Decayed Kv1.5 expression in intrauterine growth retardation rats with exaggerated pulmonary hypertension

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Lv Y, Tang L, Wei J, Xu X, Gu W, Fu L, Zhang L, Du L. Decreased Kv1.5 expression in intrauterine growth retardation rats with exaggerated pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 305: L856–L865, 2013. First published September 27, 2013; doi:10.1152/ajplung.00179.2013. —Chronic hypoxia pulmonary hypertension (CH-PHT) in adulthood is likely to be of fetal origin following intrauterine growth retardation (IUGR). Oxygen (O2)-sensitive voltage-gated potassium channels (Kv channels) in resistance pulmonary artery smooth muscle cells (PASMCs) play an important role in scaling pulmonary artery (PA) pressure. Expression and functional changes of Kv channels are determined, in part, by embryonic development. We hypothesized that O2-sensitive Kv channels play an important role in exaggerated CH-PHT following IUGR. We established a rat model of IUGR by restricting maternal food during the entire pregnancy and exposed IUGR rats and their age-matched controls aged 12 wk to hypoxia for 2 wk. We found that hypoxia exposure significantly increased PA pressure and thicker smooth muscle layer in the IUGR group relative to controls. We compared the constriction of the resistance PA to inhibitors of K+ channels, 4-aminopyridine (4-AP), tetraethylammonium, and BaCl2. Despite the thickness of the smooth muscle layer, the constriction to 4-AP was significantly reduced in the IUGR group exposed to hypoxia. Consistent with these changes in pulmonary vascular reactivity, 2 wk of hypoxia induced weaker 4-AP-sensitive Kv currents in a single IUGR PASMC. Moreover, after 2 wk of hypoxia, Kv1.5 expression in resistance PASMs decreased significantly in the IUGR group. Overexpression of Kv1.5 in cultured PASMCs could offset hypoxia-induced cell proliferation and hypoxia-inhibited Kv currents in the IUGR group. These results suggest that the inhibited expression of Kv1.5 in PASMCs contribute to the development of exaggerated CH-PHT in IUGR rats during adulthood.

chronic hypoxic pulmonary hypertension; intrauterine growth retardation; voltage-gated potassium channel; pulmonary artery smooth muscle cell

RECENTLY, INTRAUTERINE GROWTH RETARDATION (IUGR) has been considered to be associated with the fetal origin of adult disease (4). IUGR is defined as having a birth weight below the 10th percentile of the corresponding gestational age (25). Several studies have demonstrated that IUGR predicts the risk of developing various adult diseases, including obesity, Type 2 diabetes, and hypertension (12, 33). Its biological risk factors include intrauterine malnutrition and intrauterine hypoxia. Intrauterine hypoxia may also have a persistent effect on the infant pulmonary vascularature, which, when activated in adult life, predisposes to a pathological response (32). Similar to that in our recent study, exaggerated pulmonary hypertension (PHT) was observed because of exposure to chronic hypoxia in adult IUGR male rats. Additionally, intrauterine malnutrition plays an important role during this process (39). Another study showed that PHT of offspring in adulthood is associated with maternal undernutrition during pregnancy, although the offspring do not exhibit low birth weight (30). Moreover, maternal protein restriction during pregnancy limits fetal pulmonary development, a finding that may increase the susceptibility of the offspring to hypoxia after birth (14).

Pathological alterations of PHT induced by chronic hypoxia primarily occur in the resistance pulmonary artery (PA) (2). The resistance PA supplies hypoxic alveoli, thereby shunting perfusion to better-ventilated lobes. Sustained vasoconstriction elicits vascular remodeling (28), leading to V/Q (ventilation-perfusion) mismatch and systemic hypoxemia (23). In our recent study, we found that IUGR could cause exaggerated PHT through epigenetic modification on pulmonary vascular endothelial cells (39). Whether pulmonary artery smooth muscle cells (PASMCs) participate in this pathway is unknown. Oxygen (O2)-sensitive voltage-gated potassium channels (Kv channels) on PASMCs are a critical molecular target that could be directly regulated by oxidative stress in vivo and in vitro. Oxidative stress attenuated Kv channel currents and inhibited expression of the Kv channel α-subunit, which determines the characteristics of the channel (2, 13). Kv channels, including Kv1.2, Kv1.5, Kv2.1/9.3, and Kv3.1b in resistance PASMCs, are involved in the maintenance of ionic homeostasis and regulation of cell proliferation and apoptosis under hypoxic conditions during PHT development (1, 2, 22, 24, 27, 29, 38). The gene level of Kv channels, particularly that of KCNA5, also manifests decreased expression in the chronic hypoxia pulmonary hypertension (CH-PHT) cell model (36). Although the Kv β-subunit plays a role as a redox sensor and in hypoxia adaptation, its exact function is unclear.

