The role of NADPH oxidase in chronic intermittent hypoxia-induced respiratory plasticity in adult male mice

Sarah E. Drummond, David P. Burns, Karen M. O’Connor, Gerard Clarke, Ken D. O’Halloran

A. Department of Physiology, School of Medicine, College of Medicine & Health, University College Cork, Cork, Ireland
B. APC Microbiome Ireland, University College Cork, Cork, Ireland
C. Department of Anatomy & Neuroscience, School of Medicine, College of Medicine & Health, University College Cork, Cork, Ireland
D. Department of Psychiatry and Neurobehavioural Science, School of Medicine, College of Medicine & Health, University College Cork, Cork, Ireland

1. Introduction

Obstructive sleep apnoea syndrome (OSAS) is the most prevalent form of sleep-disordered breathing (SDB) (White and Younes, 2012). A common feature of OSAS is recurrent oxygen desaturation associated with obstructive events due to airway collapse. The frequency of episodic desaturations during sleep defines mild, moderate and severe sleep apnoea (Osman et al., 2018; Veasey and Rosen, 2019).

Chronic intermittent hypoxia (CIH) is widely considered to be the driving force behind the detrimental morbidities observed in patients with OSAS. This is primarily based on studies from animal models incorporating CIH (Chopra et al., 2016). It is well documented that exposure to CIH mediates cardiovascular (O’Connor et al., 2020), neurocognitive (Al Lawati et al., 2009), metabolic (Drager et al., 2011), testicular (Torres et al., 2014), pancreatic (Wang et al., 2013), hepatic (Savransky et al., 2007) and renal (Rosas, 2011) disorders associated with OSAS. These effects are likely attributable to mediators of oxidative stress called reactive oxygen species (ROS), which are produced following repeated cycles of hypoxia and re-oxygenation. Indeed, it is well described that OSAS is an oxidative stress disorder, with concomitant upregulation of inflammatory signalling in many tissues (Lavie, 2003). ROS is the collective term for a variety of reactive entities derived from molecular oxygen, including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH·). ROS have important roles in normal cell signalling at various sites related to respiratory control (Dean et al., 2004). ROS are produced by a variety of cellular complexes including the mitochondria, xanthine oxidase, phospholipase A2 and NADPH oxidase (NOX) (Di Meo et al., 2016). The NOX family of enzymes is composed of NOX1–5 inclusive and DUOX-1 and -2, which have explicit roles in ROS production (Armitage et al., 2009). NOX2 is the most widely expressed isoform, with expression of NOX2 documented throughout sites of the respiratory control network, including peripheral chemoreceptors (carotid body) (Dinger et al., 2007; Peng et al., 2009), central pattern generating and integrative sites (Glass et al., 2007), motor neurons (MacFarlane and Mitchell, 2008) and the respiratory musculature (Williams et al., 2015; Loehr et al., 2018).

Respiratory plasticity is defined as a persistent change in respiratory behaviour, based on prior experience that may involve structural and/or functional adaptations of the respiratory control network. Respiratory plasticity is defined as a persistent change in respiratory behaviour, based on prior experience that may involve structural and/or functional adaptations of the respiratory control network. The role of NADPH oxidase in chronic intermittent hypoxia-induced respiratory plasticity in adult male mice

A. Department of Physiology, School of Medicine, College of Medicine & Health, University College Cork, Cork, Ireland
B. APC Microbiome Ireland, University College Cork, Cork, Ireland
C. Department of Anatomy & Neuroscience, School of Medicine, College of Medicine & Health, University College Cork, Cork, Ireland
D. Department of Psychiatry and Neurobehavioural Science, School of Medicine, College of Medicine & Health, University College Cork, Cork, Ireland

1. Introduction

Obstructive sleep apnoea syndrome (OSAS) is the most prevalent form of sleep-disordered breathing (SDB) (White and Younes, 2012). A common feature of OSAS is recurrent oxygen desaturation associated with obstructive events due to airway collapse. The frequency of episodic desaturations during sleep defines mild, moderate and severe sleep apnoea (Osman et al., 2018; Veasey and Rosen, 2019).

Chronic intermittent hypoxia (CIH) is widely considered to be the driving force behind the detrimental morbidities observed in patients with OSAS. This is primarily based on studies from animal models incorporating CIH (Chopra et al., 2016). It is well documented that exposure to CIH mediates cardiovascular (O’Connor et al., 2020), neurocognitive (Al Lawati et al., 2009), metabolic (Drager et al., 2011), testicular (Torres et al., 2014), pancreatic (Wang et al., 2013), hepatic (Savransky et al., 2007) and renal (Rosas, 2011) disorders associated with OSAS. These effects are likely attributable to mediators of oxidative stress called reactive oxygen species (ROS), which are produced following repeated cycles of hypoxia and re-oxygenation. Indeed, it is well described that OSAS is an oxidative stress disorder, with concomitant upregulation of inflammatory signalling in many tissues (Lavie, 2003). ROS is the collective term for a variety of reactive entities derived from molecular oxygen, including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH·). ROS have important roles in normal cell signalling at various sites related to respiratory control (Dean et al., 2004). ROS are produced by a variety of cellular complexes including the mitochondria, xanthine oxidase, phospholipase A2 and NADPH oxidase (NOX) (Di Meo et al., 2016). The NOX family of enzymes is composed of NOX1–5 inclusive and DUOX-1 and -2, which have explicit roles in ROS production (Armitage et al., 2009). NOX2 is the most widely expressed isoform, with expression of NOX2 documented throughout sites of the respiratory control network, including peripheral chemoreceptors (carotid body) (Dinger et al., 2007; Peng et al., 2009), central pattern generating and integrative sites (Glass et al., 2007), motor neurons (MacFarlane and Mitchell, 2008) and the respiratory musculature (Williams et al., 2015; Loehr et al., 2018).
2. Materials and methods

2.1. Ethical approval

Procedures on live animals were performed under licence from the Government of Ireland Department of Health (B100/4498) in accordance with National and European legislation (2010/63/EU) following ethical approval by University College Cork (AEEC no. 2013/035).

2.2. Chronic intermittent hypoxia animal model

C57BL/6 J male mice (9 weeks old) were purchased from Envigo, UK, and were assigned to one of 3 groups: normoxic control (sham; n = 12), chronic intermittent hypoxia (CIH)-exposed (CIH; n = 12) and CIH + apocynin (CIH + APO; n = 12). Animals were conventionally housed in temperature- and humidity-controlled rooms, operating on a 12 h light/12 h dark cycle with food and water available ad libitum. For daily gas treatment, mice were housed in standard cages placed in commercial cages designed with hypoxia chambers (Oxyacetylene™, Biospherix, Lacona, NY, USA). Exposure to CIH consisted of the cycling of gas in environmental conditions. Mice were observed at quiet rest using whole-body plethysmography (WBP). Mice were introduced into plethysmograph chambers (Model PLY4211; volume 600 mL, Buxco Research Systems, Wilmington, NC, USA) with room air flowing through (1 L min⁻¹) to ensure adequate O₂ and CO₂ environmental conditions. Mice were allowed 60 min to acclimate to the chamber environment. Following exploration and grooming behaviours during this period, baseline recordings commenced during quiet rest. Multiple baseline measurements consisting of 15-minute bins were recorded to increase the cumulative duration of the baseline period and data were averaged for the whole period as represented in Tables 3 and 4. Segments with witnessed movement of the animal, or periods in the recorded data block with disrupted signals associated with movement or non-ventilatory behaviours (e.g. sniffing) were excluded from the analysis. Typically, following the acclimation period, mice were observed at quiet rest (immobile) throughout each of the 15-minute recording sessions included in the analysis. Respiratory variables including respiratory frequency (fR), tidal volume (Vt), minute ventilation (Ven) inspiratory time (Tins), expiratory time (Texp), peak inspiratory flow (PIF) and peak expiratory flow (PEF) were recorded every 2 s for analysis offline. Levels of O₂ and CO₂ gas entering and exiting the plethysmograph chambers were measured on a minute-by-minute basis (O₂ and CO₂ analyser; AD Instruments, Colorado Springs, CO, USA) allowing O₂ consumption (VO₂) and CO₂ production (VCO₂) to be determined (Burns et al., 2017, 2018). From this, the ventilatory equivalent for CO₂ (VE/ VCO₂) and O₂ (VE/ VO₂) were calculated.

