Long-Term Adverse Effects of Perinatal Hypoxia on the Adult Pulmonary Circulation Vary Between Males and Females in a Murine Model

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Summary

Adverse events during the perinatal period are associated with an increased risk to develop cardiometabolic diseases later in life. We established a murine model to study long-term effects of perinatal hypoxia (PH) on the pulmonary circulation. We previously demonstrated that PH led to an impaired regulation of pulmonary vascular tone in adulthood, linked to alterations in K^+ channels in males and in the nitric oxide (NO)/cyclic guanosine monophosphate pathway in females. Moreover, simultaneous administration of inhaled NO (iNO) during PH exposure prevented adverse effects of PH on adult pulmonary vasculature in females. The present study showed that PH induced a significant increase in right ventricular pressure in males and females, and an enhanced sensitivity to acute hypoxia in females. PH significantly reduced acetylcholine-induced relaxation in pulmonary artery, to a greater extent in females than in males. PH led to right ventricular hypertrophy in adulthood, appearing earlier in males than in females. Morphometric measurements showed a significant increase in the number of 25-75-µm pulmonary vessels in male lungs following PH, probably resulting in increased pulmonary vascular resistance. The effects of prolonged hypoxia in adulthood differed between males and females. Perinatal iNO during PH prevented PH-induced alterations in the cardiopulmonary system, whereas perinatal iNO alone could have some adverse effects. Therefore, PH led to long-lasting alterations in the regulation of adult pulmonary circulation, which vary between males and females. In males, the increased pulmonary vascular resistance was associated with morphological changes besides functional alterations, whereas females showed an important pulmonary vascular dysfunction.

Keywords

Perinatal hypoxia • Pulmonary circulation • Endothelium-dependent relaxation • Phosphodiesterases • Sex differences

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Introduction

Adverse events occurring *in utero* or soon after birth are associated with an increased risk of developing cardiometabolic diseases later in life [1]. Chronic pulmonary vascular diseases and abnormal pulmonary vasoreactivity in adulthood may be associated with a hypoxic insult occurring around birth [2,3]. Individuals born in a hypoxic environment show later in life an exaggerated pulmonary hypertensive response following a re-exposure to hypoxia [2,4-8].

Jan Herget and colleagues have extensively studied the effects of perinatal and adult exposure to chronic hypoxia on the pulmonary circulation in rats. They were particularly interested in the nitric oxide (NO) signaling pathway, which plays a key role in the regulation of pulmonary vascular tone and the response to hypoxia, as well as in adult pulmonary arterial hypertension (PAH) and persistent pulmonary hypertension of the newborn (PPHN) [9]. They found increased production of reactive oxygen species (ROS) [10,11] and NO, due to transient induction of inducible NO synthase (iNOS) in the pulmonary vascular wall [12,13], during early phase of chronic hypoxia in adulthood. NO can exert both protective and adverse effects, depending on the balance between NO and ROS [9]. The antioxidant N-acetylcysteine, administered during early phase of chronic hypoxia, or a combined

treatment with L-arginine and the phosphodiesterase 5 (PDE5) inhibitor sildenafil during hypoxic exposure were able to limit adverse effects of chronic hypoxia in adulthood [10,14]. They also demonstrated long-term effects of perinatal hypoxia (PH) on adult pulmonary circulation, by increasing pulmonary vessels' basal tone and reducing their reactivity to angiotensin II [15]. They showed that chronic prenatal hypoxia increased fetoplacental vascular resistance and vasoconstrictive response to angiotensin II or acute hypoxia, which could lead to placental hypoperfusion and impaired fetal nutrition and growth [16]. They were also interested in sex differences in the regulation and alterations of pulmonary circulation. They showed that females are more sensitive than males to late effects of PH on pulmonary vasculature and that these effects are blunted by the presence of ovaries during maturation [17].

Despite extensive research, the mechanisms contributing to the long-term effects of PH on the pulmonary circulation are still incompletely understood. Moreover, sex differences remain largely understudied.

We have previously established a murine model to investigate long-lasting effects of PH. We showed that PH resulted in impaired regulation of pulmonary vascular tone in adulthood [18-20]. PH triggered alterations in K^+ channels of adult pulmonary artery smooth muscle cells in males [18,19], and in the NO/ cyclic guanosine monophosphate (cGMP) pathway in females [20]. Adult females born in hypoxia displayed higher systolic right ventricular pressure (RVP) in normoxia and an increased sensitivity to acute hypoxia compared to controls [20]. Moreover, PH decreased the acetylcholine-induced endothelium-dependent relaxation of pulmonary artery (PA) in females. The vasorelaxant response to acetylcholine was restored by pirenzepine or telenzepine, selective antagonists of the muscarinic receptor M1 (M1AChR), which mediates vasoconstrictive effects of acetylcholine. The PDE1 inhibitor vinpocetine also reversed the decrease in endothelium-dependent relaxation following PH, suggesting that M1AChR-mediated impairment of acetylcholine-induced relaxation was due to activation of the calcium-dependent PDE1 [20]. Therefore, a perinatal reduction in oxygen supply leads to pulmonary vascular dysfunction, which seems associated with permanent alterations in muscarinic receptors and their effectors, in particular PDEs.

We also showed that simultaneous administration of inhaled NO (iNO) during the perinatal exposure to hypoxia was able to prevent adverse effects of PH on adult pulmonary vasculature in females [21]. Perinatal iNO restored acetylcholine-induced relaxation and prevented the development of right ventricular (RV) hypertrophy in old females [21].

A transient exposure to hypoxia during a critical period of development of the lung vasculature resulted in a definitive imprint leading to altered regulation of pulmonary vascular tone later in life. Consequently, individuals having suffered from PH could be at risk to have an altered regulation of pulmonary circulation and to develop pulmonary vascular pathologies in adulthood. This is more likely to occur during/following re-exposure to clinical conditions associated with hypoxia, such as sleep apnea, chronic or acute pulmonary diseases, or exposure to high altitude.

The present study was designed to further investigate long-term effects of PH on adult pulmonary circulation in males and females. The effects of adult (re-)exposure to acute or prolonged hypoxia and/or aging were assessed on several parameters.

Methods

Animal model

All experimental procedures were approved and carried out in accordance with the Swiss Veterinarian Animal Care Office (authorization numbers VD1454, VD2170, VD2622). C57BL/6 pregnant mice were purchased from Harlan (Horst, Netherlands). They were all fed *ad libitum* and exposed to day-night cycles. PH was induced as previously described [18,20,21]. Pregnant mice were placed under hypoxia (13 % O_2) 5 days before delivery (from D16 of gestation) and left with their litter for 5 days after birth. Pups were then bred in normoxia (21 % O_2) until adulthood. The timing of exposure to hypoxia was chosen to cover the lung vasculogenesis, during which the functional units of gas exchange develop [22]. Pups born and grown in normoxia were used as controls (Ctr). Mice were studied at 5-6 months ("young") or 12-15 months ("old"). Mice from different litters and cages were randomly assigned to the different experiments.

To investigate the effects of chronic hypoxia in adulthood, some adult mice were (re-)exposed to 13 % O_2 for 5 days and studied at the end of the exposure period.

Some mice were treated with iNO (10ppm) during the 10-day perinatal hypoxic/normoxic exposure, as previously published [21].

The different experimental groups are presented at Table 1.

