Simulating obstructive sleep apnea patients’ oxygenation characteristics into a mouse model of cyclical intermittent hypoxia

Diane C. Lim, 1,2 Daniel C. Brady, 2 Pengse Po, 2 Li Pang Chuang, 3 Laise Marcondes, 4 Emily Y. Kim, 2 Brendan T. Keenan, 2 Xiaofeng Guo, 2 Greg Maislin, 1,2 Raymond J. Galante, 2 and Allan I. Pack 1,2

1 Division of Sleep Medicine, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; 2 Center for Sleep and Circadian Neurobiology, University of Pennsylvania, Philadelphia, Pennsylvania; 3 Department of Thoracic Medicine and Department of Sleep Center, Chang Gung Memorial Hospital, Taipei, Taiwan and Graduate Institute of Clinical Medical Sciences, Chang Gung University, Taoyuan, Taiwan; and 4 Superior School of Health Sciences, Brasilia, Brazil

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RODENT MODELS of cyclical intermittent hypoxia (CIH) are widely used to assess the consequences of obstructive sleep apnea (OSA). These studies build on the seminal work of Fletcher (18, 19). Such studies are important because OSA is not only a highly prevalent chronic disease (76) but is also associated with many chronic illnesses including cardiovascular (46, 54, 60), metabolic (33, 48), and neurodegenerative (2, 75) diseases, as well as cancer (44, 52). The association between OSA and seemingly different classes of disease suggests that OSA is a systemic disorder that contributes to the development of these chronic diseases. Chronic CIH is likely one of the major pathogenic components of OSA, since CIH results in downstream effects of both hypoxia (55) and oxidative stress (34, 74). Thus investigators use rodent models of CIH to generate gene, protein, and physiological data to understand mechanisms underlying the consequences of OSA (1, 41, 51, 55).

While studies using rodent CIH models have led to major advances in knowledge, there are fundamental questions that have yet to be addressed. For example, it may be important to consider whether rodent models of CIH should better reflect oxygenation characteristics seen in OSA patients to more accurately simulate hypoxia and oxidative stress conditions. Therefore, our first aim was to use an OSA patient cohort to identify characteristics of oxygen desaturation/resaturation. We identified two features that are not currently utilized in CIH systems. First, the rate of desaturation is much faster than the rate of desaturation. Second, there is considerable variability in patients with OSA.

Simulating these characteristics represents a new approach to modeling CIH in mice that may more accurately reflect oxygenation characteristics within patients with OSA. Our second aim was to use the MSOSA data to design and implement a new CIH system that can simulate these oxygenation characteristics; this new system is the main focus of this report. We used this new model to establish arterial oxygen saturation (\(\text{SaO}_2\)) by MouseOx at different levels of fraction of inspired oxygen (\(\text{FiO}_2\)), demonstrating differences in zenith and nadir \(\text{SaO}_2\) values between four different inbred strains. Our third aim was to use this newly developed model and assess whether urinary 8,12-iso-\(i\)-PF2\(_\alpha\)-VI levels (a marker of oxidative stress) increased in mice that were exposed to a more rapid resaturation compared with a slower resaturation. We hypothesized that a shorter resaturation time would generate more oxidative stress as demonstrated in in vitro studies (40, 66) and in vivo studies (70). Our new CIH system allows us to simulate characteristics of desaturation/resaturation that were identified in patients with OSA.

METHODS

Human Studies

Molecular Signatures of Obstructive Sleep Apnea cohort. To assess characteristics of oxygen desaturation/resaturation time, we utilized data from the Molecular Signatures of Obstructive Sleep Apnea study...
MSOSA is an institutional review board-approved study at the University of Pennsylvania that prospectively recruited newly diagnosed, untreated patients with OSA from March 2008 to January 2013 at the Hospital of the University of Pennsylvania. Subjects underwent a written informed consent process and in-laboratory polysomnogram-recorded electroencephalograms; eye, chin, and pre-tibial muscle activity; electrocardiography; oximetry; chest and abdominal respiratory effort; and airflow by nasal cannula (nasal pressure) and oral thermistor (Compumedix, Safiro) that was scored using Sandman (Natus) software. Studies with less than 4 h of sleep were repeated. Only the second study was used in the analyses if studies were repeated. Automatic oxygen desaturation and respiratory modules were run, and results were manually reviewed to remove or reclassify events produced by autoscoring, add missing events, and mark bad data in 5-min epochs. Analyses presented in this manuscript are based on 47 subjects who were diagnosed with OSA (apnea-hypopnea index (AHI) > 5 events/h, an index used to indicate severity of sleep apnea and is the average number of apneas and hypopneas per hour of sleep), based on their in-laboratory study. We also calculated the oxygen desaturations index 3% (ODI3), which is the average number of desaturations events with 3% fall in oxygen saturation per hour of sleep.

MSOSA polysomnograms underwent additional measurements for the rate of oxygen desaturation (downward slope) and resaturation (upward slope) for each apnea or hypopnea. Specifically, one technician manually scored all studies for the duration of 1) oxygen desaturation, with time starting at the SaO2 where an event starts to undergo a 3% or more oxygen desaturation and ending when the SaO2 was either at the nadir or sustained a 3-s plateau; and 2) oxygen resaturation, with time starting at the SaO2 undergoing a 3% or more oxygen resaturation and time ending when the SaO2 reaches a 3-s plateau. Apneas and hypopneas without a ≥3% oxygen desaturation were not used for this analysis.

Mouse Studies

HyCon system of CIH. Savransky initially developed the HyCon-0520 to the technical requirements of Polotsky (62), which is commercially available (S. Savransky, West Des Moines, IA). The HyCon is fully automatic, using a closed-loop control mechanism to regulate air/N2 delivery by making direct comparisons between the oxygenation points set by the investigator to a Hycon O2 sensor (HO2S) placed 5 cm above the noses of sleeping mice. The HO2S samples the gas inside the home cage (Fig. 1, A and D) and is externally validated with a second external O2 sensor (EO2S), the O2 Analyzer (model 17620; VacuMed, Ventura, CA). To ensure rapid exchange of gas in the mouse cage, we delivered gasses simultaneously through six inlets (Fig. 1C), spraying the gasses downward with nozzles (Fig. 1B) and...
then directing the gasses out of the cage through 10 outlet holes in the lid (Fig. 1F). Further modifications were made to the hardware (Fig. 1E) and software to allow us to simulate oxygen desaturation/resaturation characteristics identified and measured in the MSOSA cohort. Details are outlined in RESULTS, but in short, hardware and software upgrades enabled input of the following parameters: SH (the Set High F\textsubscript{E}\textsubscript{O},); SL (the Set Low F\textsubscript{E}\textsubscript{O},); TD (desaturation time); TR (resaturation time); TPH (time during plateau at set high F\textsubscript{E}\textsubscript{O},); and TPL (time during plateau at set low F\textsubscript{E}\textsubscript{O}).