2.3. Whole-body plethysmography (WBP)

Respiratory flow recordings were examined in awake, unrestrained mice during quiet rest using WBP. Mice were introduced into plethysmograph chambers (Model PLY4211; volume 600 mL, Buxco Research Systems, Wilmington, NC, USA) with room air flowing through (1 L min⁻¹) to ensure adequate O₂ and CO₂ environmental conditions. Mice were allowed 60 min to acclimate to the chamber environment. Following exploration and grooming behaviours during this period, baseline recordings commenced during quiet rest. Multiple baseline measurements consisting of 15-minute bins were recorded to increase the cumulative duration of the baseline period and data were averaged for the whole period as represented in Tables 3 and 4. Segments with witnessed movement of the animal, or periods in the recorded data block with disrupted signals associated with movement or non-ventilatory behaviours (e.g. sniffing) were excluded from the analysis. Typically, following the acclimation period, mice were observed at quiet rest (immobile) throughout each of the 15-minute recording sessions included in the analysis. Respiratory variables including respiratory frequency (fR), tidal volume (Vt), minute ventilation (Ven) inspiratory time (Tins), expiratory time (Texp), peak inspiratory flow (PIF) and peak expiratory flow (PEF) were recorded every 2 s for analysis offline. Levels of O₂ and CO₂ gas entering and exiting the plethysmograph chambers were measured on a minute-by-minute basis (O₂ and CO₂ analyser; AD Instruments, Colorado Springs, CO, USA) allowing O₂ consumption (VO₂) and CO₂ production (VCO₂) to be determined (Burns et al., 2017, 2018). From this, the ventilatory equivalent for CO₂ (VE/ VCO₂) and O₂ (VE/ VO₂) were calculated.

2.3.1. Respiratory stability

Respiratory flow recordings for sham (n = 12), CIH (n = 12), CIH + APO (n = 12), NOX2 KO sham (n = 12) and NOX2 KO CIH (n = 12) mice during normoxia (21 % O₂) were manually examined for the measurement of respiratory stability and apnoea scoring. The breath-to-breath (Bbn) and subsequent interval (Bbn +1) of 200 breaths were analysed as previously described (Peng et al., 2011a; Souza et al., 2015). Poincaré plots expressing Bbn versus Bbn +1 for 200 consecutive breaths were plotted for expired time (Te) and total breath duration (Ttot) for all groups outlined above. Short-term variability (SD1) and long-term variability (SD2) were calculated as indices of breathing variability. Respiratory flow signals were manually analysed for the frequency of sigh events (augmented breaths), which was defined as an increase in tidal volume to at least twice the amplitude observed during a eupnoic breath, with increased inspiratory and expiratory flow. Additionally, the frequency and duration of spontaneous and post-sigh apnoeic events were manually determined by visual inspection of the recordings. The a priori criterion for apnoea was defined as a cessation in breathing greater than two missed breaths, and the a priori criterion for a post-sigh apnoea was defined as an apnoeic event occurring within 20 s following an augmented breath, as previously described (Edge et al., 2012; O’Connor et al., 2019). These criteria established clear inclusion and exclusion conditions during data analysis, which were applied to all groups. The investigator performing the analysis was not blinded to the treatment.
group. However, a second investigator confirmed inclusion and exclusion criteria that were applied uniformly and without bias to the manually scored recordings in all groups.

2.3.2. Ventilatory responsiveness to chemostimulation
Following baseline recordings, ventilatory responsiveness to chemostimulation was examined for all groups. A 10-minute hypoxic challenge (F\textsubscript{O2} = 0.1) was elicited in which mice were challenged with reduced levels of O\textsubscript{2} before returning flow to room air for 20 min. Once baseline was re-established, a 10-minute hypercapnic (F\textsubscript{CO2} = 0.05, F\textsubscript{O2} = 0.95) challenge was performed. Ventilatory parameters were also recorded every 2 s during hypoxic and hypercapnic challenges. These data were subsequently averaged and expressed on a minute-by-minute basis. Metabolic parameters (V\textsubscript{O2} and V\textsubscript{CO2}) were recorded on a minute-by-minute basis, but only data averaged for the final 3 min of the 10-minute challenge are shown to avoid transient events at the beginning of the trials. Peak ventilation for each individual animal was determined during hypoxia and hypercapnia and compared with the baseline normoxic period. Steady-state ventilatory responsiveness to hypoxia and hypercapnia (during the final 3 min of each challenge) was expressed as a change from baseline (Δ V\textsubscript{t}). The corresponding mean V\textsubscript{CO2} value was used to calculate Δ V\textsubscript{t} / V\textsubscript{CO2}.

2.4. High performance liquid chromatography (HPLC)
Monoamines and metabolites important in the neurochemical control of breathing, including 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), were quantified in brainstem samples from all groups using high performance liquid chromatography (HPLC), as previously described (Clarke et al., 2013). Frozen samples were thawed in 500 μl of chilled homogenising buffer spiked with 2 ng/20 μl of N-Methyl 5-HT (Sigma, UK) as internal standard, sonicated for 2 s bursts (Bandelin Sonoplus HD 2070) and centrifuged at 14,000 RPM for 20 min. at 8 °C (MIKRO 22 R refrigerated centrifuge). 20 μl of supernatant was injected onto the HPLC system (Electrochemical Detection). The mobile phase contained 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Fisher Scientific Ireland), 5.6 mM octane-1-sulphonic acid (Sigma) and 11 % (v/v) HPLC-grade methanol (Fisher Scientific, Ireland), and was adjusted to pH 2.8 using 4 N sodium hydroxide (Alkem/Reagecon). A reverse-phase column (Kinetex2.6 μ C18 100 × 4.6 mm, Phenomenex, UK) was maintained at 30 °C in the separation (flow rate 0.9 μl/min), the glassy carbon working electrode combined with an Ag/AgCl reference electrode (Shimadzu) was operated a +0.8 V and the chromatograms generated were analysed using Class-VP 5 software (Shimadzu, Corporation, Kyoto, Japan). Monoamines and their metabolites were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during the sample analysis. The chromatograms were processed using Class 5-VP software (Shimadzu Corporation, Kyoto, Japan). Analyte: Internal standard peak height ratios were measured and compared with standard injections and results were expressed as ng of analyte per g tissue weight.