Table 1. Experimental groups and related treatments

Group	Ctr	PH	Ctr+AH	PH+AH	Ctr+NO	PH+NO
Perinatal exposure (10 days)	21 % O_2	12 % O_2	21 % O_2	12 % O_2	21 % O_2 NO 10ppm	12 % O_2 NO 10ppm
Adult hypoxia (5days)	-	-	12 % O_2	12 % O_2	-	-

Anatomical data

The number of alive pups per litter was recorded at postnatal day 5 (P5). Body weight was recorded at P5 or in adulthood. After removal of the auricles, the right ventricle (RV) was separated from the left ventricle plus septum (LV+S) and both were weighed to determine the RV/(LV+S) ratio (Fulton index), which is used as an index of RV hypertrophy.

Hemodynamic measurements

Hemodynamic measurements were performed as previously described [20]. Adult mice were anaesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg i.p., respectively) and placed on a heating board, to prevent hypothermia, under a small Plexiglas® hood to control oxygen administration. Body temperature was monitored using a rectal probe (ADInstruments, Hastings, UK). Closed-chest measurements were performed using a Millar mikro-tip® 1.4 F catheter (Millar Instruments, Houston, TX) inserted into the right jugular vein and advanced into the RV for measurement of RVP. The catheter was connected to a pressure transducer, and pressure signals recorded using a MacLab A/D converter (ADInstruments). Systolic RVP is used as an indirect indicator of pulmonary systolic artery pressure [23]. Baseline systolic RVP was recorded in normoxia (21 % O_2) and under acute hypoxia (→2 % O_2). Gas mixture was controlled using an oxygen monitor mono2® (Roche, Bioelectronics, Switzerland). The response to iNO was also assessed by adding NO (10-60ppm) to the 12 % O_2 gas mixture. NO concetration was controlled using a SensorNox® analyzer (SensorMedics BV, Bilthoven, Netherlands).

Isolated vessel tension studies

The PA vasoreactivity was assessed as previously described [18,20]. Adult mice were administered a lethal dose of pentobarbital (1 g/kg i.p.) and the main PA was immediately harvested. Vascular rings were suspended into vertical organ chambers filled with 10 ml modified Krebs-Ringer solution maintained at 37 °C and aerated with 95 % O_2-5 % CO_2 (pH 7.4) [20]. Vessels were brought to their optimal resting tension after two 0.5g stretches. After equilibration, N^G-nitro-L-arginine (NLA, 10^{-4}M; except for dose-response to acetylcholine) and/or indomethacin (10^{-5}M) were added to exclude possible interference of endogenous NO and prostanoids. Vascular rings were then pre-constricted with phenylephrine (10^{-5}M) before addition of cumulative doses of acetylcholine or 8-bromo-cGMP. In some experiments, acetylcholine-induced relaxation was tested after preincubation with the PDE inhibitors 3-isobutyl-1-methylxanthine (IBMX, 10^{-6}M), 8-methoxymethyl-3-isobutyl-1-methylxanthine (8-MM-IBMX, $3x10^{-6}$M), sildenafil (10^{-8}M) or milrinone ($3x10^{-7}$M). Change in tension induced by the vasodilator was expressed as percentage of the initial contraction induced by phenyl-ephrine. Area under the curve (AUC) was calculated from each dose-response curve using GraphPad Prism 10.2.3 (GraphPad Software).

Cardiopulmonary parameters assessment

Cardiopulmonary parameters were recorded using a non-invasive pulse oximeter (MouseOx®, Red Box Direct Limited) during the transition from normoxia to hypoxia (13 % O_2) in anaesthetized mice placed on a heating board under a small Plexiglas® hood, or in conscious mice placed in a restraining tube. This non-invasive sensor clip, specially designed for small rodents, allowed simultaneous recordings of arterial O_2 saturation (O_2-sat), pulse rate and breath rate. The clip was placed on the leg in anesthetized mice and on tail in conscious mice.

Cardiopulmonary data were also recorded in anesthetized mice following a 30-min swim-ming challenge in normoxic or hypoxic (13 % O_2) conditions.

Table 2. Anatomical data

	Females						Males					
group	**Ctr**	**PH**	**Ctr+AH**	**PH+AH**	**Ctr+NO**	**PH+NO**	**Ctr**	**PH**	**Ctr+AH**	**PH+AH**	**Ctr+NO**	**PH+NO**
Young mice (5-6 months)												
n	25	20	30	21	6	13	42	61	20	20	5	17
Body (g)	22.8 ± 0.4	22.6 ± 0.4	21.9 ± 0.3	21.4 ± 0.2 †	24.1 ± 0.5	23.7 ± 0.4	29.9 ± 0.4 §	30.4 ± 0.4 §	29.3 ± 0.5 §	27.6 ± 0.5 †‡§	33.5 ± 1.1 *§	33.9 ± 0.8 †§
Heart (mg)	100.1 ± 1.8	97.2 ± 1.2	99.5 ± 2.6	96.2 ± 0.8	94.5 ± 1.4 *	92.5 ± 1.5 †	132.1 ± 2.6 §	127.0 ± 1.9 §	132.0 ± 3.2 §	122.0 ± 3.1 ‡§	117.5 ± 2.4 *§	121.2 ± 2.1 †§
RV (mg)	23.0 ± 0.5	22.7 ± 0.6	27.1 ± 1.0 *†	25.5 ± 0.5 †	19.2 ± 0.4 *	20.5 ± 0.6 †	31.4 ± 0.8 §	32.1 ± 0.5 §	35.3 ± 0.9 *†§	31.9 ± 1.2 ‡§	24.8 ± 0.5 *§	25.9 ± 0.6 †§
LV+S (mg)	77.1 ± 1.5	74.5 ± 0.9	72.4 ± 2.0	70.7 ± 0.9 †	75.3 ± 1.4	72.1 ± 1.3	100.7 ± 2.1 §	94.9 ± 1.5 *§	96.6 ± 2.7 §	90.2 ± 2.1 §	92.7 ± 2.0 *§	95.3 ± 1.7 §
RV/LV+S	0.300 ± 0.006	0.305 ± 0.008	0.376 ± 0.010 *†	0.362 ± 0.010 †	0.256 ± 0.008 *	0.285 ± 0.010 ‡	0.314 ± 0.006	0.341 ± 0.006 *§	0.369 ± 0.011 *†	0.353 ± 0.009	0.268 ± 0.005 *	0.273 ± 0.005 †
Old mice (12-15 months)												
n	11	15	15	15	10	17	17	16	24	18	11	
Body (g)	26.8 ± 1.1 #	28.2 ± 0.9 #	25.6 ± 0.9 #	25.2 ± 0.7 †#	27.0 ± 1.0 #	27.3 ± 0.6 #	37.8 ± 1.5 #§	39.2 ± 1.6 #§	35.7 ± 1.1 #§	36.5 ± 1.3 #§	36.2 ± 1.0 §	
Heart (mg)	106.6 ± 3.0	107.3 ± 1.9 #	102.1 ± 3.1	109.9 ± 3.7 #	102.2 ± 3.5	108.7 ± 1.8 #	140.9 ± 4.2 §	141.7 ± 3.7 #§	137.9 ± 4.1 §	135.4 ± 5.6 #§	124.8 ± 2.8 *§	
RV (mg)	23.1 ± 1.0	25.2 ± 0.4 #	24.4 ± 0.8 #	28.2 ± 1.2 †‡#	21.3 ± 0.6 #	23.2 ± 0.5 †‡#	32.1 ± 1.2 §	33.8 ± 1.2 §	33.1 ± 1.0 §	31.4 ± 1.2	27.0 ± 1.0 *§	
LV+S (mg)	83.5 ± 2.3 #	82.1 ± 1.8 #	77.7 ± 2.3	81.7 ± 2.7 #	80.9 ± 3.0	85.5 ± 1.5 #	108.9 ± 3.2 #§	108.0 ± 3.3 #§	104.8 ± 3.3 §	104.0 ± 4.6 #§	97.8 ± 2.0 *§	
RV/LV+S	0.276 ± 0.009 #	0.309 ± 0.008 *	0.314 ± 0.006 *#	0.345 ± 0.007 †‡	0.264 ± 0.006	0.271 ± 0.004 †	0.295 ± 0.008	0.316 ± 0.012	0.319 ± 0.008 *#	0.304 ± 0.007 #§	0.276 ± 0.007	