The Kv current plays a critical role in regulating the electrophysiological phenotype and pathophysiological state of PASMCs, but little is known regarding the effect of IUGR on Kv channels. Thus we hypothesized that the development of Kv channels in PASMCs might be impacted by the state of maternal nutrition during pregnancy.

In the present study, we examined the 4-AP-sensitive Kv current and expression of the Kv channel α-subunit (1, 2, 22, 24, 27, 29, 38) in an IUGR rat model, established by maternal food restriction during pregnancy with and without offspring...
hypoxia exposure at adulthood. We then focused on Kv1.5 in resistance PASMCs in IUGR rats with exaggerated CH-PHT. Here, we found that expression of the Kv1.5 α-subunit, a physiologically important component of the cellular O2 sensor, was decreased in PA rings of IUGR rats exposed to hypoxia at 12 wk of age (IUGR-hypoxia rats), and overexpression of Kv1.5 in cultured PASMCs eliminated the difference in the 4-AP-sensitive Kv current and proliferation between the IUGR-hypoxia and control-hypoxia groups. Kv1.5 appears to be a potential molecular target of IUGR-exaggerated CH-PHT.

MATERIALS AND METHODS

Rat model of IUGR and CH-PHT. The Animal Care and Use Committee of Zhejiang University approved all procedures and protocols. We established the IUGR model according to our previous study (39). Female Sprague-Dawley (SD) rats weighing 250–300 g were selected. After mating with male rats overnight, females were housed individually and randomly divided into two groups. Pregnant rats were fed with standard chow ad libitum or 50% of the ad libitum amount (determined from the amount of chow consumed by the control group) during the entire pregnancy. All females used in the experiments had not been bred previously to avoid the compounding effect of the number of pregnancies. Only male offspring were studied to avoid hormonal cycle disturbances. Offspring of nonrestricted mothers were labeled as control rats; offspring of food-restricted mothers were labeled as IUGR rats (39). At 12 wk of age, offspring rats were further divided randomly into two groups: 1) control and control-hypoxia group and 2) IUGR and IUGR-hypoxia group. The control-hypoxia and IUGR-hypoxia groups were housed in normobaric Plexiglas cages for 2 wk in which the fraction of inspired oxygen was maintained at 10%, a level known to induce hypoxic PHT and right ventricular hypertrophy in rats (30, 39). Control and IUGR groups were maintained under normoxic conditions during the same period.

Hemodynamic, histological, and morphometric measurements. Right ventricular systolic pressure (RVSP) was measured to reflect the pressure of the pulmonary artery as described previously (28). Briefly, after 2 wk of hypoxia, 14-wk-old male offspring rats were connected to a ventilator after being anesthetized with pentobarbital sodium (50 mg/kg body wt, intraperitoneally) and placed on a heating table to maintain 38°C body temperature. A PE21 catheter connected to a pressure transducer was inserted into the right ventricle via the right jugular vein. The RVSP was recorded over 1 min by use of a computer data acquisition system (RM6240B/C). Transthoracic Doppler echocardiography was performed by using a Vevo 2100 image system with a MS250 transducer (VisualSonics, Toronto, ON, Canada). Echocardiograms including B-mode, M-mode, and Pulsed Wave Doppler mode images were obtained under the same anesthesia as catheterization. The velocity time integral, diameter, and heart rate were measured in the right ventricular outflow tract. Cardiac output (CO) was derived with the formula CO = π (aortic diameter)^2/4 × (aortic velocity time integral) × heart rate (8). Mean pulmonary artery pressure (mPAP) was measured as 58.7 ± (1.21 ± PAAAT), where PAAAT is PA acceleration time (31). Left atrial pressure (LAP) was estimated as 1.24 × E/E’ + 1.9 from the ratio of Doppler mitral E flow-velocity wave and tissue Doppler mitral annulus flow E’ early diastolic velocity (21). Pulmonary vascular resistance (PVR) was estimated as (mPAP – LAP)/CO.

The heart was removed, and the right ventricle (RV) was dissected from the left ventricle and septum (LV + Sep) without any arteries. RV weights were expressed as the ratio of the RV to the LV + Sep weight (RVH) (9).

Immunohistochemical staining of α-smooth muscle actin (α-SMA) was performed on paraffin-embedded lung tissue sections by using α-SMA antibody as we reported previously (40). Under the light microscope, the area of the smooth muscle layer, expressed as a percentage of the whole vessel, was measured to assess vascular dysfunction by use of Image Pro Plus software 5.0 (Media Cybernetics, Bethesda, MD).