2.5. Statistical analysis
Data are expressed as mean ± SD or are shown in violin plots with individual data points representing each animal in the relevant groups. Data were statistically compared using Prism 8.0 (Graphpad Software, San Diego, CA, USA). Data were tested for normal distribution and equal variances. For the comparison of sham, CIH and CIH + APO groups, data sets with confirmed normal distribution were statistically compared using one-way ANOVA with Tukey’s post hoc test. Data sets which were not normally distributed were compared using a non-parametric Kruskal-Wallis with Dunn’s post hoc test. Each P value is adjusted to account for multiple comparisons. Statistical significance was taken at P < 0.05. For the comparison of NOX2 KO sham and NOX2 KO CIH groups, data sets which were normally distributed and of equal variance were statistically compared using unpaired two-tailed Student’s t test. Welch’s correction was applied in the case of unequal variance. Data which were not normally distributed were compared using Mann Whitney non-parametric tests. Statistical significance was taken at P < 0.05. Ventilatory responsiveness to chemostimulation was statistically compared by repeated measures two-way ANOVA (RMANOVA) with Bonferroni post hoc test; P < 0.05 was the criterion for statistical significance.

3. Results
3.1. Organ and muscle mass
Comparisons of organ and muscle mass in all experimental groups are shown in Tables 1 and 2. Haematocrit (Hct) was significantly increased following exposure to CIH in wild-type (P = 0.0361) and NOX2 KO mice (P = 0.0167). Administration of the putative NOX2 inhibitor, apocynin (2mM), in the drinking water during exposure to CIH, significantly reduced Hct compared with CIH-exposed mice (P = 0.035). Cardiac muscle mass was unaffected by exposure to CIH or treatment with apocynin in wild-type mice. Left cardiac ventricle (P = 0.0048) and total ventricular mass (P = 0.0075) were significantly increased following exposure to CIH in NOX2 deficient mice. The difference between wet and dry lung mass, an index of pulmonary oedema,

Table 1
Organ and muscle mass in sham, CIH-exposed and CIH + APO-exposed mice. Definition of abbreviations: Hct, haematocrit; TA, tibialis anterior; EDL, extensor digitorum longus; sol, soleus; RV, right heart ventricle; LV, left heart ventricle; sham, normoxia (21 % O\textsubscript{2})-exposed; CIH, chronic intermittent hypoxia-exposed; CIH + APO, Apocynin (2 mM) given in the drinking water for the duration of CIH exposure. Values are expressed as mean ± SD. Data sets with confirmed normal distribution were statistically compared using one-way ANOVA with Tukey’s post hoc test. Data sets which were not normally distributed were compared using a non-parametric Kruskal-Wallis with Dunn’s post hoc test. Each P value is adjusted to account for multiple comparisons. Bolded numbers indicate statistical significance (P < 0.05).

|               | Sham | CIH | CIH + APO | One-way ANOVA | CIH vs CIH + APO (P value) |
|---------------|------|-----|-----------|---------------|----------------------------|
| Hct (n = 9)   | 44 ± 1 | 47 ± 2 | 43 ± 2 | 0.0039 | 0.0361 | 0.0035 |
| Spleen (mg/g) | 2.37 ± 0.57 | 1.92 ± 0.37 | 2.63 ± 0.46 | 0.0037 | 0.1876 | 0.0025 |
| TA (mg/g)     | 1.47 ± 0.22 | 1.43 ± 0.10 | 1.56 ± 0.10 | 0.0618 | - | - |
| EDL (mg/g)    | 0.32 ± 0.06 | 0.29 ± 0.05 | 0.42 ± 0.04 | <0.0001 | 0.4775 | <0.0001 |
| Sol (mg/g)    | 0.29 ± 0.07 | 0.27 ± 0.05 | 0.31 ± 0.05 | 0.1838 | - | - |
| RV (mg/g)     | 1.16 ± 0.26 | 1.21 ± 0.21 | 1.31 ± 0.25 | 0.4118 | - | - |
| LV (mg/g)     | 3.34 ± 0.35 | 3.50 ± 0.25 | 3.41 ± 0.40 | 0.6121 | - | - |
| RV + LV (mg/g)| 4.49 ± 0.40 | 4.71 ± 0.18 | 4.71 ± 0.33 | 0.2599 | - | - |
| RV LV         | 0.35 ± 0.11 | 0.35 ± 0.11 | 0.39 ± 0.08 | 0.5751 | - | - |
| Lung Wet (mg/g)| 4.64 ± 0.34 | 5.14 ± 0.34 | 5.15 ± 0.37 | 0.0537 | - | - |
| Lung Dry      | 0.70 | - | - | - | - | - |
was significantly increased following exposure to CIH, but it was significantly increased following exposure to CIH in NOX2-null mice compared with the corresponding sham exposure (P = 0.0449).

3.2. Baseline ventilation and metabolism

Respiratory and metabolic parameters during baseline (normoxia) are shown in Tables 3 and 4. Minute ventilation (V̇t) during baseline (combined periods of normoxia) was equivalent in sham and CIH-exposed wild-type mice (P = 0.2142). Administration of the putative NOX2 inhibitor, apocynin (2mM), in the drinking water during exposure to CIH, resulted in significantly increased V̇t (P = 0.0146) and V̇I (P = 0.0171). Exposure to CIH significantly decreased fR (P = 0.0358) and increased V̇I (P = 0.0332), culminating in unaltered V̇t (P > 0.9999) in NOX2 KO mice. PIF (P = 0.5392) and PEF (P = 0.4021) were equivalent in sham and CIH-exposed wild-type mice. However, treatment with apocynin during exposure to CIH revealed a significant increase in both PIF (P = 0.0012) and PEF (P < 0.0001). Exposure to CIH resulted in an increase in PIF (P = 0.0100) and un changed PEF (P = 0.0894) in NOX2 KO mice compared with corresponding sham controls. Assessment of oxygen consumption (VO₂) and carbon dioxide production (VCO₂) revealed minimal differences between groups. Of note, VO₂ and VCO₂ were both increased for the CIH + APO group compared to exposure to CIH alone (VO₂; P = 0.0005; VCO₂; P < 0.0001). Expiratory duration (Tₑ) was unchanged following exposure to CIH in wild-type mice compared with sham; apocynin treatment throughout the CIH exposure had no effect on Tₑ. However, exposure to CIH significantly increased Tₑ in NOX2 KO mice compared with the corresponding sham exposure (P = 0.0301). Ventilatory drive (V̇t/Tₑ) was unchanged following exposure to CIH in wild-type and NOX2 KO mice. However, ventilatory drive was significantly increased in CIH + APO mice (P = 0.0003). The ventilatory equivalent for carbon dioxide (V̇t/VCO₂) did not differ between sham and CIH exposures in wild-type mice or NOX2 KO mice, while a significant reduction in V̇t/VCO₂ was evident following treatment with apocynin during exposure to CIH (P = 0.0091). Body mass (g) was significantly decreased following exposure to CIH compared with sham in wild-type mice (P = 0.0032); apocynin prevented this decrease (P = 0.0047). This well described decrease in body mass following exposure to CIH was also evident in NOX2 KO mice (P = 0.0042), reminiscent of that seen in wild-type mice. Inspiratory duration (Tᵢ), ventilatory equivalent for O₂ (V̇t/V̇O₂) and the ratio of VCO₂ to V̇O₂ (respiratory exchange ratio) were unchanged between groups.