Ctr, control mice; PH, perinatal hypoxia; AH, adult chronic hypoxia; NO, perinatal inhaled nitric oxide; RV, right ventricle; LV+S, left ventricle plus septum.
Statistical difference between two groups was determined using an unpaired t test with Welch's correction. Significant difference ($p<0.05$): * PH or Ctr+AH or Ctr+NO versus Ctr; † PH+AH or Ctr+AH or PH+NO versus PH; ‡ PH+AH versus Ctr+AH or PH+NO versus Ctr+NO; # old versus young; § males versus female

Lung morphometry

Adult mice were anaesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg i.p., respectively) and placed in a supine position. Lungs were prepared as previously published [24]. After careful punctuation of the diaphragm to collapse the lungs, the air space was filled by tracheal instillation with freshly prepared paraformaldehyde 4 % in phosphate buffered saline (PBS) at a constant pressure of 20 cm H_2O. This water pressure allows the lung to reach roughly its mid-respiratory volume. The pressure was maintained throughout the preparation to prevent the lungs from recoiling. Once the lungs were filled, the trachea was ligated, and the heart-lung unit retrieved. After a 2h fixation in paraformaldehyde, the lungs were progressively dehydrated using ethanol and Histoclear before paraffin-embedding. Five-µm lung sections were immunostained using an alpha-smooth muscle actin monoclonal antibody (Sigma, A-2457, 1:400, mouse), aminoethylcarbazol and hematoxylin. Areas of 1.5 x 1.0 mm of lung parenchyma were systematically, randomly photographed in a meandering order on the Polyvar microscope using the 10x objective [25]. After image analysis, the vessels were categorized by size. Data are expressed as number of vessels per area analyzed.

Statistical analysis

Data are expressed as mean ± SEM, unless otherwise specified. Results were analyzed using GraphPad Prism 10.2.3. The tests used for statistical analyses were mentioned in the legend of each figure or table. $P<0.05$ was considered statistically significant.

Results

Anatomical data

The number of alive pups per litter recorded at P5 was lower in PH than Ctr offspring (median (range): 6 (0-10) in Ctr (n=186) and 4 (0-8) in PH (n=265), $p<0.0001$, Mann-Whitney test). At P5, PH pups had a reduced body weight (2.90±0.03 g in Ctr (n=119) and 2.60±0.07 g in PH (n=46), p=0.0002, Mann-Whitney test) and an increased hematocrit (20.9±0.6 % in Ctr (n=8) and 26.7±0.6 % in PH (n=7), p=0.0003, Mann-Whitney test).

Table 2 presents anatomical data from 5-6-month-old ("young") and 12-15-month-old ("old") mice. The difference in body weight between Ctr and PH mice was no longer present in adulthood. At 5-6 months, the RV/(LV+S) ratio was significantly increased following PH in males, whereas no significant difference was found between PH and Ctr females. At 12–15 months however, the Fulton index was significantly higher in PH than Ctr females, probably because of significant increase in RV weight with aging in PH, but not Ctr females.

At both ages, adult hypoxia (AH) significantly increased RV weight and the Fulton index compared to normoxia in all groups except in PH males. These parameters were significantly higher in Ctr+AH than PH mice. AH significantly reduced body weight in young and old PH+AH females and young PH+AH males compared to PH mice, but not in Ctr+AH mice.

Heart weight was significantly increased with aging only in PH mice, with or without re-exposure to hypoxia.

Perinatal exposure to iNO resulted in several anatomical changes compared to Ctr or PH mice. In young mice exposed to perinatal iNO, body weight was significantly increased in males, but not in females, while heart weight and RV weight were significantly decreased in Ctr+NO and PH+NO mice. The Fulton index was significantly reduced in Ctr+NO males and females, and PH+NO males, but not in PH+NO females. In old mice however, the Fulton index and RV weight were significantly reduced in PH+NO females.

Hemodynamic measurements

RVP measured in PH mice, under normoxia or acute hypoxia, was significantly higher than in Ctr mice (Fig.1A). In normoxia, RVP was higher in Ctr females than Ctr males, but lower in PH females than PH males, whereas no significant difference was found between males and females under hypoxia (Fig.1A). A 5-day (re-)exposure to hypoxia resulted in a significant rise in RVP in Ctr+AH and PH+AH males, under normoxia or acute hypoxia (Fig.1B). In normoxia, RVP was significantly higher in Ctr+AH than PH males but not under hypoxia. An acute exposure to hypoxia during hemodynamic measurements induced in all groups a significant increase in RVP compared to normoxia (Fig.1A-B). However, this increase was significantly higher in PH females than in other groups, whereas in males, acute hypoxia led to a similar increase in all groups (Fig.1C). In males, increasing concentrations of iNO under hypoxia led to a decrease in RVP in all groups, but did not allow to reach RVP values below those measured in normoxia (Fig. 1D). When applied in normoxia, iNO did not influence RVP in all male groups (data not shown).