Vascular reactivity test. A vascular reactivity test was performed by using K+ channel inhibitors as previously reported (2). Briefly, lungs were harvested in ice-cold physiological salt solution (PSS) containing 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, and 10 mM glucose. Resistance PA rings (180–220 μm in diameter) were dissected from the lungs rapidly and gently under the anatomical microscope with the connective tissue removed at 4°C with 95% O2–5% CO2 and cut into 3-mm-long rings. The rings were mounted on stainless steel wires that passed through the lumen longitudinally (DMT 610 M; Danish Myo Technology) in the bath chambers with 5 ml of PSS solution (37°C, pH 7.40–7.43; 95% O2–5% CO2). Vascular reactivity was studied in the endothelium-denuded resistance PAs. The endothelium was removed by gently rubbing the lumen with a silk suture. Before the experiment began, a basal tension of 5 mN was applied, and vessels were allowed to equilibrate for 30 min, with washing every 15 min. The maximal constriction was elicited three times with 60 mM KCl. The failure of acetylcholine (0.1 to 10 μM) to relax vessels preconstricted with phenylephrine (1–100 μM) indicated successful denudation of the endothelium (11). Constriction to 4-aminopyridine (4-AP; 1–10 mM), tetraethylammonium (TEA; 0.2–2 mM), and barium chloride (BaCl2; 1–100 μM) was assessed sequentially in endothelium-denuded resistance PA rings. Constriction to 4-AP (10 mM), TEA (1 mM), and BaCl2 (100 μM) for 10 min was compared between the groups as previously described (2, 7). All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and transfection. PASMCs were dispersed from 12-wk-old IUGR and control rat resistance PAs as described previously (20). After enzymatic isolation, PASMCs were cultured at 37°C in DMEM supplemented with 15% FBS. All experiments were performed with cells at passages two to four. For hypoxia experiments, PASMCs were cultured in the presence or absence of CoCl2 (a classic hypoxia mimetic; 100 μmol/l; Sigma) for 48 h after transfection for 24 h as described previously (41). PASMCs were transiently transfected with X-tremeGENE HP DNA transfection reagent (Roche), which has higher transfection efficiency in difficult-to-transfect primary cells, according to the manufacturer’s instructions. In the KCNA5 construct, the coding sequence of the rat KCNA5 gene (GenBank accession no. NM_012972) was subcloned into the Xhol and Kpnl sites of the multiple cloning site (MCS) of the vector GV141 (GENECHEM). In the KCNA5-EGFP construct [which encodes Kv1.5 and enhanced green fluorescent protein (GFP)], the coding sequence of the rat KCNA5 gene was subcloned into Nhe1 and AgeI sites of MCS of the vector GV230 (GENECHEM). Cells transfected with empty vector with and without EGFP were used as negative controls to analyze the effect of the transfection on cell survival. Transfection efficiency was detected by immunoblotting with a GFP antibody (6G539; Sigma).

Electrophysiology. Whole-cell patch clamp recording was performed at room temperature (22–23°C) in Hanks’ solution with 10 mM glucose (pH 7.4). Glass pipettes (3–5 MΩ) were filled with a solution containing 140 mM potassium gluconate, 10 mM HEPES, 4 mM Mg-ATP, 0.3 mM Na-GTP, 2 mM MgCl2, and 10 mM EGTA, pH 7.2 with KOH, 295 mosmol/kg. PASMCs were voltage clamped at a holding potential of −70 mV. Currents in difficult-to-transfect primary cells were measured from −70 to +70 mV in 20-mV steps (Axopatch 700B, Axon Instruments), filtered at 1 kHz, and sampled at 2–5 kHz (2). The Kv current was defined as the difference between the whole-cell outward current recorded in the 4-AP-free bath solution and the current recorded after superfusing with the same solution contain 5 mMol/l 4-AP (15). The current density of Kv channels was calculated by dividing the average plateau phase IC by the cell capacitance (pA/PF) and was compared among the four groups (2). Electrophys-
io logical data analyses were performed by using the Clampfit subroutine of the pCLAMP software (Axon Instruments).

**Immunoblotting.** The smooth muscle layer of resistance PA rings was dissected. Total protein extracts and protein concentrations were prepared according to previously reported techniques (10, 40). The membrane protein was prepared as described previously (6). Immunoblotting was performed to detect hypoxia-related Kv channels as described previously (10, 40). Membranes were immunoblotted with antibodies to Kv1.2 (APC-010; Alomone Labs), Kv1.5 (APC-004), Kv2.1 (APC-012), Kv3.1b (APC-014), and Kv9.3 (HPA014864; Sigma) at 4°C overnight. The signal intensity of the immunoreactive Kv bands from the membrane protein was normalized to β-actin (AA128; Beyotime) expression, whereas α-SMA from total protein was compared in intact PA rings with GAPDH (ab97626; Abcam) used as the input. Fluorescent secondary antibodies (Rockland, Gilbertsville, PA) were visualized via an Odyssey scanner (LI-COR, Lincoln, NE).