3.3. Expression of sighs and apnoeas during normoxia

Fig. 1 shows representative respiratory flow traces illustrating a sigh and post-sigh apnoea (A) and a spontaneous apnoea (B) in a wild-type sham mouse during normoxia. Analysis of sighs revealed that exposure to CIH increased the frequency of sighs in wild-type (P = 0.0135; Fig. 1; C), but not in NOX2 KO mice (P = 0.2128; Fig. 1; D). No difference was observed in the frequency of post-sigh apnoeas following exposure to CIH in wild-type (Fig. 1; E) or NOX2 KO mice (Fig. 1; F). The frequency of post-sigh apnoeas was also equivalent following NOX2 blockade via apocynin administration (Fig. 1; E). The duration of post-sigh apnoeas was significantly elevated in CIH-exposed compared to sham wild-type mice (P = 0.0178; Fig. 1; G); the increase was entirely prevented by the administration of apocynin throughout the exposure to CIH (P = 0.0158; Fig. 1 G). Similarly, a significant increase in the duration of post-sigh apnoeas was observed following exposure to CIH in mice lacking the NOX2 enzyme compared with corresponding sham controls (P = 0.0385; Fig. 1; H). Analysis of spontaneous apnoeas revealed a significant increase in the frequency of spontaneous apnoeas during CIH exposure.
Table 4

Baseline breathing and metabolism measurements in sham- and CIH-exposed NOX2 KO mice. Definition of abbreviations: f, breathing frequency; Vt, tidal volume; V̇i, minute ventilation; PIF, peak inspiratory flow; PEF, peak expiratory flow; T1, inspiratory duration; Te, expiratory duration; VO2, oxygen consumption; V̇CO2, carbon dioxide production; V̇O2/V̇CO2, ventilatory equivalent for oxygen; V̇O2/V̇CO2 exchange ratio; NOX2 KO, NADPH Oxidase 2 knockout (B6.129S-Cybbtm1Din/J); sham, normoxia (21% O2)-exposed; CIH, chronic intermittent hypoxia-exposed. Values are expressed as mean ± SD. Data sets which were normally distributed were statistically compared using unpaired two-tailed Student’s t test. Welch’s correction was applied in the case of unequal variance. Data which were not normally distributed were compared using Mann Whitney non-parametric tests. Bolded numbers indicate statistical significance (P < 0.05).

| NOX2 KO sham (n = 12) | NOX2 KO CIH (n = 12) | (P value) |
|-----------------------|----------------------|-----------|
| f (brpm)              | 190.7 ± 16.0         | 175.1 ± 18.1 | 0.0358 |
| Vt (ml/g)             | 0.0057 ± 0.001      | 0.0063 ± 0.001 | 0.0332 |
| V̇i (ml/min/g)        | 1.05 ± 0.14          | 1.04 ± 0.15   | >0.9999 |
| PIF (ml/s)            | 2.60 ± 0.27          | 2.67 ± 0.25   | 0.4580 |
| PEF (ml/s)            | 1.93 ± 0.24          | 1.92 ± 0.20   | 0.9323 |
| T1 (s)                | 0.12 ± 0.006        | 0.12 ± 0.007  | 0.6994 |
| Te (s)                | 0.22 ± 0.031        | 0.26 ± 0.047  | 0.0301 |
| V̇O2 (ml/g)           | 0.049 ± 0.006       | 0.054 ± 0.006 | 0.0623 |
| VO2 (ml/min/g)        | 0.040 ± 0.01        | 0.038 ± 0.01  | 0.7982 |
| V̇CO2 (ml/min/g)      | 0.035 ± 0.01        | 0.033 ± 0.01  | 0.4568 |
| V̇CO2/V̇O2            | 29.3 ± 11.7          | 29.5 ± 8.5    | 0.7125 |
| V̇O2/V̇CO2            | 31.3 ± 7.2          | 33.8 ± 9.9    | 0.4703 |
| V̇O2/V̇CO2 exchange ratio | 0.93 ± 0.21      | 0.89 ± 0.20   | 0.6563 |
| Body Mass (g)         | 28.1 ± 2.6          | 25.4 ± 0.94   | 0.0042 |

3.4. Respiratory timing measurements during normoxia

Fig. 2 shows Poincaré plots of the breath-to-breath (Bb) and subsequent interval (Bbsub1) of 200 consecutive breaths for expiratory duration (T2; A–E) and total breath duration (Ttot; J–N) in sham (A and J), CIH (B and K), CIH + APO (C and L), NOX2-null sham (D and M) and NOX2 KO CIH-exposed mice (E and N). Assessment of the short-term (SD1) and long-term (SD2) variability of breathing revealed no evidence for respiratory instability for T2 following exposure to CIH in either wild-type (SD1 & SD2; Fig. 2; F and H, respectively) or NOX2 KO mice (SD1 & SD2; Fig. 2; G and I, respectively). However, treatment with apocynin throughout the exposure to CIH increased breathing stability as indicated by significantly lower SD1 (P = 0.0051; Fig. 2; O) and SD2 (P = 0.0015; Fig. 2; Q) compared to exposure to CIH alone.

3.5. Brainstem 5-HT and 5-HIAA concentrations

Concentrations of 5-HT and its metabolite 5-HIAA were quantified in brainstem samples of mice from all groups using HPLC. These and the ratio of 5-HIAA to 5-HT (index of serotonergic metabolism) are shown in Fig. 3 A–F. The concentration of 5-HT was not different following exposure to CIH in wild-type (Fig. 3; A) and NOX2-null mice (Fig. 3; B). Treatment with apocynin (Fig. 3; A) did not significantly alter 5-HT concentration. Analysis of 5-HIAA revealed that its concentration was equivalent in the brainstem of both wild-type (Fig. 3; C) and NOX2 KO mice (Fig. 3; D) exposed to CIH, as well as mice treated with apocynin during the exposure to CIH (Fig. 3; C). The ratio of 5-HIAA to 5-HT was significantly reduced in CIH-exposed mice compared to sham (P = 0.0006; Fig. 3; E); apocynin treatment had no effect on 5-HIAA/5-HT (P = 0.9837; Fig. 3; E). There was no difference in the ratio of 5-HIAA/5-HT between NOX2 KO mice exposed to CIH compared with NOX2 KO sham mice (P = 0.3333; Fig. 3; F).

3.6. Ventilatory responsiveness to hypoxia and hypercapnia

Fig. 4 shows the time course of the response of a range of ventilatory parameters during a 10-minute exposure to hypoxia (FIO2 = 0.1; balance N2; A–F) and hypercapnic (FICO2 = 0.05, balance N2; G–L) challenges for all groups. Minute ventilation increased rapidly across all groups at the onset of hypoxia compared to baseline ventilation values (Fig. 4; E & F, respectively). Exposure to 2 weeks of CIH increased ventilation over the duration of the hypoxic challenge compared with sham in wild-type mice (P = 0.0334; Fig. 4; E), owing to a modest increase in breathing frequency (P = 0.0857; Fig. 4; A). Apocynin treatment throughout the exposure to CIH did not ameliorate the increase in ventilation (P = 0.5349; Fig. 4; E). Similarly, exposure to CIH increased hypoxic ventilation in NOX2 KO mice (P = 0.0032; Fig. 4; F), owing to an increase in tidal volume (P = 0.0194; Fig. 4; D). Ventilation increased rapidly at the onset of hypoxia for all groups and remained elevated for the duration of the challenge (Fig. 4; K & L). Exposure to CIH had no effect on hypercapnic ventilation compared to sham-exposed wild-type mice (P = 0.3186; Fig. 4; K); treatment with apocynin throughout the exposure to CIH also resulted in no difference in hypercapnic ventilation compared with exposure to CIH alone (P = 0.8206; Fig. 4; K). In contrast, exposure to CIH significantly increased hypercapnic ventilation in NOX2 KO mice compared with NOX2 KO sham mice (P = 0.0007; Fig. 4; L), an effect underpinned by an increase in tidal volume (P = 0.0001; Fig. 4; J).