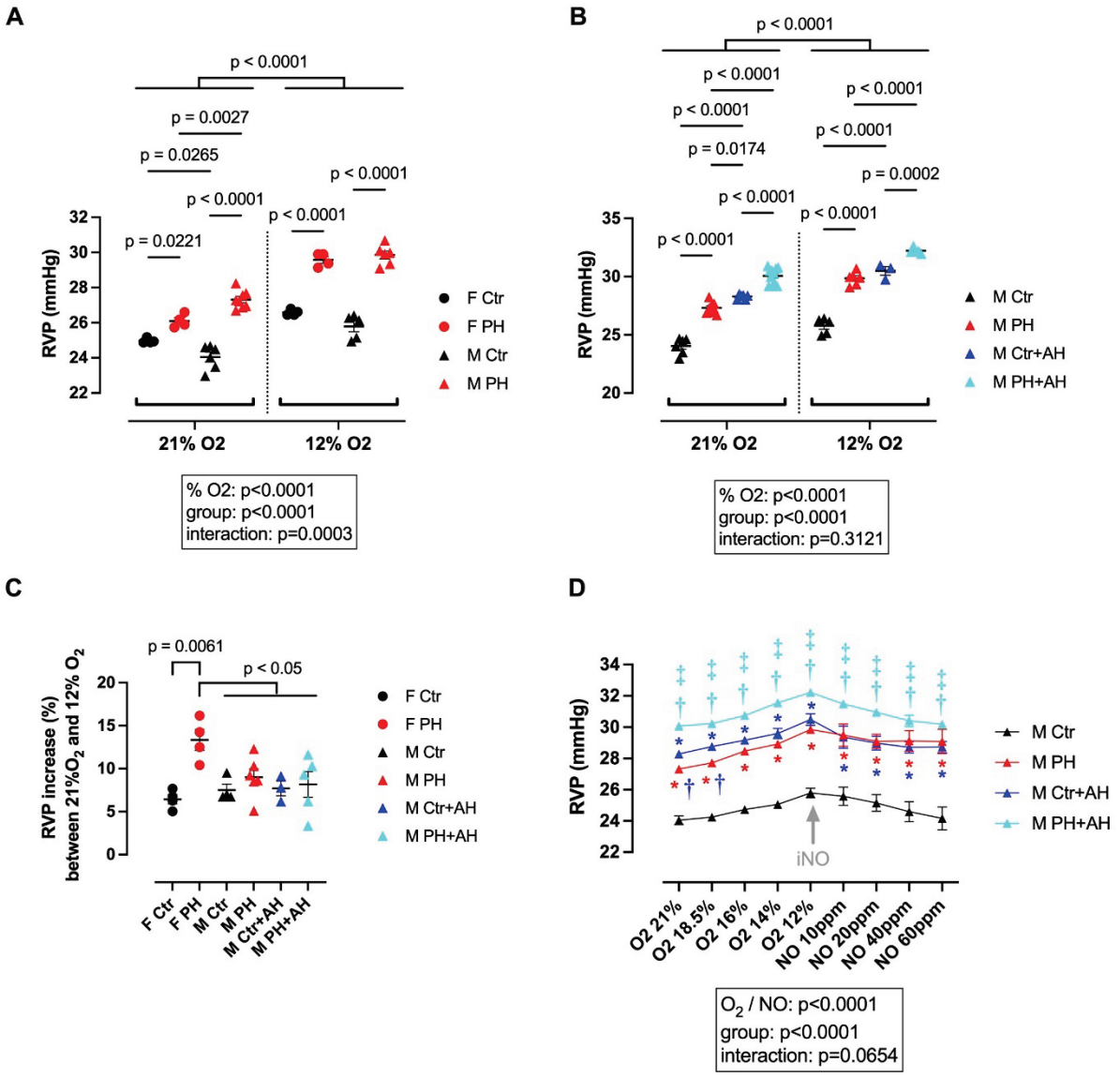

Fig. 1. Systolic right ventricular pressure (RVP) measured under normoxia (21 % O_2) or acute hypoxia (12 % O_2) in males (M) and females (F). Closed-chest measurements were performed in adult mice born in normoxia (Ctr) or hypoxia (PH), with or without a 5-day exposure to chronic hypoxia in adulthood (AH). Graphs **A-C** present individual values with bar at mean ± SEM. (**A**) RVP in Ctr and PH males and females (n=4-8); (**B**) RVP in Ctr and PH males with or without AH (n=3-11); data were analyzed by two-way ANOVA (results are shown below) with Sidak's multiple comparison test (significant p values are reported on the graph). (**C**) RVP increase between normoxia and acute hypoxia, expressed as percentage of RVP in normoxia (n=3-6); data were analyzed by one-way ANOVA and unpaired t tests with Welch's correction to compare two groups (significant p values are reported on the graph). (**D**) RVP measured in males during the transition from normoxia to acute hypoxia, followed by addition of increasing concentrations of inhaled nitric oxide (iNO) in the 12 % O_2 gas mixture; data are expressed as mean ± SEM (n=3-11) and were analyzed by two-way ANOVA (results are shown below) with Sidak's multiple comparison test; significant difference (p<0.05): * PH or Ctr+AH versus Ctr; † PH+AH or Ctr+AH versus PH; ‡ PH+AH versus Ctr+AH.

Isolated vessel tension studies

The resting tension measured in PA rings was similar between Ctr and PH mice (0.38±0.02 g in Ctr females (n=7), 0.37±0.02 g in PH females (n=6), 0.38±0.02 g in Ctr males (n=7), 0.40±0.01 g in PH males (n=16)). Acetylcholine-induced relaxation was significantly reduced in PA of PH versus Ctr mice (Fig. 2A-B). The decrease observed in AUC between Ctr and PH mice was greater in females (-28 %) than in males (-14 %). A 5-day (re-)exposure to hypoxia led to similar dose-response curves in Ctr+AH and PH+AH mice (Fig. 2A-B). The resulting relaxation was significantly improved in PH+AH versus PH mice, and Ctr+AH versus PH mice (Fig. 2A-B); in contrast, the response to low concentrations of acetylcholine was reduced in Ctr+AH versus Ctr females (significant difference at $3x10^{-8}$ and 10^{-7} M) (Fig. 2A), although AUC did not significantly differ between the two groups (p=0.0519).