Validation of HyCon modifications: MouseOx study. All experiments were approved by the IACUC at the University of Pennsylvania. Mice were routinely housed on a 12:12-h light:dark cycle (7 am:7 pm) and given food and water ad libitum. To validate our modified HyCon system, Sa\textsubscript{O}, was first measured via a MouseOx in eight C57BL/6 (National Institute on Aging, Bethesda, MD) using a sinusoidal desaturation/resaturation program. Analysis of these data using a power analysis revealed that five mice per group were needed to give 80% power to observe a mean difference of at least 5% oxygen saturation (for details, see Data Analysis and Statistical Approaches: Mouse Studies). We then measured Sa\textsubscript{O}, in four of the founder strains from the Collaborative Cross (8). Therefore, 10 C57BL/6 (National Institute on Aging, Bethesda, MD), 10 129SH/SvImJ (Jackson Lab, ME), 10 A/J (Jackson Lab, ME), and 10 NZO/HILJ (Jackson Lab, ME) male mice, aged 4 mo, were used to explore the Sa\textsubscript{O}, levels in response to a short and a long resaturation program. Specifically, five mice per strain were scheduled to receive a nonsinusoidal short resaturation (TR of 15 s) program, and five mice per strain were scheduled to receive a nonsinusoidal long resaturation (TR of 90 s) program. To measure Sa\textsubscript{O}, a MouseOx collar sensor was placed over the carotid artery (Rev 6.3.10 MouseOx software) of an anesthetized mouse, which was then gently placed inside a clear tuberestrainer (Starr Life Sciences). The MouseOx sensor relays data to STARR-Link, a device outside the home cage that transmits an analog voltage representation of digital data to a computer that displays Sa\textsubscript{O}, heart rate, respiratory rate, and other measurements. Within the tube, the mouse was allowed to walk back-and-forth for 10–15 min to acclimate to its new surroundings before recording started. An external oxygen sensor (EO\textsubscript{O},S) was placed directly at the level of the mouse’s nose right outside the restrainer. Oxygen saturation was recorded throughout continuous CIH cycles that desaturated from an F\textsubscript{E}\textsubscript{O}, of Set High F\textsubscript{E}\textsubscript{O}, (SH) to Set Low F\textsubscript{E}\textsubscript{O}, (SL) over Time Desaturation (TD) seconds, then resaturated from SL to SH over Time Resaturation (TR) seconds. Table 1 outlines the sinusoidal and nonsinusoidal schedules that were used, with the nonsinusoidal TD and TR determined from the data on subjects with OSA. After obtaining at least 10 acceptable cycles (e.g., minimal movement artifact) the F\textsubscript{E}\textsubscript{O}, at SL was stepped down by 0.01. For the nonsinusoidal CIH schedule, TD and TR were changed with each step down in SL, with time during plateau at SH (TPH) held constant at 3 s and time during plateau at SL (TPL) was 0.

Effects of short vs. long resaturation times on oxidative stress. To explore whether CIH schedules differentially affected levels of oxidative stress, we measured urinary 8,12-iso-IP\textsubscript{F2α}-VI, a validated measure of oxidative stress that is elevated in patients with OSA (36, 57). We exposed mice to one of three conditions: 1) a short resaturation (SR) time (TR = 15 s), a characteristic of the MSOSA cohort; 2) a long resaturation (LR) time (TR = 90 s), which has been used in other published mouse models of CIH (9, 12, 32, 39, 50, 56, 68, 69); and 3) sham, continuous air at flow rates similar to SR and LR. We examined the effect of each condition on urinary 8,12-iso-IP\textsubscript{F2α}-VI over a 3-h period of CIH. Based on the observed variability and effect sizes seen in an independent pilot sample using pooled groups of mice undergoing each condition (for details, see Data Analysis and Statistical Approaches: Mouse Studies), 40 new C57BL/6 (National Institute on Aging) male mice, 6 mo old were used to measure urinary 8,12-iso-IP\textsubscript{F2α}-VI levels. Twenty mice underwent all three conditions (sham, SR, and LR, with SR and LR separated by 1 wk). Five mice underwent sham, then SR (separated by 1 day); five mice underwent sham and LR only (separated by 1 day); five mice underwent SR only; and five mice underwent LR only. In total, 90 urine samples were obtained: 30 sham, 30 SR, and 30 LR samples. Two-hundred microliters of each urine sample is required for measurement of 8,12-iso-IP\textsubscript{F2α}-VI, and 10 µl is required for creatinine; therefore, samples of <210 µl were excluded from further analyses.

HyCon settings for 8,12-iso-IP\textsubscript{F2α}-VI study were selected to ensure results reflected resaturation time, compatible with human studies. We used data from the MouseOx study to select a schedule where the short and long resaturation programs resulted in similar Sa\textsubscript{O}, nadirs. Settings for SR schedule were F\textsubscript{E}\textsubscript{O}, 0.21 to 0.12 (Sa\textsubscript{O}, 65%) over 38 s and from 0.12 to 0.21 over 15 s for 3 h, a total of 204 cycles; settings for LR schedule were F\textsubscript{E}\textsubscript{O}, 0.21 to 0.11 (Sa\textsubscript{O}, 65%) over 32 s and from 0.13 to 0.21 over 90 s for 3 h, a total of 89 cycles. The sham condition involved placing a mouse in a HyCon home cage exposed to continuous room air at a flow similar to that in the CIH cages.

