PERIPHERAL VASOCONSTRICTION AND ABNORMAL PARASYMPATHETIC RESPONSE TO SIGHS AND TRANSIENT HYPOXIA IN SICKLE CELL DISEASE

Suvimol Sangkatumvong, Ph.D., Michael C.K. Khoo, Ph.D., Roberta Kato, M.D., Jon A. Detterich, M.D., Adam Bush, B.S., Thomas G. Keens, M.D., Herbert J. Meiselman, Sc.D., John C. Wood, M.D.,Ph.D., and Thomas D. Coates, M.D.

ONLINE DATA SUPPLEMENT

Methods

All experiments were carried out at Children's Hospital Los Angeles, California under the auspices of a protocol that was approved by the Committee on Clinical Investigation.

Subject eligibility:

Eligibility criteria were: 1) thirteen years or older; 2) homozygous sickle cell disease (HbSS), hemoglobin SC disease, hemoglobin $S\beta^0$-thalassemia; 3) no transfusions in the past three months; 4) no sickle cell-related symptoms in the past month; 5) no chronic anti-inflammatory therapy; 6) no infections or acute medical problems within four weeks; 7) no pregnancy at time of experiment; 8) signed informed consent. Control (CTL) subjects met the same criteria, except for not having SCD.

Enrollment and Hypoxia induction:

The experimental protocol was designed to measure the physiologic responses to transient hypoxia such as may occur naturally during sleep. To accomplish this, single episodes of hypoxia were induced by breathing five breaths of 100% nitrogen (N_2).

All subjects were interviewed at enrollment and on the day of each experiment by a physician experienced in SCD (TDC) to make sure that the eligibility criteria were met and no other issues in the subject's medical history would preclude participation. Hemoglobin electrophoresis was done on all subjects at enrollment to confirm the hemoglobin genotype. A licensed physician was in attendance throughout all procedures.

The subject was placed comfortably in a supine position with the head of the bed adjusted at a 30-degree angle. Room temperature was controlled between 20°C and 23°C. A standard fitted anesthesia mask was held over the nose and mouth, sealed in place by elastic bands. A non-rebreathing valve that vented expired air to the room was attached directly to the mask. The input side of this valve was connected to a second valve that allowed switching the inspired gas between room air and 100% N_2. This valve was not visible to the subject and made no noise during the switching process. The subject was connected to the devices noted below and allowed to rest quietly at least fifteen minutes until all vital signs had stabilized. Without the subject's knowledge, the inspired gas was switched to 100% N_2 at the beginning of inspiration and back to room air after the subject had taken five tidal volume breaths of N_2. The protocol was repeated so that each subject experienced two episodes of hypoxia per visit, separated by an approximately 15 minute-interval. Hypoxic episodes with significant movement artifacts were excluded from analysis.

Respiration, SaO_2 in the right index finger, and electrocardiogram (ECG) were recorded continuously using the LifeShirt® physiological monitoring system (VivoMetrics, Ventura, California). Microvascular perfusion was recorded at the nail capillary bed of the right index finger using the PeriFlux® laser Doppler monitoring system (Perimed, Sweden). The laser probe was mounted in a temperature-controlled collar and held in place using double-sided clear tape, preventing any pressure on the nail bed capillaries. The collar kept the skin at 33°C throughout the session. The ECG r-wave-to-r-wave interval (RRI), respiratory rate (RR), and tidal volume (V_T) were derived from the basic data using VivoLogic® software (VivoMetrics, Ventura, California). Parameters were recorded digitally between once a second (SaO_2) and 200

times/second (ECG) and resampled at 30 Hz prior to analysis using MATLAB (MathWorks Inc., Natick, Massachusetts). The details of the mathematical techniques have been reported elsewhere[10].

Safety Protocol

The research protocol was accepted by the local ethics committee as "minimal risk". Nonetheless, because of concerns that inducing hypoxia might lead to a VOC, each subject was interviewed about his/her symptoms prior to and at 1 hour, 12 hours, 24 hours, and 1 week following the experiment. An external data safety monitoring committee, in addition to the principle investigator, monitored the study progress. We used the same five-breaths of N_2 and safety monitoring protocol for separate experiments using magnetic resonance imaging (MRI). The MRI data are not reported in this paper; however, we will report safety data from all 52 experimental episodes (19 CTL, 33 SCD) from both studies. Note that some subjects participated in both studies and thus have more than one experimental episode represented in the safety data.