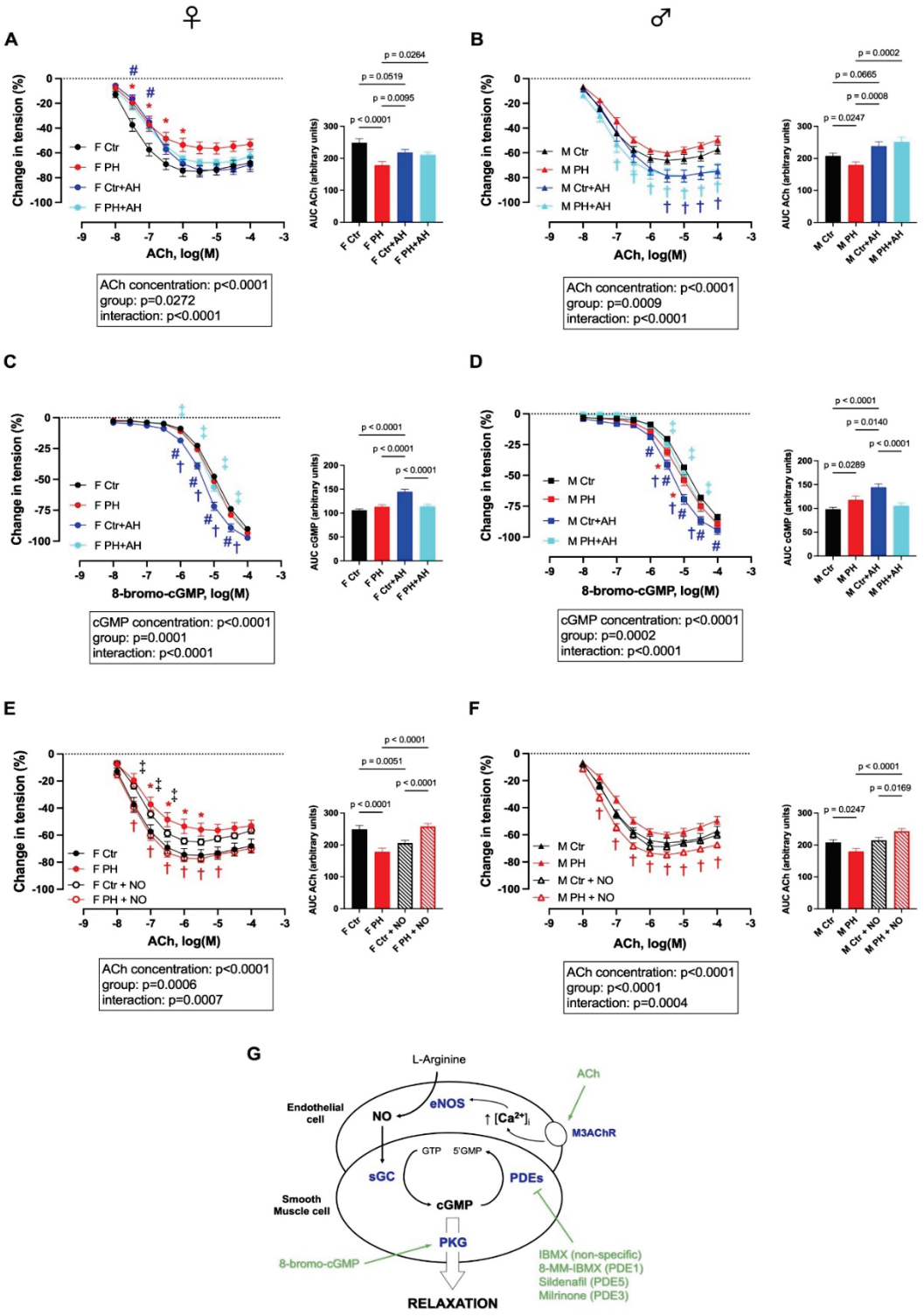

Fig. 2. Relaxation induced by cumulative doses of acetylcholine (ACh) or 8-bromo-cGMP in pulmonary arteries of males (M) and females (F) born in normoxia (Ctr) or hypoxia (PH), with or without a 5-day exposure to chronic hypoxia in adulthood (AH), or with perinatal exposure to inhaled nitric oxide (NO). Vascular rings were pre-constricted with phenylephrine 10^{-5}M. Data are expressed as mean ± SEM of the percentage of change in tension induced by the vasodilator (**A** n=6-8; **B** n=7-16; **C** n=5-9; **D** n=7-9; **E** n=6-14; **F** n=7-16). Data were analyzed by two-way ANOVA (results are shown below each graph) with Sidak's multiple comparison test; significant difference ($p<0.05$): * PH versus Ctr; # Ctr+AH vs Ctr; † PH+AH or Ctr+AH or PH+NO versus PH; ‡ PH+AH versus Ctr+AH or PH+NO versus Ctr+NO. The corresponding AUC were presented next to each graph; data are expressed as mean ± SEM and were analyzed by unpaired t tests with Welch's correction to compare two groups (significant p values are reported on each graph) (**G**). Schematic representation of the NO/cGMP relaxing pathway and interactions with pharmacological agents. eNOS, endothelial nitric oxide synthase; M3AChR, muscarinic acetylcholine receptor M3; PDEs, phosphodiesterases; PKG, cGMP-dependent protein kinase; sGC, soluble guanylyl cyclase. Dose-response curves to ACh assessed in Ctr and PH females were previously published in Peyter *et al.* [20]. Dose-response curves to ACh assessed in Ctr, PH and PH+NO females were previously published in Peyter *et al.* [21].

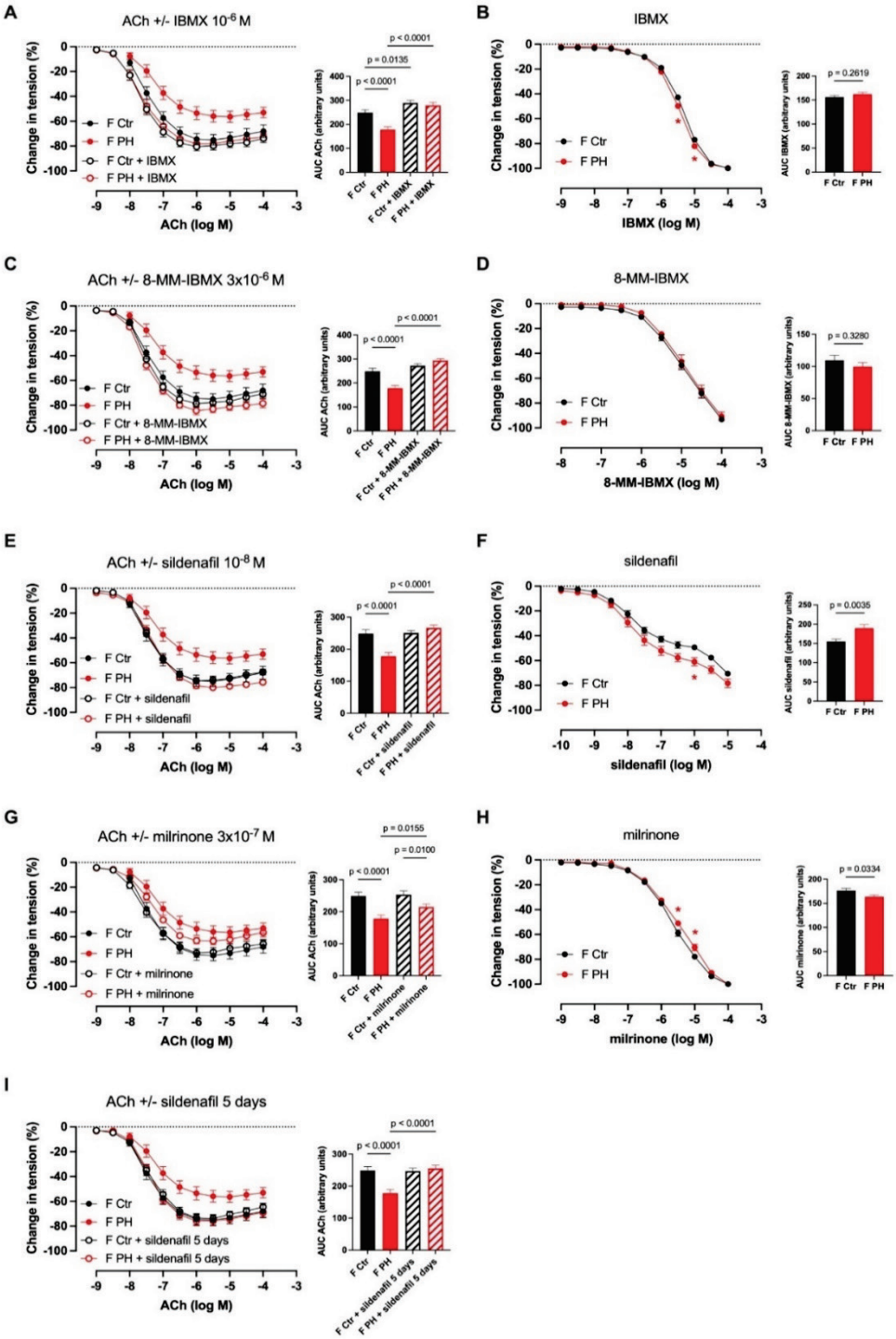

Fig. 3. Relaxation induced by cumulative doses of acetylcholine (ACh) in pulmonary arteries of females (F) born in normoxia (Ctr) or hypoxia (PH). Vascular rings were pre-incubated with phosphodiesterase (PDE) inhibitors (**A**, IBMX; **C**, 8-MM-IBMX; **E**, sildenafil; **G**, milrinone) and pre-constricted with phenylephrine 10^{-5}M. Some mice were treated with sildenafil during 5 days before testing the reactivity of their pulmonary arteries in the absence of PDE inhibitor (**I**). Data are expressed as mean ± SEM of the percentage of change in tension induced by ACh (**A** n=6-8; **C** n=6-7; **E** n=6-11; **G** n=6-11; **I** n=6-8). Data were analyzed by two-way ANOVA (results are shown below each graph) with Sidak's multiple comparison test; significant difference ($p<0.05$): * PH versus Ctr; † PH+PDE inhibitor versus PH. The corresponding AUC were presented next to each graph; data are expressed as mean ± SEM and were analyzed by unpaired t tests with Welch's correction to compare two groups (significant p values are reported on each graph). Dose-response curves to the PDE inhibitors (**B**, IBMX; **D**, 8-MM-IBMX; **F**, sildenafil; **H**, milrinone) were established in pulmonary arteries pre-constricted with phenylephrine 10^{-5}M. Data are expressed as mean ± SEM of the percentage of change in tension induced by the PDE inhibitor (**B** n=8; **D** n=5-11; **F** n=8; **H** n=7-8). Data were analyzed by two-way ANOVA with Sidak's multiple comparison test; * significant difference between PH and Ctr mice. The corresponding AUC were presented next to each graph; data are expressed as mean ± SEM and were analyzed by unpaired t tests with Welch's correction. Dose-response curves to ACh assessed in Ctr and PH females were previously published in Peyter *et al.* [20].

The PDE-resistant cGMP analog 8-bromo-cGMP induced similar relaxation in Ctr, PH and PH+AH females, but a significantly better relaxant response in Ctr+AH females (Fig. 2C). In males, 8-bromo-cGMP-induced relaxation was significantly increased in PH versus Ctr males, whereas Ctr+AH males relaxed significantly more than the other groups (Fig. 2D).

