ongoing and future studies in genomics, epigenomics, other -omics, and environmental/clinical phenotypes measured across species and across the lifespan must be integrated to fully understand how the challenge of hypoxia impacts various physiological systems.

AUTHOR CONTRIBUTIONS

All authors are participants of the Center for Physiological Genomics of Low Oxygen (CPGLO), an interdisciplinary research team established at the University of California, San Diego in 2016 that aims to maximize information from formerly isolated and highly informative social and biological (anthropological, physiological, -omic, clinical) research efforts. All co-authors of this review are CPGLO participants and contributed sections to the manuscript and approved the final version. JJY, ALN, ECH, and TSS prepared and edited the manuscript. CPGLO is co-directed by TSS, ALN, FLP, AM, and PJP at UC San Diego and the Scripps Institution of Oceanography.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.885295/full#supplementary-material

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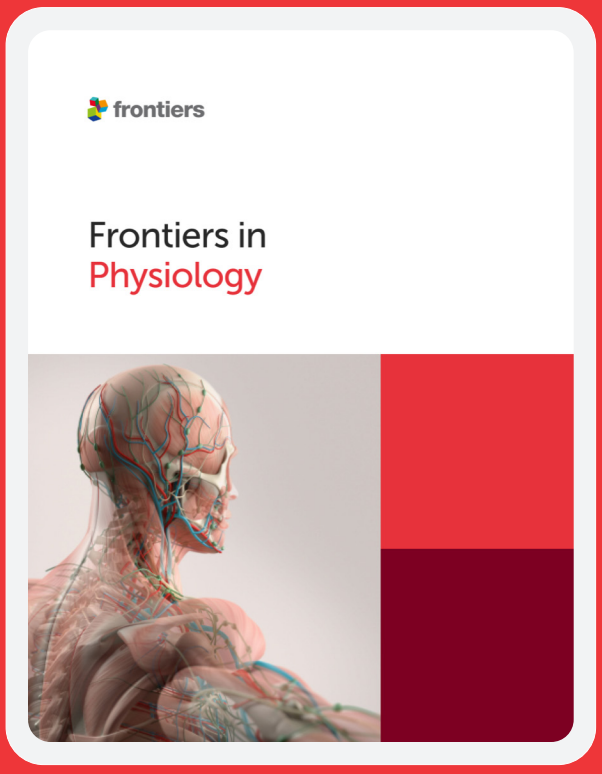

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