3.7. Peak ventilatory responsiveness to hypoxia and hypercapnia

Fig. 5 shows grouped ventilatory data from all groups of mice in response to ventilatory challenge with hypoxia (10 % O2, balance N2; Fig. 5; A and B, respectively) and hypercapnia (5% CO2, 95% O2; Fig. 5; C and D, respectively) in order to assess chemoreceptor responsiveness. The peak hypoxic ventilatory response (HVR; delta V̇) was unchanged following exposure to CIH compared with sham-exposed mice (P = 0.0699; Fig. 5; A). Apocynin treatment throughout the exposure to CIH did not affect the ventilatory response to hypoxia (P = 0.6159; Fig. 5; A). Peak HVR was significantly increased in NOX2 KO mice exposed to CIH compared with NOX2 KO sham mice (P = 0.0003, Fig. 5; B). The peak ventilatory response to hypercapnia (HCVR; delta V̇) for all groups is shown in Fig. 5; C and D, respectively. Exposure to CIH had no significant effect on the peak HCVR compared with sham in wild-type mice (Fig. 5; C); apocynin did not significantly alter the peak HCVR compared with exposure to CIH alone (Fig. 5; C). Exposure to CIH resulted in a significant increase in the peak HCVR in mice lacking the NOX2 enzyme compared with the corresponding sham exposure (P = 0.0016; Fig. 5; D).
3.8. Metabolic responsiveness to hypoxia and hypercapnia

Fig. 6 shows group data for carbon dioxide production (delta $\dot{V}CO_2$; Fig. 6; A, B, E & F, respectively) and the ventilatory equivalent for carbon dioxide (delta $\dot{V}CO_2/\dot{V}CO_2$; Fig. 6; C, D, G & H, respectively) from all groups of mice in response to ventilatory challenge with hypoxia (10% $O_2$), balance $N_2$ (Fig. 6; A, B, C & D respectively) and hypercapnia (5% $CO_2$; Fig. 6; E, F, G & H, respectively). $\Delta VCO_2$ was unchanged following exposure to CIH in wild-type (Fig. 6; A) or NOX2 KO (Fig. 6; B) mice during hypoxia; treatment with apocynin throughout the CIH exposure also had no effect on $\Delta VCO_2$ during hypoxia (Fig. 6; C). Similarly, $\Delta V_{I}/VCO_2$ was unchanged following exposure to CIH in wild-type (Fig. 6; C) and NOX2 KO (Fig. 6; D) mice during hypoxia; treatment with apocynin throughout the CIH exposure also had no effect on $\Delta VCO_2$ during hypoxia (Fig. 6; C). $\Delta VCO_2$ was unchanged following exposure to CIH in wild-type (Fig. 6; A) or NOX2 KO (Fig. 6; B) mice during normoxia; treatment with apocynin throughout the CIH exposure also had no effect on $\Delta VCO_2$ during normoxia in mice lacking the NOX2 enzyme when compared with the corresponding sham exposure ($P = 0.3478$; Fig. 6; F). $\Delta V_{I}/VCO_2$ was unchanged following exposure to CIH or treatment with apocynin in wild-type mice during hypercapnia (Fig. 6; G). However, $\Delta V_{I}/VCO_2$ was significantly increased during hypercapnia in NOX2 KO mice following exposure to CIH ($P = 0.0029$; Fig. 6; H).

4. Discussion

4.1. CIH and respiratory plasticity

It is well established that IH holds the capacity to induce plasticity at multiple levels of the respiratory control system, with the magnitude of this effect largely dependent on the time, intensity and duration of the IH stimulus (Navarrete-Opazo and Mitchell, 2014). Ventilatory adaptation following exposure to CIH, which can manifest as a progressive increase in normoxic ventilation following chronic exposure to IH, has previously been reported in rats (Reeves and Gozal, 2006; Skelly et al., 2012). Increased propensity for central apnoea has been reported in...
Fig. 2. Respiratory timing measurements in awake mice during normoxia. A B, C, D and E, Poincaré plot of breath-to-breath (BBn) and subsequent breath-to-breath (BBn+1) interval of expiratory duration (Te) over 200 consecutive breaths for sham (A), CIH (B), CIH + APO (C), NOX2 KO sham (D) and NOX2 KO CIH (E) groups. F, G, H & I, group data of short-term (F, G; SD1) and long-term (H, I; SD2) variability of breathing based on Te for all groups. J, K, L, M, N, Poincaré plots of BBn and BBn+1 of total breath duration (Ttot) over 200 consecutive breaths for sham (J), CIH (K), CIH + APO (L), NOX2 KO sham (M) and NOX2 KO CIH (N) groups. O, P, Q, R, group data of short-term (O,P; SD1) and long-term (Q,R; SD2) variability of breathing based on Ttot for all groups. Data are shown in violin plots with individual data points representing each animal in the relevant groups; median values and interquartile ranges are shown by horizontal lines. For F, H, O, Q, data were statistically compared using one-way ANOVA with Tukey’s post hoc test. Statistical significance was taken at P < 0.05. For G, I, P, R, data sets which were normally distributed were statistically compared using unpaired two-tailed Student’s t test. Welch’s correction was applied in the case of unequal variance. Data which were not normally distributed were compared using Mann Whitney non-parametric tests. Statistical significance was taken at P < 0.05. Relevant comparisons denoted as follows; # denotes APO + CIH significantly different from corresponding CIH values, ## P < 0.01.
patients with OSAS (Salloum et al., 2010). In light of this, it has been demonstrated that exposure to CIH results in central apnoea in neonatal rat pups (Julien et al., 2008), adult rats (Edge et al., 2012; Souza et al., 2015; Joseph et al., 2020) and mice (Elliot-Portal et al., 2018). Exposure to CIH has also been shown to increase the frequency of sighs and post-sigh apnoeas in rats (Edge et al., 2012; Souza et al., 2015; Joseph et al., 2020). Of interest, the administration of antioxidants has proven beneficial in ameliorating a range of IH-induced respiratory anomalies, highlighting the role of ROS in CIH-induced respiratory morbidity (Peng et al., 2003; Edge et al., 2012). Moreover, evidence suggests that NOX2 may be the source of CIH-induced ROS which may act to facilitate some of the mechanisms, which underpin the alterations in respiratory control outlined above (Peng et al., 2009; Edge et al., 2012; Joseph et al., 2020). We hypothesised that 2 weeks of exposure to CIH would result in mal-adaptation in respiratory control, which would manifest as enhanced normoxic ventilation, altered reflex chemoresponsiveness, and the manifestation of respiratory instability and increased apnoea index. Furthermore, we postulated that these alterations would be underpinned by a NOX2-derived ROS-dependent mechanism, amenable to blockade by supplementation with apocynin (putative NOX2 inhibitor) and NOX2 knock-out.