Simultaneous exposure to iNO and PH was able to preserve the relaxant response to acetylcholine in females and males, thus resulting in a significantly increased relaxation compared to PH mice (Fig. 2E-F). However, perinatal exposure to iNO alone resulted in a decreased acetylcholine-induced relaxation in Ctr+NO versus Ctr females (Fig. 2E), but did not affect Ctr+NO males (Fig. 2F).

Several PDE inhibitors were studied in females. The non-specific PDE inhibitor IBMX significantly improved acetylcholine-induced relaxation in Ctr and PH females, which then showed similar dose-response curves (Fig. 3A). The PDE1 inhibitor 8-MM-IBMX completely restored acetylcholine-induced relaxation in PH females but had no effect in Ctr females (Fig. 3C). The PDE5 inhibitor sildenafil also completely reversed the alteration in acetylcholine-induced relaxation observed in PH females but did not influence the relaxation in Ctr females (Fig. 3E). A 5-day treatment with sildenafil led to similar results (Fig. 3I). Finally, the PDE3 inhibitor milrinone significantly increased the response to acetylcholine in PH females, but did not restore complete acetylcholine-induced relaxation, and had no effect in Ctr females (Fig. 3G). The dose-response curves to IBMX and 8-MM-IBMX were similar between both groups (Fig. 3B-D). The relaxation induced by sildenafil was biphasic and significantly increased in PH versus Ctr females (Fig. 3F). In contrast, milrinone induced a lower relaxation in PH than Ctr females (Fig. 3H).

Cardiopulmonary parameters at rest and after exercise

In anesthetized females at rest, the transition from normoxia to acute hypoxia induced a significant decrease in O_2-sat and increase in pulse rate and breath rate in all study groups (Fig. 4A-C). In normoxia, O_2-sat was significantly lower in PH than Ctr females, whereas pulse rate and breath rate were similar between both groups (Fig. 4A-C). Under acute hypoxia, breath rate was significantly higher in PH than Ctr females and lower in PH+NO than PH females, without any significant difference in O_2-sat or pulse rate (Fig. 4A-C).

In conscious females at rest, transition from normoxia to hypoxia induced a significant decrease in O_2-sat in all groups (Fig. 4D), but a significant increase in pulse rate in PH and Ctr+NO females (Fig. 4E), and in breath rate in Ctr+NO females (Fig. 4F). No significant difference was found between Ctr and PH females in these conditions. Ctr+NO females showed a significant decrease in O_2-sat, pulse rate and breath rate compared to Ctr females in normoxia, as well as in O_2-sat under hypoxia (Fig. 4D-F). In normoxia, pulse rate and breath rate were significantly higher in PH+NO than Ctr+NO females (Fig. 4E-F); under hypoxia, O_2-sat was significantly higher in PH+NO than Ctr+NO females (Fig. 4D).

A swimming challenge under hypoxia led, in all groups, to significantly lower O_2-sat than exercise in normoxia (Fig. 4G), without change in pulse rate and breath rate (Fig. 4H-I). O_2-sat was significantly higher in PH+NO than PH females (Fig. 4G).

Lung morphometry

Morphometric measurements on adult male lungs showed a significant increase in the number of 25-75-µm pulmonary vessels per area in PH versus Ctr males, whereas no significant difference was found for smaller vessels (<25 µm) or larger vessels (>75 µm) (Fig. 5A). The total number of vessels per area did not significantly differ between groups (Fig. 5B). The number of small and medium vessels tended to be higher in PH than Ctr+AH males, although the difference was not significant, probably due to the small number of mice analyzed. A similar trend was found when comparing total pulm-onary vessels per area in PH males with the other groups.

Discussion

The present study showed that PH leads to long-term adverse effects in adult pulmonary circulation, which vary between males and females (Table 3).

The reduction in litter size and pup weight observed at P5 showed that PH resulted in fetal growth restriction and increased perinatal mortality, which is consistent with previous reports [16].

The higher RVP measured in 5-6-month-old PH versus Ctr mice likely reflects the development of pulmonary hypertension. RVP was further increased by acute hypoxia, to a greater extent in PH females than in other groups, suggesting that PH induced an increased sensitivity to acute hypoxia in the pulmonary vasculature of adult females.