Urine collection for 8,12-iso-IP\textsubscript{F2α}-VI study was performed such that each sample represented one mouse exposed to 3 h (0800 to 1100) of SR, LR, or sham. Before placing a single mouse into one cage, each mouse was held by the scruff of the neck and a light bladder massage was performed (20, 42, 72) to obtain an initial void (hour 0). Three clear 96-well plates were placed on the bottom of each HyCon home cage to collect urine and collected within 5 min to prevent evaporation, then stored on ice in the dark. In addition, hourly bladder massage (at hours 1, 2, and 3) was performed and this urine was pooled with urine collected within the cage for that hour. After each hourly urine collection was completed, urine was immediately put on dry ice and stored at −80°C. We combined urine (not including the initial void) so that one sample reflected a 3-h urine collection during CIH or sham. Pooled hourly samples was required to obtain a sufficient volume of urine for analyses.

Results for 8,12-iso-IP\textsubscript{F2α}-VI were normalized to creatinine and both were measured by LC-MS/MS as previously described (58, 64). In short, samples for 8,12-iso-IP\textsubscript{F2α}-VI underwent solid-phase extraction (SPE) and were analyzed using Acquity UPLC and Waters Xevo TQ-S tandem quadrupole mass spectrometer (Waters, Milford, MA) used in the electrospray negative (ES−), multiple reaction monitoring (MRM) mode.

Table 1. HyCon CIH schedule for MouseOx study (sinusoidal and nonsinusoidal) and urinary 8,12-iso-IP\textsubscript{F2α}-VI study (nonsinusoidal only)
Clinical characteristics. Continuous characteristics are summarized using means, standard deviations (SDs), and medians and compared among Apnea-Hypopnea Index (AHI) groups in the MSOSA data using a parametric analysis of variance (ANOVA) or nonparametric Kruskal-Wallis test, where appropriate. Categorical variables are summarized using frequencies and percentages and were compared among AHI groupings using chi-square or Fisher’s exact tests. AHI groups were defined based on clinically relevant definitions as controls (AHI < 5), mild OSA (AHI 6 –15), moderate OSA (AHI 16 –30), and severe OSA (AHI > 30). If a significant difference was observed in omnibus tests, we subsequently performed post hoc pairwise comparisons between groups to identify the specific pairwise differences contributing to overall significance.

MSOSA: oxygen desaturation and resaturation characteristics. Within the MSOSA sample, each individual oxygen desaturation/resaturation cycle was assessed for the amplitude, nadir, and duration. These measures were then used to 1) visually compare the characteristics of desaturations and resaturations and 2) assess the within-subject variability of desaturation nadirs and the association with the total number of desaturations. To compare characteristics of desaturation/resaturation, event duration was plotted using the least squares mean and 95% confidence interval (CI) derived from a repeated-measures ANOVA model. The events were grouped in two ways: 1) to maintain the paired combinations of desaturations and resaturations, events were grouped based on the desaturation amplitude; and 2) to make the estimates comparable within a given amplitude, desaturations and resaturations were unpaired and grouped by their respective individual amplitudes. To assess desaturation variability, we ranked each subject based on their total number of desaturation events and plotted the subject-specific mean and SD of the desaturation nadirs against this ranking. We also calculated the coefficient of variation of the nadirs (CV = (SD of nadir)/mean nadir)) and assessed its correlation with the total number of events using Spearman’s rank correlation coefficients. After filtering out desaturation/resaturation events with amplitude <3%, a total of 7,232 events were included in these analyses, with the median number of events per subject equal to 91 (range: 2 –639 events).

Assessment of changes in SaO2 to different FIO2 using the new CIH system: Mouse Studies. Using the analysis from the sinusoidal desaturation/resaturation program, across FIO2 values relevant to our SR and LR schedules (e.g., 21-16 to 21-10), the SD of the zenith values ranged from 2.2 to 3.2 (mean = 2.7) and the SD of the nadirs ranged from 3.7 to 6.0 (mean = 4.9). For studies in different inbred strains, given these estimates, a total of five mice per group result in >80% power to observe a mean difference of at least 5% in zenith O2 saturation values between strains and a mean difference of at least 9% in nadir O2 saturation levels at an α = 0.05 using a standard t-test. Given that these analyses were meant to be exploratory and descriptive, we decided that powering the analysis based on this magnitude of O2 differences was sufficient. Data were carefully reviewed for cycles deemed as acceptable (i.e., without poor signal detection). Program-specific (SR and LR) least squares mean and standard error (SE) SaO2 values for both the zenith and nadir of all cycles were estimated and compared using a repeated-measures ANOVA model, accounting for multiple measures per mouse, separately for each FIO2 cycle and mouse strain. Given no strong a priori hypothesis regarding the differences in the two programs, two-sided P values were computed to assess whether the zenith or nadir were significantly different between the SR and LR programs.