**MTT assay.** For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, PASMCs were seeded onto 96-well plates (~2 × 10^4 cells/well, by using a cell counting chamber) for 24 h before transfection. PASMCs were then cultured in the presence or absence of CoCl2 (a classic hypoxia mimetic; Sigma) for 48 h after transfection for 24 h. MTT assays (Sigma) were performed to assess cell proliferation every day for 3 days after transfection (0, 24, 48, and 72 h) according to the manufacturer’s instruction.

**Confocal microscopy.** Confocal microscopy imaging was performed to identify the expression level of Kv1.5 in cultured cells before and after transfection as described previously (5), by using an Olympus confocal laser scanning microscope (BX61W1-FV1000, Japan). The Kv1.5 primary antibody (1:100; APC-150; Alomone Labs), and α-SMA (1:150; AA132; Beyotime) was used as described previously (10, 19). Secondary antibodies of tetramethylrhodamine isothiocyanate (1:200; ZSGB) and fluorescein isothiocyanate (1:150; A0568; Beyotime) were used in cells transfected with plasmids that do not express GFP. Nuclear staining was performed with use of 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; 100 nM; Sigma) at 4°C overnight. The signal intensity of the immunoreactive Kv bands from the membrane protein was normalized to β-actin (AA128; Beyotime) expression, whereas α-SMA from total protein was compared in intact PA rings with GAPDH (ab97626; Abcam) used as the input. Fluorescent secondary antibodies (Rockland, Gilbertsville, PA) were visualized via an Odyssey scanner (LI-COR, Lincoln, NE).

**RESULTS**

**Effects of chronic hypoxia on the PA of IUGR rats in vivo.** The offspring of mother rats that were fed a restricted diet had lower birth weights compared with the offspring of mothers who ate ad libitum (5.0 ± 0.577 vs. 7.0 ± 0.858 g, respectively; P < 0.05). Litter sizes were not different among the two groups (10 ± 2/litter). At 12 or 14 wk old, no significant differences in body weight were observed between the control and IUGR groups (12 wk: 444 ± 12.7 vs. 438 ± 8.2 g, respectively; P > 0.05; 14 wk: 457 ± 9.1 vs. 479 ± 8.4 g, respectively; P > 0.05).

To investigate the function of the PA of IUGR rats during adulthood, we assessed the RVSP, mPAP, and RVHI of 14-wk-old rats in vivo. PHT is defined as a mPAP > 25 mmHg (at rest) (31). RVSP and RVHI values were similar between the control and IUGR groups (RVSP: 22.88 ± 0.38 mmHg vs. 23.76 ± 0.28 mmHg, respectively; P = 0.09, Fig. 1, A and B; RVHI: 0.26 ± 0.01 vs. 0.28 ± 0.01, respectively; P = 0.16, Fig. 1C), as well as mPAP from echocardiograms provided in Table 1. After 2 wk of hypoxia exposure, RVSP, mPAP, and RVHI values increased. The increase was more significant in the IUGR-hypoxia group than its age-matched control-hypoxia group (RVSP: 32.50 ± 0.30 mmHg vs. 37.80 ± 0.63 mmHg, respectively; P < 0.05, Fig. 1, A and B; mPAP: 27.13 ± 1.58 mmHg vs. 34.50 ± 2.05 mmHg, respectively; P < 0.05, Table 1; RVHI: 0.32 ± 0.01 vs. 0.40 ± 0.02, P < 0.05, respectively; Fig. 1C). There were no significant differences in heart rate among all the groups (control: 351 ± 10.41 bpm; IUGR: 330 ± 19.01 bpm; control-hypoxia: 368 ± 9.94 bpm; IUGR-hypoxia: 350 ± 9.73 bpm; Fig. 1A). The measurements of
cardiac output were similar in all the groups (Table 1). Thus these results reflected an elevation of PVR in the IUGR-hypoxia group compared with the control-hypoxia group (Table 1).