Conversion of SaO_2 to partial pressure of oxygen (PaO_2)

To estimate the change of PaO_2 during the N_2 challenge, we measured the oxyhemoglobin dissociation curves in 5 normal subjects and 5 SCD subjects with a Hemox-Analyzer (TCS Scientific Corporation, New Hope, Pennsylvania) and fitted the data to a sigmoid equation. The respective fitting parameters for the oxyhemoglobin dissociation curves of the SCD and CTL subjects were then used to convert SaO_2 to PaO_2[10]. As the oxygen dissociation curves are right-shifted in SCD subjects, the same levels of blood PaO_2 could result in a lower SaO_2 in SCD

subjects than in CTL subjects. The conversion from SaO_2 to PaO_2 allowed the comparison of the blood PaO_2 level between the two subject groups to be made continually following hypoxia stimuli.

Assessment of the ANS responses

ANS status was evaluated non-invasively by frequency domain analysis of cardiac beat-to-beat variability, also known as heart rate variability (HRV). Sympathetic and parasympathetic inputs of the ANS innervate the sinoatrial node, leading to fluctuation of the heart rate. The magnitude of the HRV in a given frequency range can be expressed as a number, or "power," a measure that quantifies the magnitude of parasympathetic or sympathetic activity. Parasympathetic activity is a major contributor to high-frequency components of HRV (0.15 – 0.4 Hz) whereas the low-frequency components of HRV (0.04 – 0.15 Hz) may be due to both parasympathetic and sympathetic activity[11]. The ratio of low-frequency to high-frequency spectral power, termed LHR, is a broad index of "sympathovagal balance"[12,13]. Note that both high frequency power (HFP) and LHR represent degrees of autonomic regulation rather than absolute levels of autonomic tone.

The time-courses of HFP and LHR were computed using a recursive time-varying autoregressive model[14-16]. Moreover, since respiration causes sinusoidal variations in heart rate[17], we compensate for the effects of changes in breathing patterns on HRV by using the respiratory waveform as an input in the time-varying autoregressive with exogenous input model[10]. Consequently, the respiratory-adjusted ANS data presented here represent all autonomic

inputs other than respiration. We denoted respiratory-adjusted HFP and LHR as HFP_{ra} and LHR_{ra}. We have published the details of these computations elsewhere[18-20].

Detection of sighs and decreases in perfusion

Spontaneous sighs and drops in peripheral perfusion were detected automatically from the recorded V_T tracing and the laser-Doppler flow data using a threshold-based algorithm developed in MATLAB. A sigh was defined as an inspiration with a volume greater than 2.5 times a subject's average inspired tidal volume ($V_{T,inspired}$). A perfusion drop was defined as any decrease in perfusion that was 0.5 times below a subject's average perfusion unit (PU) value obtained from the laser-Doppler device.

The numbers of sighs with and without perfusion drop were used to calculate the probability of a perfusion drop event for each sigh. Only perfusion drops occurring immediately (i.e., within 5 seconds of a sigh) were considered to be related to a sigh.

Signal alignment

In order to study the physiological responses among multiple subjects, the timing of all recordings from all subjects was aligned with respect to the nadir of hypoxia or the onset of a sigh. For the response to N_2 breathing, the time at which SaO_2 reached its nadir value following a hypoxia stimulus and the onset of sighs were marked as time=0 (Figure 1). Other measurements from the same subject were then aligned according to this reference time.

All sighs for each subject were aligned based on the respiration waveform. The time at the beginning of the n^{th} sigh inspiration was used as the reference time t_n=0 to align all of the sighs

for each subject. This process is demonstrated in Figure 2 where four time segments (shaded regions, panel A) of the four signals are all aligned at the onset of the sighs (Panel B), and the median of the signal from all sighs at each time point is then displayed in column C for each of the simultaneously measured signals. This allowed calculation of the median response (PU, RRI, HFP_{ra}) with respect to the onset of sigh for each subject.

Statistical Analyses

Rank-sum test

To statistically assess differences between the SCD and CTL subjects, we used rank-sum test to compare the following measurements between the two groups: their baseline physiological and HRV parameters, probabilities of perfusion drops for each sigh, and frequencies of sigh breaths.

Chi-square test

The association between sighs and perfusion drops was analyzed using the Chi-square test. Each breath was treated as an event, and was categorized as either a sigh or a non-sigh breath. If a breath was immediately followed by a PU drop, that breath was marked as a PU drop event. The number of occurrences of each event (breath with sigh/no sigh, PU drop/no PU drop) were used to construct a 2x2 contingency table for each subject, reflecting the relation from sighs to perfusion drops. This information was subsequently used in a Chi-square test for the possible association between sighs and PU drops for each subject.

2W RMANOVA

Two-way repeated measures analysis of variance (2W RMANOVA) was performed on all HRV indices to compare the SCD and CTL groups. One set of measurements from a baseline period and another during a stimulus period were obtained from each HRV time-course. Post-hoc pairwise comparisons using the Holm-Sidak method were conducted to determine whether: a) each HRV index following a stimulus (sigh or hypoxia) differed from its baseline; b) the values of the HRV index in response to a stimulus from the two groups of subjects differed from each other.