Assessment of effect of short and long desaturation times on oxidative stress. The number of mice used in analyses comparing urinary 8,12-iso-iPF2α,VI levels among CIH conditions was based on estimates of urinary 8,12-iso-iPF2α,VI collected in an independent pilot study (unpublished), collecting pooled urine from five mice undergoing each of the three conditions in 2-h bins across an 8-h period. Using these data, we estimated the mean urinary 8,12-iso-iPF2α,VI levels for each condition based on the first two 2-h bins, and treated all data as replicates to estimate the pooled SD used in the pairwise comparison. Based on these estimated mean differences and pooled SDs, and using a one-sided α = 0.05, we estimated that 15 mice per group were required to have 90% power to observe the same difference between LR and SR as seen in the pilot data, 8 mice to have 90% power to observe the same difference between SR and sham, and 7 mice for 90% power to find a difference between LR and sham. Therefore, 15 samples per condition is an appropriate sample size to maintain power across all comparisons. Since we determined that on average a minimum of ~3 h was required to obtain a sufficient amount of urine from an individual mouse, rather than a pooled sample, we then doubled this number per condition to ensure we would have at least 15 samples with a large enough urine volume for 8,12-iso-iPF2α,VI calculations. Samples where volumes were <210 µl, the volume required for accurate assessment of 8,12-iso-iPF2α,VI, were excluded (n = 24; 12 sham, 8 LR, and 4 SR) from final analyses. Program-specific (SR, LR, and sham) least squares mean and SE urinary 8,12-iso-iPF2α,VI levels were estimated using repeated-measures ANOVA models. In addition, a linear mixed model analysis was performed to assess whether there was a linear dose response in the levels of 8,12-iso-iPF2α,VI across the three programs, i.e., whether 8,12-iso-iPF2α,VI SR > 8,12-iso-iPF2α,VI LR > 8,12-iso-iPF2α,VI sham. While studying higher frequency and faster rate of desaturation/resaturation has not been systematically performed in animal or in vitro models of CIH, assuming that relevant enzymes that are oxygen dependent (65) are still generating superoxide or hydrogen peroxide (45), the rate by which the electron acceptor (oxygen) is introduced should influence the rate of production of free radicals. Since the SR program introduces oxygen at a faster rate, we hypothesized that the rate of reoxygenation will affect the amount of free radicals produced, such that urinary 8,12-iso-iPF2α,VI levels will be higher in the SR program than the levels in the LR program, and levels in the LR program will be higher than in sham. Given the directionality of this hypothesis, one-sided P values were computed to test the hypothesis of a linear dose response across programs, as well as to examine pairwise comparisons between SR, sham, and LR conditions. As an exploratory analysis based on the observed results of these a priori pairwise comparisons, we also compared the 8,12-iso-iPF2α,VI levels in the short resaturation group vs. the average in the combined sham and long resaturation groups (discussed further in RESULTS, Mouse Studies).

RESULTS

Human Studies

MSOSA clinical characteristics. MSOSA subjects (n = 47) had a mean (±SD) age of 47.0 ± 11.3 yr, a body mass index (BMI) of 31.8 ± 4.7 kg/m², and 68.1% were male. They had an AHI of 34.2 ± 24.0 events/h. The oxygen-desaturation index (ODI) (the number of times per hour of sleep that the blood’s oxygen level drops by 3% or more from baseline), SaO2 nadir, and % time SaO2 < 90% were on average 17.2 ± 19.5 events/hour, 82.9 ± 7.7%, and 7.1 ± 17.1% (Table 2). There were no statistically significant differences among the mild (AHI 6 –15), moderate (AHI 16–30), and severe (AHI > 30) groups in sex (P = 0.226) or age (P = 0.790). There was a borderline nonsignificant difference in BMI (P = 0.060), with more severe OSA subjects being more obese on average.
MSOSA: obstructive events have a resaturation time that stays relatively constant while oxygen desaturation time increases with increasing magnitude of desaturation. When looking at oximetry data, we can qualitatively see that an obstructive event results in an oxygen desaturation/resaturation cycle that is not sinusoidal (Fig. 2A). Using the MSOSA data, we provide quantitative measurements for these nonsinusoidal cycles. Figure 3A shows the result with desaturation and resaturation grouped by the desaturation amplitude for every desaturation/resaturation pair; Fig. 3B shows this relationship for desaturations and resaturations grouped by their respective amplitudes (i.e., unpaired). The data shown in these figures reveal two important characteristics: 1) as desaturation amplitude increases, time for desaturations also increases in an almost linear fashion; e.g., as the amplitude increases, average desaturation time increased from 16 to 54 s; 2) in contrast, resaturation time remains relatively constant regardless of the degree of desaturation or resaturation amplitude; e.g., ranging from 11 to 19 s in the two figures. Thus the shape of the oxygen desaturation/resaturation cycle is not sinusoidal. Therefore, to more accurately simulate the human desaturation/resaturation curve in a mouse model of CIH, desaturation time should be longer than resaturation. For example, based on our data, if we wanted to program a schedule with an SaO₂ desaturation of 25% and a corresponding SaO₂ resaturation of 25% in a mouse CIH model, the desaturation time should be 40 s, while the resaturation should be 17 s.

MSOSA: within- and between-subject variability in a night of sleep. Based on sleep study data, no subject desaturates to the same nadir all night; there is variability in the oxygen saturation levels over the course of a night of study in an OSA patient with severe OSA [apnea-hypopnea index (AHI) = 83.7 with arterial oxygen saturation (SaO₂) nadir 53%], time lapse 6 h 45 min. There is considerable variability in the oxygen saturation nadirs across the night, and there are periods of normoxia, even if it is during wake.

### Table 2. Demographics of 47 MSOSA subjects

<table>
<thead>
<tr>
<th>Measure</th>
<th>Overall (N = 47)</th>
<th>AHI 6–15 (N = 11)</th>
<th>AHI 16–30 (N = 16)</th>
<th>AHI &gt;30 (N = 20)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>32 (68.1%)</td>
<td>5 (45.5%)</td>
<td>12 (75.0%)</td>
<td>15 (75.0%)</td>
<td>0.2255</td>
</tr>
<tr>
<td>Age, yr</td>
<td>47.0 ± 11.3 [47.6]</td>
<td>46.4 ± 12.6 [50.0]</td>
<td>45.8 ± 11.3 [47.6]</td>
<td>48.3 ± 11.1 [49.1]</td>
<td>0.7900</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>31.8 ± 4.7 [31.6]</td>
<td>29.4 ± 3.9 [28.8]</td>
<td>31.4 ± 4.9 [31.1]</td>
<td>33.5 ± 4.5 [33.5]</td>
<td>0.0596</td>
</tr>
<tr>
<td>AHI</td>
<td>34.2 ± 24.0 [24.7]</td>
<td>10.8 ± 17.1 [11.4]</td>
<td>21.3 ± 4.3 [20.6]</td>
<td>57.4 ± 18.8 [57.8]&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>ODI</td>
<td>17.2 ± 19.5 [10.2]</td>
<td>2.1 ± 3.1 [0.9]</td>
<td>7.9 ± 4.4 [7.7]</td>
<td>32.9 ± 21.0 [28.9]&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>SaO₂ nadir, %</td>
<td>82.9 ± 7.7 [86.0]</td>
<td>89.6 ± 2.9 [90.0]</td>
<td>85.1 ± 3.8 [86.0]</td>
<td>77.4 ± 8.2 [78.5]&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>% Time SaO₂ &lt; 90</td>
<td>7.1 ± 17.1 [0.4]</td>
<td>1.7 ± 5.2 [0.0]</td>
<td>1.3 ± 3.2 [0.3]</td>
<td>14.7 ± 24.1 [4.3]0.0003</td>
<td></td>
</tr>
</tbody>
</table>