To further investigate exaggerated PHT, we performed immunohistochemical staining and immunoblotting in 14-wk-old control rats and IUGR rats. The thickness of the resistance PA smooth muscle layer in artery slices was similar between normoxia groups [55.69 ± 5.77% (control) vs. 57.90 ± 8.56% (IUGR); \(P = 0.62, \text{Fig. 2, A and B}\)]. Additionally, there were no differences in the normalized expression of α-SMA in the intact resistance PA between normoxia groups [1.0 (control) vs. 1.086 ± 0.252 (IUGR); \(P = 0.74, \text{Fig. 2, C and D}\)]. Treatment with hypoxia also produced a significant difference in the thickness of the smooth muscle layer and expression of α-SMA [thickness of smooth muscle layer: 61.70 ± 12.21% (control-hypoxia) vs. 79.02 ± 13.46% (IUGR-hypoxia); \(P < 0.05, \text{Fig. 2, A and B}\)] [expression of α-SMA: 1.338 ± 0.218 (control-hypoxia) vs. 2.130 ± 0.180 (IUGR-hypoxia); \(P < 0.05, \text{Fig. 2, C and D}\)].

4-AP-sensitive Kv channels contribute to aggravated constriction of the resistance PA in IUGR-hypoxia rats. To evaluate the effect of hypoxia on Kv channels in PASMCs between the control and IUGR groups, we isolated resistance PA rings from the lungs of the rat model and compared their functional responses to different K⁺ channel blockers. Effective denudation of the endothelium eliminated acetylcholine relaxation (Fig. 3A). In the same small PA rings, we found that 10 mM 4-AP (a specific K⁺ channel blocker) (16) caused strong constriction at low concentrations (1–10 mM). TEA (0.2–2 mM), a K⁺Ca channel blocker, did not significantly constrict PA rings, whereas 1–100 μM BaCl₂ (an inward rectifier K⁺ channel blocker) caused weaker constriction of PA rings than 4-AP. The concentration of drugs was used sequentially according to previous reports (2, 7). The constriction to 4-AP (10 mM), TEA (1 mM), and BaCl₂ (100 μM) was similar between the control and IUGR groups. After 2 wk of hypoxia, the constriction to 4-AP (10 mM) was inhibited, and the inhibition was more severe in the IUGR-hypoxia groups (Fig. 3, B–E). However, the constriction to TEA and BaCl₂ was not different between the control-hypoxia and IUGR-hypoxia groups and was not inhibited significantly by hypoxia in endothelium-denuded PA rings. Taken together, these data suggest that 4-AP-sensitive Kv channels were more significantly inhibited by chronic hypoxia in IUGR resistance PA rings.

Increased 4-AP-sensitive Iₖᵥ in resistance PASMCs of IUGR-hypoxia rats. To ascertain whether the difference in response to the K⁺ channel blocker between the control and IUGR groups only correlated with the proliferation of PASMCs, we performed whole-cell voltage-clamp recordings on single PASMCs dispersed from IUGR rats and their age-matched controls. The whole-cell currents and current density were similar between the normoxia groups (Fig. 4, A and B). However, the outward currents and current density of the

### Table 1. Doppler echocardiographic measurements in rats with or without hypoxia

<table>
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<tr>
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<th>Control</th>
<th>IUGR</th>
<th>Control-Hypoxia</th>
<th>IUGR-Hypoxia</th>
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<tr>
<td>mPAP, mmHg</td>
<td>19.04 ± 1.40</td>
<td>19.71 ± 2.80</td>
<td>27.13 ± 1.58</td>
<td>34.50 ± 2.05*</td>
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<tr>
<td>CO, ml/min</td>
<td>140.20 ± 7.11</td>
<td>164.64 ± 11.26</td>
<td>140.27 ± 11.53</td>
<td>154.87 ± 3.54</td>
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<tr>
<td>PVR, mmHg·min·ml⁻¹</td>
<td>100.90 ± 10.36</td>
<td>81.01 ± 20.04</td>
<td>154.18 ± 4.71</td>
<td>179.71 ± 8.33*</td>
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IUGR, intrauterine growth retardation; mPAP, mean pulmonary artery pressure; CO, cardiac output; PVR, pulmonary vascular resistance; values are expressed as means ± SE; *\(P < 0.05\) compared to control-hypoxia group; \(n = 4\) SD rats/group.

![Fig. 2](http://aijplung.org/)

**Fig. 2.** The smooth muscle layer of resistance pulmonary artery is thicker in IUGR rats than control rats with exaggerated PHT. A: representative immunohistochemical staining images for α-SMA (smooth muscle actin; brown signal; scale bar = 75 μm). Arrows show the smooth muscle layer. B: summary plots of the area ratio [medial/(medial + lumen) area] show the wall thickness of the smooth muscle layer in resistance pulmonary artery between IUGR and control rats with or without hypoxia. C: representative immunoblots of α-SMA expressed in the intact resistance pulmonary artery (PA) rings. D: summary plots of relative expression of α-SMA compared with GAPDH from each group. Data are presented as means ± SE. *\(P < 0.05\) compared with control-hypoxia group; \(n = 8\) SD rats/group.
A significant mediator of IUGR more sensitive to hypoxia (Fig. 5, to the control-hypoxia group, suggesting that Kv1.5 may be an exaggerated PHT had decreased expression of Kv1.5 relative the hypoxia groups. However, only IUGR-hypoxia rats with normoxia groups, the expression of Kv channels was lower in between the control and IUGR groups. Compared with the expression of the channels was not statistically different be-