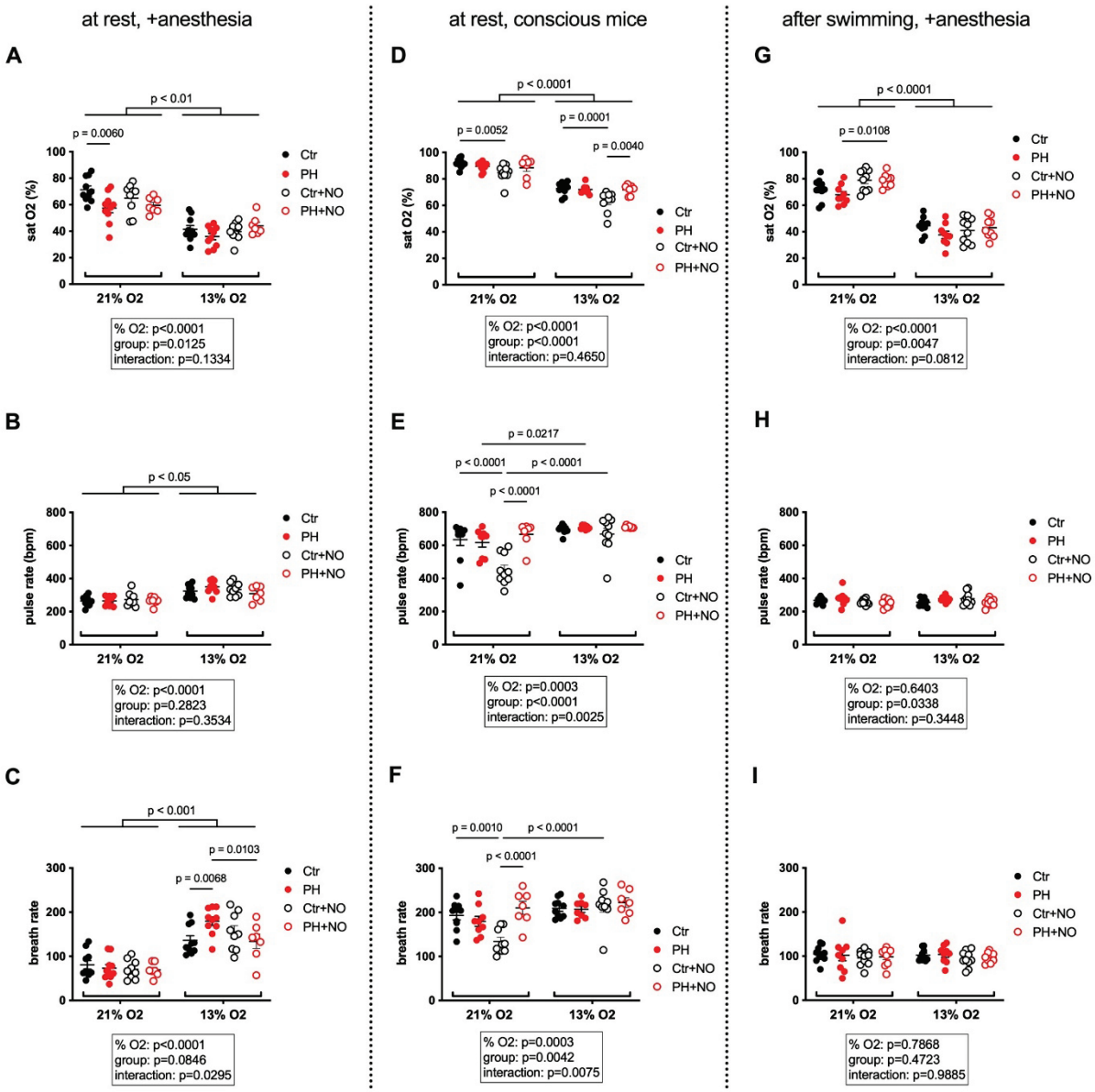

Fig. 4. Cardiopulmonary parameters at rest and after a 30-min swimming challenge in females born in normoxia (Ctr) or hypoxia (PH), with or without perinatal exposure to inhaled nitric oxide (NO). Oxygen saturation (sat O2) (**A, D, G**), pulse rate (**B, E, H**) and breath rate (**C, F, I**) were recorded in anesthetized females at rest (**A-C**), conscious females at rest (**D-F**) and females anesthetized after 30-min swimming in normoxic or hypoxic conditions (**G-I**). For females at rest, cardiopulmonary data were recorded during the transition from normoxia (21 % O_2) to acute hypoxia (12 % O_2); for females submitted to exercise, the recordings were performed in the same condition (normoxia or hypoxia) as the swimming challenge. Individual values are presented with bar at mean ± SEM (**A-C** n=7-10; **D-F** n=7-10; **G-I** n=9-10). Data were analyzed by two-way ANOVA (results are shown below each graph) with Sidak's multiple comparison test (significant p values are reported on the graph).

Acute iNO did not modify RVP in normoxia and simply reversed the effect of acute hypoxia on RVP in adult males, without lowering RVP below the value measured in normoxia.

Ex vivo investigation of PA vasoreactivity showed that endothelium-dependent relaxation was significantly reduced in PH versus Ctr mice, to a greater extent in females than in males. We previously demonstrated that this pulmonary vascular dysfunction was linked to a reduced eNOS protein content in PH versus Ctr females and alterations in M1AChR and PDE1 [20]. Here, we showed that preincubation with the non-specific PDE inhibitor IBMX, the PDE1 inhibitor 8-MM-IBMX or the PDE5 inhibitor sildenafil completely restored the relaxant response to acetylcholine in PH females. Similarly, a 5-day treatment with sildenafil also abolished the alteration of acetylcholine-induced relaxation in PH females. These data suggest that PDEs contribute to the pulmonary vascular dysfunction following PH, and that PDE1 and PDE5 inhibitors could

be promising agents to counterbalance adverse effects of PH in adulthood.

Our pharmacological and hemodynamic data suggested that PH had a greater impact on the adult pulmonary circulation in females than in males. Nevertheless, at 5-6 months, the Fulton index was significantly increased only in PH males, whereas at 12-15 months this index was significantly higher in PH than Ctr females, suggesting that the functional alterations observed in young females could lead to RV hypertrophy later in life. Therefore, PH leads to RV hypertrophy in adulthood, which appeared earlier in males than in females. Given that RVP was significantly increased in both young PH males and females, sex hormones could contribute to the delayed development of RV hypertrophy in females. This is supported by the observation that female rats were more sensitive than males to late effects of PH on pulmonary circulation and that neonatal – but not adult – gonadectomy exacerbated pulmonary hypertensive effects of PH only in females [17]. In humans, incidence of PAH is higher in young women than in men, although women have a better prognosis and survival, while these differences disappear after menopause [26].

Morphometric measurements were performed to explain RV hypertrophy already found in young PH males, despite lower vascular dysfunction in PH males than females. We observed a significant increase in the number of 25-75-µm pulmonary vessels in adult male lungs following PH, which could result in increased pulmonary vascular resistance, thus leading to RV hypertrophy. Studying angiogenic factors in neonatal lungs could be useful to characterize pulmonary vascular remodeling following PH. Investigation of pulmonary vascular density over time could help to determine whether PH directly induced an increase in number of pulmonary vessels or rather a decrease followed by an increase in vessel formation as a compensatory mechanism. Comparison with other studies is difficult because hypoxia-induced pulmonary vascular remodeling is a complex process, depending on the species, sex, and developmental stage at which the exposure to hypoxia occurred, but also on the site along the pulmonary vasculature [27].

Prolonged (re-)exposure to hypoxia in adulthood resulted in a significant increase in the Fulton index in all groups except in PH males - which already had a high index without re-exposure – and modified PA vasoreactivity. Long-term consequences of PH significantly differed from the effects of AH, which is consistent with previous reports [7,17], probably because PH occurred during a plastic phase of development thus leading to permanent adaptations/alterations.

Simultaneous administration of iNO during PH was able to prevent adverse effects of PH on endothelium-dependent relaxation of PA and the development of RV hypertrophy in females [21]. Here we showed that perinatal iNO also normalized some cardiopulmonary parameters in PH females, and protected males from impaired acetylcholine-induced relaxation and RV hypertrophy. However, perinatal iNO alone induced some changes in anatomical and cardiopulmonary parameters in adult mice, and decreased endothelium-dependent relaxation in Ctr+NO versus Ctr females, but not in males. Therefore, anything affecting cardiopulmonary hemodynamics during the perinatal period may have long-term consequences.

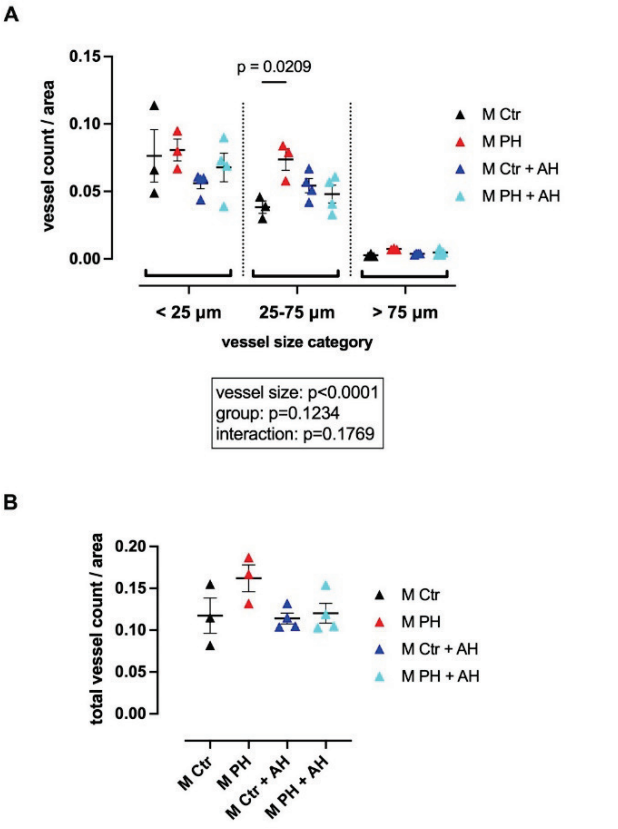

Fig. 5. Morphometric analysis of adult lungs from males (M) born in normoxia (Ctr) or hypoxia (PH), with or without a 5-day exposure to chronic hypoxia in adulthood (AH). Individual values are presented with bar at mean ± SEM (n=3-4). (**A**) The counted vessels were categorized by size; data are expressed as number of vessels per area analyzed; data were analyzed by two-way ANOVA (results are shown below) with Sidak's multiple comparison test (significant p values are reported on the graph). (**B**) Total number of vessels per area analyzed; data were analyzed by two-way ANOVA followed by Sidak's multiple comparison test.