Results are presented as N (%) or means ± SD [median]. MSOSA, Molecular Signatures of Obstructive Sleep Apnea Cohort; BMI, body mass index; AHI, apnea-hypopnea index; ODI, oxygen desaturation index. †P value from exact test (categorical outcomes) and ANOVA or Kruskal-Wallis test (continuous variables).
desaturation/resaturation characteristics across the night within a subject, including periods of normoxia (Fig. 2B) from, e.g., nocturia. We quantified this variability for each OSA subject in the MSOSA cohort. The mean and SD of desaturation nadirs for each of the 47 subjects is shown in Fig. 4A, ordered based on the total number of desaturation events throughout the night. In general, subjects with a fewer number of total desaturations had a higher mean nadir (largely above 90%) with a smaller SD; subjects with many total desaturations had lower mean nadirs with a larger SD. For example, in the most severe subject with the lowest SaO2 nadir, not all events were to the same nadir, but varied widely. This is illustrated in Fig. 4B, which shows subject-specific coefficients of variation for desaturation nadirs against the total number of desaturations. We observe a significant positive correlation between these two measures (Spearman’s rho = 0.668, P < 0.0001) (Fig. 4B). In addition to quantifying variability within one subject, we see variability between subjects. There are subjects with few events that have little variability and other subjects with few events with a wide variability. Conversely, there are subjects with many events with little variability and subjects with many events with wide variability in SaO2 nadir.

**Mouse Studies**

Changes in HyCon hardware and software allow programming of oxygen desaturation/resaturation slopes and variability similar to those in OSA subjects. To accommodate both the mixing of gasses at a higher FIO2 and faster cycling, a two-valve system is required (Fig. 1E). Software upgrades enabled input of the following parameters: SH (the Set High FIO2, the desired “normoxia”); SL (the Set Low FIO2, the desired “hyp-
oxia” nadir); TD (desaturation time, time from SH to SL); TR (resaturation time, time from SL to SH); TPH (time during plateau at SH); and TPL (time during plateau at SL). Figure 5A is a schematic of how each cycle can have a unique SH, SL, TD, TR, TPH, and TPL. Figure 5B is a real-time strip demonstrating our ability to program oxygen desaturation and resaturation slopes similar to that seen in subjects (Figs. 2A and 3A). Specifically, this modified system allows slower desaturation with more rapid resaturation. Software upgrades in the HyCon also allow variation in O₂ nadirs across sleep (Fig. 5C), similar to that seen in OSA subjects (Fig. 2B), including, if desired, periods of normoxia. Currently, it is possible to program up to 100 periods (P) per 24 h, with each period capable of delivering unique values of SH, SL, TD, TR, TPH, and TPL. This will

Fig. 5. HyCon oxygen desaturation, resaturation, and variability can be programmed to reflect patients with OSA. A: schematic defining fraction of inspired oxygen (FIO₂) at the Set High Point (SH) as the FIO₂ at the High set point, and Set Low Point (SL) as the FIO₂ at the Low set point. TD is the time of desaturation (s), TR is the time of resaturation (s), TPH is the plateau at SH (s), and TPL is the plateau at SL (s). We are currently able to set up to 100 different periods (P), with each period having unique settings (SH, SL, TD, TR, TPH, TPL) and ranging from minutes to hours. P1 is period 1 and P2 is period 2. B: real-time strip of the HyCon demonstrating SH = 20.9%, SL = 9%, TD = 39 s, TR = 14 s, TPH = 5 s, and TPL = 2 s. C: programming variability of desaturation and resaturation throughout sleep within the HyCon. The HyCon is able to deliver variability throughout sleep as seen by the EO₂S tracing; here the start time was 0900 and the end time was 1600, with a time lapse of ~7 h.
allow investigators to address whether variability in frequencies, amplitude, nadir, and durations of oxygen desaturation play differential roles in influencing changes in gene and protein expression.

Measurement of changes in oxygen saturation with this new system: MouseOx study. We first investigated the temporal relationship between the various measures of oxygen. Figure 6 displays, in real time, the overlap of the HyCon settings (top panel, straight line), the HyCon O₂ sensor (top panel, dotted line), the external O₂ sensor (middle panel), and the saturation from the MouseOx sensor (bottom panel). The external O₂ sensor signal lags behind that of the HyCon because gasses are vacuumed through a tube (3 ft in length) before the oxygen concentration is measured and a chemical reaction transpires prior to evaluating the oxygen concentration. The MouseOx readings are also delayed behind the HyCon sensors, most likely due to the physiology of oxygen delivery. However, after accounting for these expected delays there is a cycling of 1:1:1, meaning that one cycle delivered by the HyCon corresponds to one cycle measured by the external O₂ sensor, which corresponds to one cycle recorded by the MouseOx.

We evaluated oxygen saturation with the MouseOx at different FiO₂ values. We report the oxygen saturation averages (at the zenith and nadir) at each “dose” of CIH for both the sinusoidal (Table 3) and nonsinusoidal (Table 4) schedule. Our sinusoidal oxygen saturation measurements are similar to that described by Jun et al. (31). At CIH levels with very low nadirs, the oxygen saturation did not return back to the baseline of normoxia if the mouse was sleeping (observation). However, when the mouse woke up, i.e., had an arousal as reflected by movement artifact on the MouseOx, then there was a return to normoxia. This likely occurs because hypoxia was so severe at the lower FiO₂ levels that allowing only 30 s to come back to normoxia may not be adequate to completely normalize blood oxygen levels, unless there is a compensatory further increase in breathing and heart rate to increase oxygen delivery. Thus, with progressive decreases in FiO₂, there is not only a progressive decrease in SaO₂ nadir, but also a small effect on the zenith (from 94% to 90%). In our nonsinusoidal oxygen saturation programs within the C57Bl/6 strain, there were statistically significant differences in both the SaO₂ zenith and nadir between the SR and the LR schedules at different levels of FiO₂. In general, we see that the LR program resulted in