In our model, normoxic ventilation and corresponding metabolic measurements were unaffected by 2 weeks of exposure to CIH in both wild-type and NOX2 KO mice. This lack of a CIH-induced effect on resting ventilation is in agreement with previous studies in guinea pigs (Lucking et al., 2018), rats (McGuire et al., 2003; Edge et al., 2012; Souza et al., 2015; Joseph et al., 2020) and mice (Elliot-Portal et al., 2018). However, previous research by our group demonstrated increased normoxic ventilation in male rats following 9 days of exposure to CIH (Skelly et al., 2012). This difference may be explained by the intensity of the IH stimulus, which was more severe in the latter study, as the CIH profile utilised 20 hypoxia-reoxygenation cycles/hour compared with the 12 cycles/hour employed in the current study. Indeed, ventilatory adaptation to intermittent hypoxia has been reported in rats following 30 days (Reeves and Gozal, 2006) and 21 days of exposure (Morgan et al., 2016a), with both groups highlighting that the duration and paradigm of IH exposure appears pivotal in determining the phenotypical respiratory response. Thus, we suggest that we did not observe evidence of enhanced normoxic ventilation due to the relatively mild and short duration of CIH used in this study. A CIH-induced decrease in metabolic rate has previously been reported in rat models (Morgan et al., 2016a; Joseph et al., 2020). However, we report no alteration to VO2 or VCO2 following exposure to CIH in either wild-type or NOX2 KO mice, consistent with previous studies in mice (Elliot-Portal et al., 2018). Differences in metabolic responses during normoxic breathing following exposure to CIH are likely due to species differences between rat and mouse models, as differential metabolic responses to hypoxia between small and large mammals have been described (Hauouzi et al., 2009).

4.2. The role of NOX-2 in CIH-induced respiratory plasticity

Many forms of IH-induced plasticity are ROS-dependent (MacFarlane and Mitchell, 2008), and more specifically these ROS appear to be NOX2-derived (MacFarlane et al., 2009; Peng et al., 2009). Whilst obstructive apnoeas are the predominant apnoea type in OSAS patients due to collapse of the upper airway, central or spontaneous apnoeas also occur in patients due to respiratory control instability. This reveals a common overlap in obstructive and central sleep apnoea. OSAS patients receiving CPAP treatment regularly present with periodic breathing and central apnoea, with some patients consequentially being diagnosed with complex apnoea (Morgenthaler et al., 2006). Complex apnoea in OSAS patients may be a result of CIH-induced remodelling in the central respiratory network governing respiration, serving to perpetuate the severity of OSAS (O’Halloran, 2016). A key finding in the current study is that exposure to CIH produced a significant increase in the frequency of spontaneous apnoeas in wild-type mice during resting ventilation. Studies in female rats (Souza et al., 2015; Joseph et al., 2020), male rats (Edge et al., 2012) and male mice (Elliot-Portal et al., 2018) have revealed similar CIH-induced increases in apnoea index. To determine if NOX2 was a potential source of ROS underlying increased apnoea index, we administered apocynin throughout the exposure to CIH. This was efficacious in preventing the CIH-induced increase in the frequency of spontaneous apnoeas. This result is largely reminiscent of findings reported by (Edge et al., 2012), where 9 days of exposure to CIH induced a similar rise in the frequency of spontaneous apnoeas, an effect ameliorated by the same dose of apocynin (2 mM) used in the current study. While apocynin is commonly used as a putative NOX2 inhibitor, evidence suggests that apocynin’s pharmacological action may be through more general antioxidant action (Wind et al., 2010). To examine the...
specificity of the apocynin-induced effect, we utilised NOX2 KO mice which were exposed to CIH. Somewhat surprisingly, we observed a near identical increase in the frequency of spontaneous apnoeas following exposure to CIH in mice deficient in NOX2 compared to wild-type controls. These data reveal either a non-NOX2 dependent source of ROS in the mechanism underlying the development of increased propensity for apnoea following exposure to CIH, or an emergent mechanism driving increased apnoea index in NOX2 KO mice (e.g. increased hypercapnic ventilatory response in NOX2 KO mice; see section 4.4).

While we report no alteration to the frequency of post-sigh apnoeas following exposure to CIH, the duration of post-sigh apnoeas was increased following exposure to CIH, which was ameliorated by apocynin administration. This suggests that CIH-exposed mice develop a delayed ability to regulate respiratory cycles following deep breaths, characterised by sighs or post-sigh apnoeas, similar to previous observations in female rats (Souza et al., 2015). However, our study implicates a role for non-NOX2-derived ROS, evidenced by the persistence of this effect in CIH-exposed NOX2 KO mice.

We reasoned that there is a capacity for exposure to CIH to induce breathing instability, likely through alterations to the rhythm and

Fig. 4. Ventilatory responsiveness to hypoxia and hypercapnia. Group data (mean ± SD; n = 10-12 per group) for breathing frequency (A, B; fR), tidal volume (C, D; VT), and minute ventilation (E, F; V˙i), during baseline and 10 min of exposure to hypoxia (10 % inspired oxygen; balance N2) for sham, CIH, CIH + APO (A, C, E) and NOX2 KO sham and NOX2 KO CIH (B, D, F) groups. Group data (mean ± SD; n = 10-12 per group) for breathing frequency (G, H; fR), tidal volume (I, J; VT), and minute ventilation (K, L; V˙i), during baseline and 10 min of exposure to hypercapnia (5% inspired CO2; balance O2) for sham, CIH, CIH + APO (G, I, K) and NOX2 KO sham and NOX2 KO CIH (H, J, L) groups. Sham, normoxia (21 % O2) exposed; CIH, chronic intermittent hypoxia exposed; CIH + APO, Apocynin (2 mM) given in the drinking water for the duration of CIH exposure; NOX2 KO, NADPH Oxidase 2 knock-out (B6.129S-Cybbtm1Din/J), sham- or CIH-exposed. Data were statistically compared by repeated measures two-way ANOVA with Bonferroni post hoc test. For repeated measures two-way ANOVA; CIH denotes sham vs. CIH; APO denotes CIH vs. CIH + APO for A, C, E, I, K. Int denotes the interaction between two factors for A–L. For Bonferroni post hoc test; * denotes sham vs. CIH, *P < 0.05; * denotes CIH vs. CIH + APO, *P < 0.05, **P < 0.01; $ denotes NOX2 KO sham vs. NOX2 KO CIH, $P < 0.05, $$P < 0.01, $$$P < 0.0001.
In the current study, administration of apocynin throughout approaches as well as relative contributions from the peripheral chemoreceptors. In the current study, administration of apocynin throughout the exposure to CIH produced a significant decrease in breathing variability to levels below that of controls. The outcome may be related to the pronounced metabolic effect of apocynin, which resulted in increased ventilatory drive, albeit inadequate to fully compensate for the pronounced metabolic effect of apocynin, which resulted in ability to levels below that of controls. The outcome may be related to receptors. In the current study, administration of apocynin throughout approaches as well as relative contributions from the peripheral chemo-receptors. In the current study, administration of apocynin throughout the exposure to CIH produced a significant decrease in breathing variability to levels below that of controls. The outcome may be related to the pronounced metabolic effect of apocynin, which resulted in increased ventilatory drive, albeit inadequate to fully compensate for increased VCO₂ (Table 5).