and Kv9.3, as well as

-subunit-specific antibodies to Kv1.2, Kv1.5, Kv2.1, Kv3.1b,

and Kv1.5 after transfection can also be inhibited by hypoxia in resistance PASMCs. Although the normalized expression of Kv1.5 after transfection can also be inhibited by hypoxia in both groups, there was no difference between the control-hypoxia and IUGR-hypoxia groups. For electrophysiology tests, cells were transfected with KCNA5-EGFP. Immunoblotting showed that GFP is present only in transfected cells (Fig. 7A). Only PASMCs expressing GFP were selected to be patch-clamped based on their green fluorescence. 4-AP-sensi-

tive Kv currents in both hypoxia groups with KCNA5-EGFP were almost the same as in cells cultured under normoxic conditions (Fig. 8). There was no effect of the empty vector with or without GFP on proliferation and the current. This result suggested that Kv1.5 overexpression in cultured PASMCs could offset hypoxia-induced cell proliferation and hypoxia-inhibited Kv current in the IUGR group.

DISCUSSION

Although many diseases are considered to originate from IUGR (12, 33), the fetal origin of CH-PHT has not yet been

control and IUGR groups were both inhibited after exposure to hypoxia and lowered in the IUGR-hypoxia than in the control-hypoxia group (Fig. 4, C and D). Because a previous study showed that hypoxia inhibits the same channels as 4-AP (2), 10 mM 4-AP was applied to investigate the differences in currents among the four groups. The whole-cell outward currents after application of 4-AP within 1 min were inhibited, and the remaining currents and current density exhibited no difference between the control and IUGR groups (Fig. 4, A, B and F). 4-AP had a reduced ability to inhibit the outward currents in the hypoxia groups (Fig. 4E). However, subtraction analyses showed that the density of 4-AP-sensitive Kv currents in resistance PASMCs was similar between the normoxia groups (Fig. 4, A, B and E) and were reduced more significantly in the IUGR-hypoxia group than in the control-hypoxia group (Fig. 4, C-E), indicating that 4-AP-sensitive Kv currents were inhibited more severely in IUGR single PASMCs by hypoxia.

Expression of Kv1.5 is inhibited in the development of exaggerated PHT in IUGR rats. Considering that 4-AP-sensitive Kv currents were inhibited in resistance PASMCs by hypoxia, we further investigated the expression levels of the Kv channel α-subunit in vivo, which is the major component of the channel (23). To examine the expression of Kv channel α-subunits, we performed immunoblotting experiments using α-subunit-specific antibodies to Kv1.2, Kv1.5, Kv2.1, Kv3.1b, and Kv9.3, as well as β-actin as a control (Fig. 5, A and B). The expression of the channels was not statistically different between the control and IUGR groups. Compared with the normoxia groups, the expression of Kv channels was lower in the hypoxia groups. However, only IUGR-hypoxia rats with exaggerated PHT had decreased expression of Kv1.5 relative to the control-hypoxia group, suggesting that Kv1.5 may be an important mediator of IUGR more sensitive to hypoxia (Fig. 5, A and B).

Kv1.5 overexpression reduces the hypoxia effect on cultured PASMCs of IUGR rats. To assess the functional significance of inhibited Kv1.5 expression in PASMCs that contribute to exaggerated PHT in IUGR-hypoxia rats, we established CH-PHT (mimic by CoCl2) on PASMCs from IUGR and control rats (Fig. 6A) and labeled Kv1.5 in PASMCs (Fig. 7B). Fluorochrome detection and the MTT assay suggest that exposure of IUGR PASMCs to CoCl2 caused increased proliferation (Fig. 6, A and B) and inhibited Kv1.5 expression (Fig. 7, B and C) compared with the control-hypoxia group. Incubation with only the primary or secondary antibodies revealed no signal, supporting the specificities of the antibodies. As shown in Fig. 6, we observed that the overexpression of Kv1.5 (without GFP) reverses overproliferation in both control-hypoxia and IUGR-hypoxia PASMCs. Although the normalized expression of Kv1.5 after transfection can also be inhibited by hypoxia in both groups, there was no difference between the control-hypoxia and IUGR-hypoxia groups. For electrophysiology tests, cells were transfected with KCNA5-EGFP. Immunoblotting showed that GFP is present only in transfected cells (Fig. 7A). Only PASMCs expressing GFP were selected to be patch-clamped based on their green fluorescence. 4-AP-sensi-