![Fig. 6. HyCon settings overlap with External O₂ sensor and MouseOx demonstrates dynamic 1:1:1 cycling rate. Top: simultaneous HyCon settings (solid line denotes HyCon settings and dashed line denotes what the HyCon O₂ sensor measures to feedback to the software which then regulates the valves). Middle trace: external O₂ sensor placed at the level of the mouse’s nose. Bottom trace: oxygen saturation measured by MouseOx. All tracings are overlapped in time and demonstrate a 1:1:1 cycling rate.]

| Table 3. Assessment of changes in oxygen saturation in the new system: MouseOx study using a sinusoidal schedule (C57Bl/6 mice) |
|-----------------|-----------------|------------------|
| FiO₂ | Zenith | Nadir |
| 21-16 | 92.4 ± 2.7 | 84.1 ± 2.9 |
| 21-15 | 94.0 ± 1.3 | 81.6 ± 1.4 |
| 21-14 | 94.1 ± 0.8 | 78.1 ± 1.0 |
| 21-13 | 94.5 ± 0.9 | 76.2 ± 2.1 |
| 21-12 | 92.5 ± 1.1 | 67.9 ± 2.8 |
| 21-11 | 92.7 ± 0.8 | 62.8 ± 1.7 |
| 21-10 | 92.5 ± 0.7 | 61.0 ± 1.4 |
| 21-9 | 91.8 ± 0.8 | 54.7 ± 2.5 |
| 21-8 | 90.5 ± 1.1 | 48.9 ± 1.8 |
| 21-7 | 89.8 ± 1.2 | 44.0 ± 2.0 |
| 21-6 | 89.6 ± 1.2 | 38.7 ± 1.4 |
| 21-5 | 90.7 ± 1.7 | 36.6 ± 1.7 |

Values are means ± SE SaO₂ of zenith and nadir.
Table 4. Assessment of changes in oxygen saturation in the new system: MouseOx study using a nonsinusoidal schedule

<table>
<thead>
<tr>
<th>Fio2</th>
<th>Zenith</th>
<th>Nadir</th>
<th>P</th>
<th>Zenith</th>
<th>Nadir</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SR</td>
<td>LR</td>
<td></td>
<td>SR</td>
<td>LR</td>
<td></td>
</tr>
<tr>
<td>21-18</td>
<td>97.6 ± 0.6</td>
<td>97.0 ± 0.6</td>
<td>0.430</td>
<td>92.6 ± 1.5</td>
<td>89.6 ± 1.5</td>
<td>0.154</td>
</tr>
<tr>
<td>21-17</td>
<td>96.4 ± 0.7</td>
<td>96.2 ± 0.7</td>
<td>0.830</td>
<td>87.7 ± 1.0</td>
<td>84.9 ± 1.1</td>
<td>0.057</td>
</tr>
<tr>
<td>21-16</td>
<td>95.0 ± 0.5</td>
<td>95.4 ± 0.4</td>
<td>0.504</td>
<td>83.2 ± 0.7</td>
<td>81.0 ± 0.7</td>
<td>0.003</td>
</tr>
<tr>
<td>21-15</td>
<td>94.3 ± 0.4</td>
<td>92.4 ± 0.4</td>
<td>0.066</td>
<td>78.9 ± 0.8</td>
<td>76.1 ± 0.8</td>
<td>0.017</td>
</tr>
<tr>
<td>21-14</td>
<td>93.8 ± 0.5</td>
<td>93.1 ± 0.5</td>
<td>0.072</td>
<td>74.7 ± 1.1</td>
<td>70.5 ± 1.1</td>
<td>0.009</td>
</tr>
<tr>
<td>21-13</td>
<td>94.0 ± 0.6</td>
<td>94.5 ± 0.6</td>
<td>0.606</td>
<td>70.8 ± 1.4</td>
<td>65.2 ± 1.4</td>
<td>0.004</td>
</tr>
<tr>
<td>21-12</td>
<td>93.8 ± 0.8</td>
<td>94.1 ± 0.8</td>
<td>0.283</td>
<td>65.8 ± 1.6</td>
<td>60.4 ± 1.6</td>
<td>0.022</td>
</tr>
<tr>
<td>21-11</td>
<td>92.1 ± 1.0</td>
<td>94.8 ± 1.0</td>
<td>0.044</td>
<td>61.4 ± 1.9</td>
<td>55.3 ± 1.9</td>
<td>0.025</td>
</tr>
<tr>
<td>21-10</td>
<td>91.8 ± 0.7</td>
<td>93.9 ± 0.7</td>
<td>0.036</td>
<td>56.7 ± 1.2</td>
<td>52.3 ± 1.3</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Values are means ± SE SaO2 of zenith and nadir in both short (SR) and long (LR) resaturation times. P < 0.05 between SR and LR at zenith or nadir in boldface.

significantly lower nadirs at Fio2 levels of 0.21–0.16 and lower, and the SR program resulted in lower SaO2 zenith values at Fio2 of 0.21–0.11 and 0.21–0.10. These differences were not seen in the 129SH/SvImJ, A/J, and NZO/HilJ strains; there was only a borderline significant difference (P = 0.045) in the SaO2 nadirs at Fio2 of 0.21–0.16 in the NZO. Not unexpectedly, delivering the same Fio2 to different strains does not result in the same SaO2; there were significant differences among the four strains at every level of Fio2 for both the SR (all P ≤ 4.7 × 10−7) and LR (all P ≤ 0.006).