Respiratory neurons at multiple brainstem sites are known to have key roles in generating homeostatic respiratory rhythm with inspiratory neurons at the preBötC coming to the fore as key players in this process. Exposure to CIH has been shown to alter the electrophysiological properties of these neurons (Moraes et al., 2013) and respiratory circuits at the level of the preBötC, in turn affecting transmission to the hypoglossal motor nucleus leading to increased transmission failure to the hypoglossal nerve (Garcia et al., 2016). Furthermore, complete ablation of the preBötC increases the propensity for apnoea in sleeping rats (McKay and Feldman, 2008). ROS are implicated in multiple forms of IH-induced plasticity, with H₂O₂ shown to be capable of modulating rhythmogenesis by the preBötC (Garcia et al., 2011). Furthermore, antioxidant therapy has been shown to successfully reduce CIH-mediated irregularities of the network rhythm improving transmission to the hypoglossal nerve (Garcia et al., 2016). Thus, in the current study we suggest that exposure to CIH may promote a pro-oxidant state which destabilises rhythmogenesis originating from the preBötC. This may represent part of a central mechanism that promotes apnoea and respiratory instability. Additionally, due to the similar effect of exposure to CIH on measures of respiratory stability in wild-type and NOX2 KO mice, it appears that apocynin functions as a non-specific antioxidant at higher centres of respiratory control in a similar manner to that previously shown in the vascular system (Heumüller et al., 2008). It is also plausible to suggest that increased propensity for apnoea arises from altered peripheral control of breathing. CIH-induced sensitisation of the carotid bodies may lead to ventilatory overshoot and in this way may destabilise breathing, providing the substrate for apnoea.

### 4.3. CIH and the hypoxic ventilatory response

Ventilatory responsiveness to hypoxia following exposure to CIH varies greatly depending on the pattern and duration of hypoxic exposure. A diverse range of observations arise as a result of an intricate system of molecular mechanisms that underlie plasticity in the respiratory control reflex circuits and ultimately control the physiology of breathing in response to oxygen desaturation. Augmented HVRs and concomitant oxidative stress have been observed in healthy individuals following exposure to CIH (Pialoux et al., 2009) and in patients with OSAS (Narkiewicz et al., 1999). Exposure to CIH evokes carotid body sensory facilitation ex vivo and in anaesthetised animals (Peng et al., 2001; Rey et al., 2004; Peng et al., 2006b). Carotid body responses to hypoxia are enhanced following exposure to CIH (Peng et al., 2001, 2006b).
Fig. 6. Ventilatory and metabolic responsiveness to hypoxia and hypercapnia. Group data for carbon dioxide production (A, B; V\dot{\text{CO}_2}) and ventilatory equivalent for carbon dioxide (C, D; V\dot{\text{I}}/V\dot{\text{CO}_2}) expressed as a change from baseline to the final 3 min (ΔV\dot{\text{CO}_2} & Δ V\dot{\text{I}}/V\dot{\text{CO}_2}) of exposure to hypoxia (10 % inspired oxygen; balance N\_2) for sham, CIH, CIH + APO (A & C) and NOX2 KO sham and NOX2 KO CIH (B & D) groups. Group data for carbon dioxide production (E, F; V\dot{\text{CO}_2}) and ventilatory equivalent for carbon dioxide (G, H; V\dot{\text{I}}/V\dot{\text{CO}_2}) expressed as a change from baseline to the final 3 min (ΔV\dot{\text{CO}_2} & Δ V\dot{\text{I}}/V\dot{\text{CO}_2}) of exposure to hypercapnia (5% inspired CO\_2; balance O\_2) for sham, CIH, CIH + APO (E & G) and NOX2 KO sham and NOX2 KO CIH (F & H) groups. Data are shown in violin plots with individual data points representing each animal in the relevant groups; median values and interquartile ranges are shown by horizontal lines. Sham, normoxia (21 % O\_2)-exposed; CIH, chronic intermittent hypoxia-exposed; CIH + APO, Apocynin (2 mM) given in the drinking water for the duration of CIH exposure; NOX2 KO, NADPH Oxidase 2 knock-out (B6.129S-Cybbtm1Din/J), sham- or CIH-exposed. For A, C, E, G, data were statistically compared using one-way ANOVA with Tukey’s post hoc test. Statistical significance was taken at P < 0.05. For B, D, F, H, data sets which were normally distributed were statistically compared using unpaired two-tailed Student’s t test. Welch’s correction was applied in the case of unequal variance. Data which were not normally distributed were compared using Mann Whitney non-parametric tests. Statistical significance was taken at P < 0.05. Relevant comparisons denoted as follows; # denotes CIH vs CIH + APO, $$$ P < 0.0001, $ denotes NOX2 KO sham vs. NOX2 KO CIH, ** P < 0.01.
Nevertheless, an enhancement (Reeves et al., 2003; Reeves and Gozal, 2003) to account for the decrease in metabolic rate that occurs when small animals are acutely exposed to hypoxia (Frappell et al., 1992). This is an especially crucial consideration in mouse models, such as that employed in the current study, as mice exhibit a greater hypometabolic response to hypoxia than rats (Jochmans-Lemoine et al., 2015). When ventilation is normalised for VCO₂ production, the true hyperventilatory response to hypoxia in mice is revealed (i.e. an increase in V̇I/V̇CO₂). Failure to report on metabolic measures in several previous studies, limits the capacity to determine the true hyperventilatory response to hypoxia (Morgan et al., 2016a). In the current study, analysis of the delta V̇I/V̇CO₂ response to hypoxia revealed equivalent responses in sham and CIH-exposed mice. Our results support a central mechanism driving CIH-induced apnoea.

CIH-induced increases in ventilatory responsiveness to hypoxia are attenuated in rats treated with apocynin, as evidenced by a reduction in V̇I/V̇CO₂ (Morgan et al., 2016b). This implicates ROS, which may be NOX2-derived (depending on the specificity of apocynin), in altered chemoreflex sensitivity following exposure to CIH, possibly due to a reduction in nitrotyrosine production in the carotid body (Del Rio et al., 2010; Morgan et al., 2016b). NOX subunits including p22phox, gp91phox, p47phox, and p67phox have been localised to type I cells of guinea pig, rat, and human carotid bodies (Kummer and Acker, 1995). Exposure to CIH significantly increases the mRNA expression of NOX1, NOX2 and NOX3 in peripheral chemoreceptors (Peng et al., 2009; Khan et al., 2011; Prabhakar et al., 2015). Moreover, there is ample evidence to suggest that CIH-induced sensory plasticity of the carotid body is NOX2-dependent (Peng et al., 2006a; Peng et al., 2009; Yuan et al., 2011; Nanduri et al., 2013, 2015). However, in the current study, we observed no CIH-induced increase in HRV and no difference in HRV in apocynin-treated mice or NOX2 KO mice. This highlights the complexity in understanding the precise mechanisms and conditions underlying CIH-induced ROS-dependent hypoxic sensitivity and as such it warrants further attention.

4.4. CIH and the hypercapnic ventilatory response

Although CO₂ has a pivotal role in ventilatory control during sleep, there is a paucity of information examining the effects of exposure to CIH on hypercapnic sensitivity. Chemoreflexes are essential during sleep as a protective mechanism to stimulate arousal and terminate apnoeic events. Failure to do so may contribute to an increased propensity of apnoeic events, thus leading to an increased severity of OSAS. Conversely, an exaggerated response to CO₂ contributes to ventilatory instability by reducing the CO₂ reserve towards apnoeic threshold (Dempsey et al., 2010). An increase in the hypercapnic ventilatory response (HCVR) has been observed in OSAS patients, as a result of episodic hypoxia (Appelberg and Sundström, 1997; Khodadaeh et al., 2006). Consistent with this, a CIH-induced increase in the ventilatory response to hypercapnia has been reported in mice (Elliot-Portal et al., 2018) and dogs (Katayama et al., 2007). Conversely, evidence supports an unaltered HCVR following CIH exposure in a range of models including guinea pigs (Lucking et al., 2018) and rats (Greenberg et al., 1999; Edge et al., 2012). In the current study, we report no evidence in support of a CIH-induced augmentation to the chemoreflex response to hypercapnia in wild-type mice. However, the ventilatory response to hypercapnia was enhanced following exposure to CIH in NOX2 KO mice.