tive Kv currents in both hypoxia groups with KCNA5-EGFP were almost the same as in cells cultured under normoxic conditions (Fig. 8). There was no effect of the empty vector with or without GFP on proliferation and the current. This result suggested that Kv1.5 overexpression in cultured PASMCs could offset hypoxia-induced cell proliferation and hypoxia-inhibited Kv current in the IUGR group.
widely demonstrated. In the present study, we confirmed that IUGR, in conjunction with chronic hypoxia later in life, causes resistance PA dysfunction and exaggerated PHT as previously reported (39). Furthermore, we studied upstream, Kv1.5-related events leading to PHT and PASMC proliferation with and without IUGR. Together, our data support a critical role for Kv1.5 in the mechanism of IUGR-exaggerated CH-PHT.

IUGR predisposes human infants to an increased risk of chronic lung disease after birth (3). This is known as the “fetal origin of adult diseases” hypothesis (4). Whether CH-PHT is related to IUGR has not been widely recognized. In the present study, we found that RVSP, mPAP, PVR, RVHI, and the expression of α-SMA were higher in IUGR-hypoxia rats than in control-hypoxia rats, indicating that IUGR can cause exag-

Fig. 4. Chronic hypoxia inhibited 4-AP-sensitive voltage-gated potassium (Kv) currents more prominently in IUGR rats. A and B: representative traces of the whole-cell currents in resistance pulmonary artery smooth muscle cells (PASMCs) in the control (A) and IUGR (B) rats before (top, left traces) and after (top, right traces) treatment with 4-AP (5 mM). Analyses of current subtraction, which represents 4-AP-sensitive Kv currents, are shown at the bottom of A and B. C and D: representative traces of the whole-cell currents in resistance PASMCs in the IUGR-hypoxia and control-hypoxia rats before (top, left) and after (top, right) treatment with 4-AP (5 mM). Analyses of current subtraction, which represents 4-AP-sensitive Kv currents, are shown at the bottom of C and D. E: plots of the whole-cell 4-AP-sensitive Kv currents’ density of PASMCs of control, IUGR, control-hypoxia, and IUGR-hypoxia groups after treatment with 4-AP were analyzed. F: plots of remaining currents’ density between control, IUGR, control-hypoxia, and IUGR-hypoxia groups. Data are presented as means ± SE. *,†,‡P < 0.05; n = 18 cells from 6 rats per group.

Fig. 5. Decreased expression of Kv1.5 in IUGR-hypoxia PA rings. A: representative immunoblots of the α-subunit of Kv1.2, Kv1.5, Kv2.1, Kv3.1b, and Kv9.3 in resistance PASMCs. β-Actin was used as a loading control. B: summary histogram of immunoblot analyses of the α-subunit of Kv1.2, Kv1.5, Kv2.1, Kv3.1b, and Kv9.3 in resistance PASMCs. Data are presented as means ± SE. *P < 0.05 compared with their respective control-hypoxia group; n = 4 SD rats/group.
gerated CH-PHT and pulmonary vascular smooth muscle layer reconstruction. Cardiac output was slightly but not significantly decreased in the IUGR-hypoxia than in the control-hypoxia groups, indicating that our model of IUGR focuses more on the peripheral PA rather than on cardiac function. In addition, no significant differences were detected between age-matched rats of the IUGR and control groups maintained under normoxic conditions. These findings were similar to those in a previous study that found that maternal food restriction during pregnancy was related to the development of CH-PHT in adulthood (30, 39). In contrast to Rexhaj’s study (30), we found that the offspring of rats that were food restricted during the entire pregnancy had birth weights below the 10th percentile of normal birth weights, meeting the criteria of IUGR. Our study and that of Rexhaj et al. differed in that they restricted maternal food during mid and late pregnancy, whereas we began intervention from an early period. The age-related development of body size or degree of food reduction could explain the difference in results (26), and the findings might also suggest that dietary restriction in later pregnancy could be an important determinant of PA development in the offspring. However, future studies are required to more precisely define how and when maternal food restriction affects pulmonary vascular dysfunction in the offspring. These findings are relevant to human diseases. For example, maternal food restriction is correlated with obesity and diabetes in human offspring (17), and transient intrauterine insults are associated with pulmonary vascular dysfunction later in life (32).