Differences in oxidative stress with short and long resaturation times. We assessed whether the three conditions had differential effects on oxidative stress, measured as urinary 8,12-iso-iPF2α-VI. After removing urine samples where volume was not >210 μl, the final analysis included 18 sham, 22 long resaturation, and 26 short resaturation samples. Untransformed least squares mean ± SE urinary 8,12-iso-iPF2α-VI levels normalized to milligram creatinine across the three conditions were 0.36 ± 0.08 ng/mg, 0.29 ± 0.04 ng/mg, and 0.64 ± 0.19 ng/mg, for sham, LR, and SR, respectively. These levels were natural log transformed for our statistical analyses, to satisfy assumptions of normality. When comparing the three conditions, we find that the data are consistent with our a priori hypothesis that the SR program results in the greatest levels of urinary 8,12-iso-iPF2α-VI (Table 5). When looking at pairwise comparisons between conditions, short resaturation increases mean 8,12-iso-iPF2α-VI relative to sham by 1.30-fold (90% CI: 1.00–1.69, P = 0.049), while long resaturation is not elevated relative to sham (P = 0.676). We observed a borderline, but nonsignificantly elevated 8,12-iso-iPF2α-VI for the SR compared with LR (P = 0.067); we note that the estimated difference was larger between SR and LR and between SR and sham. Given that there was no difference between LR and sham, we combined these groups and compared against SR. In this comparison, the SR had 8,12-iso-iPF2α-VI levels 1.36 times larger (90% CI: 1.02–1.82, P = 0.039) than the combined sham + LR group.

**DISCUSSION**

We demonstrate with data from the MSOSA study of OSA subjects that a cycle of intermittent hypoxia is not sinusoidal with the desaturation time increasing in an almost linear relationship to the degree of hypoxia (based on amplitude), whereas resaturation time is somewhat constant and much faster (~15 s), independent of the amplitude. There is also within-subject variation in the degree of desaturations across the night. We implemented these characteristics into our new mouse model of CIH by modifying the Hycon to deliver a shorter resaturation time and allow for variability. The shorter resaturation time may have functional significance, as we demonstrated that even after only 3 h of CIH there is an increase in urine 8,12-iso-iPF2α-VI in the short resaturation

Table 5. Effects of Sham, LR, and SR on oxidative stress using a mixed-model analysis comparing natural log-transformed 8,12-iso-iPF2α-VI measurements among conditions in samples with >210 μl

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Estimate ± SE</th>
<th>90% CI</th>
<th>1-Proportional difference (90% CI)</th>
<th>P1-sided</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose Response (Sham &gt; LR &gt; SR)</td>
<td>0.12 ± 0.10</td>
<td>-0.05, 0.28</td>
<td>0.1211</td>
<td></td>
</tr>
<tr>
<td>LR-Sham</td>
<td>-0.09 ± 0.19</td>
<td>-0.42, 0.24</td>
<td>0.91 (0.66, 1.27)</td>
<td>0.6760</td>
</tr>
<tr>
<td>SR-Sham</td>
<td>0.27 ± 0.16</td>
<td>0.00, 0.53</td>
<td>1.30 (1.00, 1.69)</td>
<td>0.0478</td>
</tr>
<tr>
<td>SR-LR</td>
<td>0.35 ± 0.23</td>
<td>-0.04, 0.75</td>
<td>1.43 (0.97, 2.11)</td>
<td>0.0666</td>
</tr>
</tbody>
</table>

*Calculated as e^estimate and interpreted as the between-group multiplicative magnitude of difference in untransformed results; e.g., the value in the first group is e^estimate times larger than that in the second.*
schedule compared with long resaturation plus sham. By designing a rodent model of CIH that is capable of simulating oxygenation characteristics seen in humans, we have a tool that can implement these and other oxygenation characteristics to study the effects of cyclic intermittent hypoxia on various outcomes of CIH.

Lessons From the Clinical Cohort

The MSOSA data illustrate several observations about apnea/hypopnea events. First, we demonstrate quantitatively that an event’s cycle of intermittent hypoxia is not sinusoidal, but has a much more rapid time for oxygen resaturation compared with that for oxygen desaturation. Furthermore, our measurements of desaturations/resaturation time support the idea that for obstructive apneic/hypopneic events, desaturation time (time to reach the nadir) is variable and is a function of the SaO2 nadir; but resaturation time is relatively constant (about 15–20 s) and rapid. Desaturation of obstructive events is terminated by an arousal at the end of the obstructive event. What stimulates the arousal is a combination of hypoxia and hypercapnia sensed both peripherally and centrally as well as increasing ventilatory effort (4, 21, 27). Whether there is genetic variation in determining one’s arousal threshold which shapes the duration of apnea is an exciting and active area of inquiry (14). Resaturation time of obstructive events is determined by ventilatory drive such that the longer the apneic episode the greater is the ventilatory drive at the end of the apneic episode. For example, a long apnea will result in a larger (faster) increase in ventilation when airway patency is reestablished. Conversely, a short apnea will result in a smaller (slower) increase in ventilation. Thus the relatively constant time for resaturation is likely to be explained by this relationship between apnea length and ventilatory response. The results we present, however, are specific to obstructive events. Central events in Cheyne-Stokes breathing are different in etiology in that they result from the unstable operation of a closed-loop feedback system with a sleep-dependent CO2 apneic episode threshold (13). The arousal, when it occurs, is not at the end of the apnea but rather at the peak of ventilation during the ventilatory cycle. Given the difference in etiology, it is unclear if resaturation time in Cheyne-Stokes respiration would also be constant and more rapid than desaturation time and requires further study. Ataxic respiration (damage to the medulla oblongata) and Biot’s respiration (damage to the pons that can be caused by opioid use) being of an altogether different etiology is another area that requires further study to assess whether resaturation time is constant and more rapid than desaturation.

Another characteristic illustrated by MSOSA is that there is considerable within-subject variability in SaO2 nadir throughout a given night sleep, as well as clear variability between subjects, even with similar number of events. In general, the higher the number of events a subject experiences, the higher the variability of the SaO2 nadir. However, there are subjects with few events that have large variability in nadirs, and there are also subjects with a large number of events but low variability. Whether variability of oxygen nadir has an impact differentially on molecular consequences and disease outcome measures remains an open question for future study.