The potentiated ventilatory response to hypercapnia in NOX2 KO mice suggests a suppression of hypercapnic drive by a NOX2-dependent mechanism. An exaggerated response to alterations in CO₂ may serve to destabilise breathing and may have contributed to the increased propensity for apnoea in NOX2 KO mice. Our results support the notion that NOX2-dependent ROS exert a tonic inhibition on sites important for central chemoreception such as the NTS, locus coeruleus and the retrotrapezoid nucleus (Nattie and Li, 2012), acting to suppress hypercapnic drive. This NOX2-dependent inhibition may have a regulatory function in generating an appropriate ventilatory response to a perturbation in CO₂ levels, as exposure to CIH further exacerbates the chemoreflex response in NOX2 KO mice, whereas it was without effect in wild-type animals. The mechanisms underpinning this response warrant further investigation.

4.5. Conclusion

Our results reveal the capacity for 2 weeks of exposure to a mild-to-moderate paradigm of CIH to evoke plasticity within the respiratory control system, predominantly evidenced by increased propensity for apnoea. Oxidative stress plays a causal role in the ventilatory mal-adaptations and sympathetic nervous system overactivity observed in animal models of OSAS, consistent with the human condition (Peng et al., 2009, 2013). Our results implicate ROS, which may not be NOX2-derived, in the development of CIH-induced respiratory mal-adaptation, as we observe what may be general antioxidant effects of apocynin ameliorating the CIH-induced increase in apnoea index. Our results may have implications for OSAS patients diagnosed with complex apnoea (Morgenhaler et al., 2006). Patients with complex apnoea exhibit spontaneous or central apnoeas, in addition to obstructive apnoeas, which may be the result of CIH-induced remodelling with the central respiratory network (O’Halloran, 2016), such as the PreBötC (Garcia et al., 2016). Therefore, respiratory system mal-adaptation following exposure to CIH may exacerbate OSAS severity, serving to perpetuating a vicious cycle of respiratory morbidity. Our results reveal that antioxidant therapies may be a useful adjunctive therapy for the treatment of patients with OSAS and other respiratory control disorders. However, further experimentation is necessary to delineate the source of ROS, mechanism of action and specific sites throughout the respiratory control network at which they exert their effects in order to develop precise therapeutic strategies. Our study was restricted to the assessment of male mice, which we acknowledge is a limitation of the study. Sex differences in pathophysiological responses to CIH have been described (O’Halloran et al., 2017), however, CIH-induced increased propensity for apnoea has been described in male and female rodents. As such, it is likely that our findings apply to both sexes, but this remains to be determined.

Funding

Department of Physiology, UCC.

Author contributions

Conceptualisation of experimental design: SD, DB and KOH. Experimental procedures: SD, DB, KOC, GC. Data analysis: SD, KOC. Drafting of the original manuscript: SD, KOH. All authors approved the final draft of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.
effects on oxidative status, sympathetic tone, plasma glucose and insulin levels, and arterial pressure. J. Appl. Physiol. 117 (1985), 706–719.

Osman, A.M., Carter, S.G., Carberry, J.C., Eckert, D.J., 2018. Obstructive sleep apnea: current perspectives. Nat. Sci. Sleep 10, 31–34.

Peng, Y.J., Kline, D.D., Dick, T.E., Prabhakar, N.R., 2001. Chronic intermittent hypoxia enhances carotid body chemoreceptor response to low oxygen. Adv. Exp. Med. Biol. 499, 23–36.

Peng, Y.J., Overholt, J.L., Kline, D., Kumar, G.K., Prabhakar, N.R., 2003. Induction of sensory long-term facilitation in the carotid body by intermittent hypoxia: implications for recurrent apneas. Proc. Natl. Acad. Sci. U. S. A. 100, 10073–10078.

Peng, Y.J., Yuan, G., Jaccono, F.J., Kumar, G.K., Prabhakar, N.R., 2006a. 5-HT evokes sensory long-term facilitation of rodent carotid body via activation of NADPH oxidase. J. Physiol. 576, 289–295.

Peng, Y.J., Yuan, G., Ramakrishnan, D., Sharma, S.D., Bosch-Marce, M., Kumar, G.K., Semenza, G.L., Prabhakar, N.R., 2006b. Heterozygous HIF-1a deficiency impairs carotid body-mediated systemic responses and reactive oxygen species generation in mice exposed to intermittent hypoxia. J. Physiol. 577, 705–716.

Peng, Y.J., Nanduri, J., Yuan, G., Wang, N., Deneris, E., Pendyala, S., Natarajan, V., Kumar, G.K., Prabhakar, N.R., 2009. NADPH oxidase is required for the sensory plasticity of the carotid body by chronic intermittent hypoxia. J. Neurosci. 29, 4903–4910.

Peng, Y.J., Nanduri, J., Khan, S.A., Yuan, G., Wang, N., Kinsman, B., Vaddi, D.R., Kumar, G.K., Garcia, J.A., Semenza, G.L., Prabhakar, N.R., 2011a. Hypoxia-inducible factor 2alpha (HIF-2a) heterozygous-null mice exhibit exaggerated carotid body sensitivity to hypoxia, breathing instability, and hypertension. Proc. Natl. Acad. Sci. U. S. A. 108, 2065–2067.

Peng, Y.J., Raghuraman, G., Kumar, S.A., Kumar, G.K., Prabhakar, N.R., 2011b. Angiotensin II evokes sensory long-term facilitation of the carotid body via NADPH oxidase. J. Appl. Physiol. 111 (1985), 964–970.

Peng, Y.J., Nanduri, J., Raghuraman, G., Wang, N., Kumar, G.K., Prabhakar, N.R., 2013. Role of oxidative stress-induced endothelin-converting enzyme activity in the alteration of carotid body function by chronic intermittent hypoxia. Exp. Physiol. 98, 1620–1630.

Perin, R.R., Feldth, D.P., Mitchell, G.S., 2018. Cross-talk inhibition between 5-HT(2B) and 5-HT(7) receptors in phrenic motor facilitation via NADPH oxidase and PKA. Am. J. Physiol. Regul. Integr. Comp. Physiol. 314, R709–R715.

Pialoux, V., Hanly, P.J., Foster, G.E., Brugniaux, J.V., Beaudin, A.E., Hartmann, S.E., Drummond, E., 2017. Role of NADPH oxidase and ROS in the sensory long-term facilitation of chemosensory responses to intermittent hypoxia in the adult rat. Br. J. Pharmacol. 161, 885–896.

Veasey, S.C., Rosen, I.M., 2019. Obstructive sleep apnea in adults. N. Engl. J. Med. 380, 1422–1434.

White, D.P., Younes, M.K., 2012. Obstructive sleep apnea. Compr. Physiol. 2, 1041–1054.