The mechanisms associated with IUGR and adult diseases are complex and likely to result from disruptions in multiple pathways (12). Although the endothelium modulates vascular constriction, endothelium-independent mechanisms regulate vascular constriction more directly (18). To investigate whether an endothelium-independent mechanism was involved with IUGR-related CH-PHT, the vessel tension was evaluated with the endothelium denuded. The O2-sensitive Kv channel in resistance PASMCs, as an endothelium-independent mediator, can regulate the vascular response to hypoxia (2, 28). Although this role for Kv channels is widely conserved, its susceptibility may vary among individuals, such as those with IUGR or a low birth weight. The selectively reduced responses of PASMCs to the Kv channel-specific blocker 4-AP relative to other K+...
channel blockers could have resulted from reduced Kv channel expression to hypoxia. Meanwhile, 4-AP had a reduced ability to constrict artery rings in the IUGR-hypoxia group compared with the control-hypoxia in the same PA rings, indicating that Kv channels are inhibited more significantly in IUGR-hypoxia rats. This finding was further supported by whole-cell voltage-clamp experiments performed on a single PASMC. The strong parallel between the inhibited current of 4-AP and hypoxia also suggests that Kv channels are physiologically relevant to the effect of chronic hypoxia on IUGR. These findings are consistent with those in studies showing that Kv current inhibition could increase the proliferation of smooth muscle cells, decrease apoptosis, and induce vascular remodeling (28, 29, 42).

Considering that many members of the Kv channel subfamilies in resistance PASMCs, such as Kv1.2, Kv1.5, Kv2.1, Kv3.1b, and Kv9.3, can be regulated by chronic hypoxia and affect the resting membrane potential of PASMCs (1, 2, 22, 27, 29, 38), it would be interesting to determine whether they are also susceptible to IUGR. All Kv channels we examined were downregulated by 2 wk of hypoxia treatment. However, only the expression of Kv1.5 was significantly decreased in IUGR-hypoxia rats compared with control-hypoxia rats, implying that Kv1.5 inhibition is important in IUGR-induced, more exaggerated PHT.

We further investigated whether overexpression of Kv1.5 could reverse this established PHT in vitro. A previous description of the downregulation of Kv channels in PHT, particularly Kv1.5, suggested that these channels are dysfunctional, perhaps with a genetic basis (42). Our work suggests that Kv1.5 overexpression by transfection with the normal rat gene could reverse the phenotype of IUGR-hypoxia and con-

Fig. 7. Expression of Kv1.5 in cultured PASMCs from IUGR and control rats exposed to hypoxia in vitro. A: representative immunoblots of GFP in cultured PASMCs demonstrate the transfection efficiency. B: representative immunocytochemistry photographs show Kv1.5 (red) expression in control and IUGR rat PASMCs with or without transfection of KCNA5 exposed to 48 h hypoxia or normoxia (scale bars = 40 μm). C: summary plots of Kv1.5 fluorescence units were normalized to the α-SMA fluorescence units (green). Data are presented as means ± SE. *P < 0.05 compared with their respective control-hypoxia group. Cells were dispersed from 4 SD rats/group.

Fig. 8. Kv1.5 overexpression restored Kv currents in IUGR-hypoxia PASMCs. 4-AP-sensitive Kv currents of PASMCs of IUGR-Kv1.5-hypoxia and control-Kv1.5-hypoxia rats transfected with plasmid KCNA5-EGFP were analyzed. Data are presented as means ± SE; n = 18 cells from 6 rats per group.
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trol-hypoxia PASMCs to normal and eliminate the difference between the two groups. Additionally, current restoration in IUGR-hypoxia PASMCs was induced by Kv1.5 overexpression in PASMCs. Together, these suggested that gene therapy might be a target therapy in human IUGR with PHT. Whether the upregulation of Kv1.5 in PASMCs is beneficial or mal-adaptive throughout the life span remains unclear. Archer and colleagues (28) demonstrated that rats overexpressing Kv1.5 by airway-specific administration using gene transfer were resistant to PHT for 28 days.

Our findings that exogenous gene transfer could elevate the expression of Kv1.5 in IUGR PASMCs not only provided evidence for a molecular target underlying vascular dysfunction in offspring of restrictive diet pregnancy but also suggested that this mechanism may be related to abnormal DNA modification during pregnancy (30). In rats, maternal food restriction during pregnancy increases oxidative stress in the placenta (34), a condition that is known to alter DNA methylation (37) and may result in changes in gene expression, which are maintained throughout the life span (12).

In conclusion, our observations show that Kv1.5 is a molecular target in exaggerated CH-PHT originating from IUGR. The inhibition of Kv1.5 in regulating the vascular response to chronic hypoxia at adulthood is enhanced in IUGR. However, the upstream mechanism in this phenomenon requires further investigation. Future studies regarding how gene expression of Kv1.5 is regulated in IUGR of exaggerated CH-PHT will be needed to address this issue in more detail.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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