Use of Mouse Models of CIH to Simulate Characteristics of Desaturation/Resaturation Found in Subjects with OSA

Cyclical intermittent hypoxia systems are not standardized. To understand the variability and clinical relevance of current rodent models of CIH we performed a PubMed search (search terms “sleep,” “intermittent hypoxia,” and “rodent” with filters “Journal Articles,” “English”) identifying 326 articles resulting in 21 distinct rodent systems of CIH that correlated FiO2 to blood arterial oxygen levels (5–7, 9, 12, 15, 17, 22, 24, 32, 37–39, 43, 50, 53, 56, 62, 68, 69, 73). In short, there are many different mechanical delivery CIH systems being used to conduct OSA research in rodent models with no standardizations. CIH systems have a considerable variation in SaO2/PaO2 at a given FiO2. For example, at an FiO2 of 0.05, SaO2 can range from 32.5% (61) to 80–85% (50, 68). CIH systems also have considerable variation in schedule with desaturation time, ranging from 12 s (38) to 3 min (9), and considerable variation in resaturation time, ranging from 18 s (38) to 6 min (9). Most schedules use sinusoidal desaturation and resaturation times, e.g., 30 s-30 s (13, 15, 21, 37, 61, 62); 60 s-60 s (5, 43); 90 s-90 s (32, 39); or 120 s-120 s (56). In addition to mechanical differences of gas delivery between CIH systems, there are differences in assessing the performance of CIH systems, i.e., using either arterial blood gasses or continuous MouseOx (Starr Life Sciences, Oakmont, PA). Given mechanical differences between systems, consistently providing details regarding the SaO2 at a given FiO2 would allow data from different laboratories with different systems to be compared.

Shorter resaturation results in increased 8,12-iso-iPF2α-VI.

It is well known that a consequence of cellular reoxygenation after sustained hypoxia (e.g., as seen in a stroke or heart attack) is a net increase in ROS (3, 16), with increases in superoxide observed instantly (40, 66) and reaching significance as early as 1 min after reoxygenation (26). Clinical studies indicate that subjects with OSA exhibit oxidative stress (29, 35), as well as the downstream effects of oxidative stress, like the activation of the transcription factor NfκB (63, 71) and cytokine release (49). Animal studies also provide evidence that CIH leads to oxidative stress (30, 51, 59). Here, we present data that a CIH schedule that includes a faster resaturation time may affect the rate of free radicals produced, consistent with the notion that relevant enzymes are generating superoxide or hydrogen peroxide in response to the rate by which the electron acceptor (oxygen) is introduced into the cell. Thus, to more accurately simulate the effects of OSA, rapid resaturation is likely to be more relevant.

Given that more rapid cycling occurs with faster resaturation, there is a difference in the number of cycles between the short resaturation schedule (n = 209) and the long resaturation schedule (n = 89). We had considered keeping the number of cycles constant by increasing the long resaturation schedule to 6 h and 40 min and comparing it to a short resaturation schedule for 3 h. This, however, would have confounding effects in that we would be comparing effects over much different time scales. The reason we chose to keep duration of 3 h constant was that, practically speaking, when investigators use a long resaturation schedule of 90 s they do not double the total duration of CIH exposure (per day) to accommodate their decision to do a long resaturation, e.g., they do not “match” the number of cycles to investigators that use a shorter resaturation...
of the mitochondria, releases superoxide during hypoxia, allo-
owing ROS to cross into the cytosol to either trigger the
release of intracellular calcium or inhibit prolyl hydroxylase
from degrading Hif1α. With so many potential CIH-associated
mechanisms to explore, our new rodent model of CIH will
allow us to study the effects of oxygen desaturation/resatura-
tion frequency, hypoxic nadir, and duration on cellular oxygen
sensors.

Limitations

Human studies. Data surrounding very low oxygen satu-
ration should be acknowledged with caution due to the intrinsic
and known limitations of the pulse oximeter at very low
oxygen saturations. We believe that our results can be gener-
alized to most OSA populations. However, additional studies
with larger numbers and among populations with various
ethnic, age, comorbidity, and BMI distributions would be
useful to both better generalize and either confirm or refute the
desaturation/resaturation characteristics observed here. Al-
though the MSOSA was not designed to assess the impact of
variable oxygen characteristics on differences in outcome mea-
sures, e.g., hypertension, stroke, etc., this will be an interesting
future direction.

Mouse studies. Mice have metabolic rates normalized to
body weight that is 7–8 times less efficient than a human. Mice
have a 7- to 8-fold increase in heart rates and respiratory rate,
although most mice are housed 10°C below their thermoneu-
trality and therefore the difference in heart rate may only be
5-fold (25). These scalar differences between mice and humans
make it impossible to exactly replicate the patterns of desatura-
tion/resaturation in individual subjects with OSA. However,
one would argue that the major characteristics of oxygenation
found in OSA subjects should be simulated in mouse models.
By incorporating these characteristics, we may find new mol-
ecular consequences of CIH that help us better understand the
pathophysiological consequences of OSA.

When using continuous oxygen saturation devices like the
MouseOx, the technology calculates oxygen saturation based
on oxygen dissociation curves for mice or rats, not curves
based on humans (23). Because Starr Life Sciences has im-
proved their technology over the years, it is important to report
which generation of hardware (sensors) and software are used
due to notable differences (E. Ayers, Starr Life Sciences,
personal communication). Evolving technology that allows
continuous measurement of oxygenation would seem to be the
optimal method of assessing the performance of a CIH system,
allowing standardization of CIH systems. The MouseOx rec-
ordings in Tables 3 and 4 are specific to the strains of mice we
tested, and specific to the CIH schedules we selected within the
Hycon system. We recommend that SaO2 as well as Pio2, data
be reported to allow comparison of the results with different
schedules and systems when exposed to various conditions.

Conclusions

Rodent models of CIH are extremely valuable in helping us
understand the mechanism for the consequences of OSA.
Rather than assume that SaO2 nadir is the only important
variable, we have questioned this premise and present quanti-
tative characteristics of oxygen desaturation/resaturation and
variability in subjects with OSA. We have implemented these
characteristics into a mouse model of CIH that is practical and commercially available. One characteristic seen in OSA sub-
characteristics into a mouse model of CIH that is practical and incorporating them into mouse models of CIH may reveal novel pathways and mechanisms.

ACKNOWLEDGMENTS

We thank Dr. V. Y. Polotsky for introducing us to the HyCon and S. Savransky for expertise and willingness to implement the numerous requests to modify the HyCon. We thank the Penn team behind the MSOSA data [Trial name: Biomark-
mechanisms. into mouse models of CIH may reveal novel pathways and developing the MSOSA data [Trial name: Biomark-
mechanisms. into mouse models of CIH may reveal novel pathways and mechanisms.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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