Table 3. Summary of the main results

	Females	**Males**
RV/LV+S	PH = Ctr	PH > Ctr
in young mice	Ctr+AH > Ctr, PH+AH > PH, Ctr+AH > PH	Ctr+AH > Ctr, PH+AH = PH, Ctr+AH > PH
(Table 2)	Ctr+NO < Ctr, Ctr+NO < PH+NO	Ctr+NO < Ctr, PH+NO < PH
RV/LV+S	PH > Ctr	PH = Ctr
in old mice	Ctr+AH > Ctr, PH+AH > PH	Ctr+AH > Ctr, PH+AH = PH
(Table 2)	Ctr+NO = Ctr, PH+NO < PH	Ctr+NO = Ctr
RVP	PH > Ctr	PH > Ctr
	RVP ↗ by acute hypoxia	Ctr+AH > Ctr, PH+AH > PH, PH+AH >
	Increased sensitivity to acute hypoxia in PH	Ctr+AH, Ctr+AH > PH
(Fig.1)	females	RVP ↗ by acute hypoxia
ACh-induced relaxation	PH < Ctr	PH < Ctr
(Fig.2)	Ctr+AH < Ctr, PH+AH > PH, Ctr+AH > PH	PH+AH > PH, Ctr+AH > PH
	Ctr+NO < Ctr, PH+NO > PH, PH+NO > Ctr+NO	PH+NO > PH, PH+NO > Ctr+NO
cGMP-induced	PH = Ctr	PH > Ctr
relaxation	Ctr+AH > Ctr, Ctr+AH > PH+AH, Ctr+AH > PH	Ctr+AH > Ctr, Ctr+AH > PH+AH, Ctr+AH > PH
(Fig.2)		
Effects of PDE	IBMX, 8-MM-IBMX and sildenafil completely	–
inhibitors on response	restore ACh-induced relaxation in PH	
to ACh		
(Fig.3)		
O_2 saturation	↘ by acute hypoxia in all groups and conditions	–
	At rest, anesthesia: PH < Ctr in normoxia	
	At rest, conscious: Ctr+NO < Ctr in normoxia and	
	hypoxia; Ctr+NO < PH+NO in hypoxia	
(Fig.4)	After exercise: PH+NO > PH in normoxia	
Pulse rate	At rest, anesthesia: ↗ by acute hypoxia in all	–
	groups	
	After exercise: no difference between normoxia	
(Fig.4)	and hypoxia	
Breath rate	At rest, anesthesia: ↗ by acute hypoxia in all	–
	groups	
	After exercise: no difference between normoxia	
(Fig.4)	and hypoxia	
Lung morphometry	–	medium vessels density: PH > Ctr
(Fig.5)		

In vivo assessment of O_2-sat, pulse rate and breath rate, at rest or after a swimming challenge, showed that PH impacted the whole cardiopulmonary system. In females, perinatal exposure to hypoxia and/or to iNO differentially impacted cardiorespiratory parameters at rest or after exercise, probably as a result of functional alterations induced by PH and/or iNO in female pulmonary circulation. Acute hypoxia induced a decrease in O_2-sat in all groups and conditions, and an increase in pulse rate and breath rate at rest but not after swimming.

Taken together, our data demonstrated that PH led to long-lasting alterations in the regulation of pulmonary vascular tone. Males and females are differentially impacted by several factors acting on the cardiopulmonary system, like PH, perinatal iNO, adult prolonged or acute hypoxia, and aging (Table 3).

There are few reports directly comparing long-term effects of PH between males and females. In

humans, reanalysis of data of [2] showed that hypoxic exposure at high altitude led to exaggerated pulmonary vasoconstriction in males but not in females; however, number of females was very low (n=3-4 females, n=6-7 males) [28]. Neonatal exposure to hypoxia in swine induced a more severe pulmonary vascular disease in male than female piglets [28], and an impaired endothelium-dependent relaxation in pulmonary small arteries, but male and female data were not separately analyzed [29]. In contrast, female rats were more sensitive than males to late effects of PH on pulmonary circulation and these effects are blunted by the presence of ovaries during maturation [17].

Limitations and perspectives

Our study has several limitations. First, not all parameters were assessed in both males and females. Missing data need therefore to be completed to provide a comprehensive overview. Namely, lung morphometry should be performed in females, but also following perinatal iNO to determine if iNO during PH can prevent/reverse pulmonary vascular changes.

Then, some experiments were performed on a limited number of animals, in particular lung morphometry. Increasing sample size would provide more accurate and probably more significant results.

Another limitation was that estrous cycle was not monitored. However, mice were randomly assigned to the different experiments over time, thus mitigating variability due to litters, cages, and estrous cycle stages. The similar variability between male and female data strongly suggests that randomization was sufficient to limit the effects of estrous cycle on measurements.

Moreover, due to size limitation, mice only allow to use the main PA for isolated vessel tension studies. However, PH likely impacts the whole pulmonary vascular tree, including pulmonary veins (PVs), thus contributing to the increase in RVP and Fulton index observed in PH mice. PH-induced alterations may vary among the different kinds of pulmonary vessels [27,30]. Another animal model like rat could help to better investigate such alterations in PA and PV. Indeed, PVs play a crucial role in bringing freshly oxygenated blood to the heart [22,30]. Investigating alterations occurring in both PA and PV would help to better understand the mechanisms implicated and to design effective therapeutic strategies with limited side-effects. Indeed, if a treatment only improves PA or PV reactivity, it may lead to an imbalance between arterial and venous pulmonary circulation, resulting in altered hemodynamics.

Our pharmacological experiments highlighted PDE inhibitors as promising drugs to improve pulmonary vascular relaxation, in particular PDE5 and PDE1 inhibitors. This is consistent with clinical data in humans, where combined therapies using iNO and/or PDE inhibitors showed encouraging results in the treatment of pulmonary vascular diseases [31,32]. Another way to improve NO/cGMP-mediated relaxation will be to limit cGMP efflux, which appeared as important as cGMP hydrolysis in cGMP clearance [33]. The multidrug resistance-associated protein 4 (MRP4) was found to contribute to cGMP efflux and MRP4 inhibitors are currently under investigation to treat several vascular disorders like erectile dysfunction and pulmonary hypertension [34-36]. It would be therefore interesting to study the contribution of cGMP efflux in our model and to test whether MRP4 inhibitors could help to reverse pulmonary alterations following PH. Other stimulators of the NO/cGMP-mediated relaxation, like riociguat, could also be studied [32,37].

Cardiopulmonary parameters and exercise tolerance should be assessed after treatment with PDE or MRP4 inhibitors. Early interventions using PDE or MRP4 inhibitors, or Resveratrol, an antioxidant with PDE inhibition properties, during PH exposure could be of interest, to inhibit pulmonary vascular remodeling and prevent functional alterations. It would be interesting to test whether perinatal iNO at a lower dose would also be beneficial for PH mice, without adverse effects in Ctr animals.

Studying several proteins implicated in the NO/cGMP pathway is needed to better characterize the molecular alterations and elucidate the mechanisms implicated.

In conclusion, a transient exposure to hypoxia during the perinatal period results in long-term adverse effects on the adult pulmonary circulation, which vary between males and females. In males, the increased pulmonary vascular resistance seems to be associated with morphological changes besides functional alterations, whereas females have an important pulmonary vascular dysfunction. The effects of chronic hypoxia in adulthood also vary between males and females, and along life. Moreover, simultaneous administration of iNO during the PH exposure has a protective effect against long-term alterations induced by PH on the cardiopulmonary system. However,

perinatal iNO alone could have some adverse effects in Ctr mice. Therefore, any intervention in the perinatal period can leave a definitive imprint that could influence the regulation of the cardiovascular system later in life.

Further investigations are needed to better understand the mechanisms contributing to the differential alteration of adult pulmonary circulation following PH in males and females.

More broadly, our data emphasize the need to consider sex as an important biological variable in cardiovascular research.

Conflict of Interest

There is no conflict of interest.

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