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Gene expression profiling of sex differences in HIF1-dependent adaptive cardiac responses to chronic hypoxia

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Bohuslavová R, Kolář F, Kuthanová L, Neckář J, Tichopád A, Pavlinkova G. Gene expression profiling of sex differences in HIF1-dependent adaptive cardiac responses to chronic hypoxia. *J Appl Physiol* 109: 1195–1202, 2010. First published July 15, 2010; doi:10.1152/japplphysiol.00366.2010.—Although physiological responses to chronic hypoxia, including pulmonary hypertension and right ventricular hypertrophy, have been well described, the molecular mechanisms involved in cardiopulmonary adaptations are still not fully understood. We hypothesize that adaptive responses to chronic hypoxia are the result of altered transcriptional regulations in the right and left ventricles. Here we report results from the gene expression profiling of adaptive responses in a chronically hypoxic heart. Of 11 analyzed candidate genes, the expression of seven and four genes, respectively, was significantly altered in the right ventricle of hypoxic male and female mice. In the transcriptional profile of the left ventricle, we identified a single expression change in hypoxic males (*Vegfa* gene). To directly test the role of HIF1, we analyzed the expression profile in *Hif1a* partially deficient mice exposed to moderate hypoxia. Our data showed that *Hif1a* partial deficiency significantly altered transcriptional profiles of analyzed genes in hypoxic hearts. The expression changes were only detected in two genes in the right ventricle of *Hif1a*$^{+/-}$ males and in one gene in the right ventricle of *Hif1a*$^{+/-}$ females. First, our results suggest that hypoxia mainly affects adaptive expression profiles in the right ventricle and that each ventricle can respond independently. Second, our findings indicate that HIF1a plays an important role in adaptive cardiopulmonary responses and the dysfunction of HIF1 pathways considerably affects transcriptional regulation in the heart. Third, our data reveal significant differences between males and females in cardiac adaptive responses to hypoxia and indicate the necessity of optimizing diagnostic and therapeutic procedures in clinical practice, with respect to sex.

hypoxia inducible factor-1α; hypoxia; gene expression profiling; vascular endothelial growth factor-A; cardiopulmonary adaptations; transcriptional regulation; quantitative RT-PCR

CHRONIC HYPOXIA is associated with many cardiopulmonary diseases or with prolonged stay at high altitude. Hypoxia induces adaptive changes at systemic and cellular levels, which have a profound effect on the morphology and function of the cardiopulmonary system, including pulmonary hypertension with the remodeling of the pulmonary arterioles and right ventricular (RV) hypertrophy. These changes may ultimately lead to heart failure. Whether the adaptive myocardial changes are beneficial or detrimental is determined by the duration and severity of hypoxic exposure.

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Although physiological and pathophysiological responses to hypoxia have been described quite well (10, 23, 25), the molecular mechanisms involved in cardiac adaptations are still not fully understood. A variety of experimental studies have demonstrated that hypoxia affects many processes and pathways in the heart, including apoptosis (35), regulation of protein synthesis (9, 12), metabolism (18, 32), and transcriptional regulation, mainly by hypoxia-inducible factors (HIF) (30). HIF1 is a primary transcriptional regulator of hypoxia-induced cellular and systemic responses, and it activates a large number of target genes (~70) that are involved in many different cellular processes, such as cell proliferation, angiogenesis, metabolism, and apoptosis (31). Apart from the HIF family, hypoxia activates a number of other transcription factors (reviewed in 15), such as p53, AP-1, NF-κB, and GATA2 (37).

The regulation of gene expression and physiological responses to hypoxia are tissue specific. Hypoxia has opposite effects on systemic and pulmonary circulation, leading to vasodilatation in the systemic but vasoconstriction in the pulmonary vascular bed. Although two ventricles are influenced by tissue hypoxia, only the RV is exposed to increased pressure load. Consequently, chronic hypoxia is associated with the structural remodeling of pulmonary vessels and the development of RV hypertrophy. These adaptive changes are associated with altered gene expression within the heart (9, 12). Furthermore, clinical as well as experimental data show a significant difference between males and females in the cardiovascular responses (24, 26, 27). Although new experimental data analyzing sex differences in cardiovascular responses have recently been published, the underlying molecular mechanisms are still unknown.

To identify differences in the regulation of gene expression in the heart and differences between males and females adapted to a relatively moderate degree (12% O_2) of chronic continuous hypoxia (CCH), we have used mouse models and quantitative RT-PCR. To analyze the role of HIF1 pathways in responses to chronic hypoxia, we also performed gene expression profiling of *Hif1a* partially deficient male and female mice. Our results show that gene expression was differently regulated in the right and left ventricles and that it was significantly affected by hypoxia, sex, and *Hif1a* partial deficiency.

MATERIALS AND METHODS

Animal model. The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals,* published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996). The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of

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Sciences of the Czech Republic. All experiments were performed with male and female littermate mice that were either wild type (*wt*) or heterozygous *Hif1a* knock-out ($Hif1a^{+/-}$) that were on average 13 wk old at the start of the study. The heterozygous *Hif1a* mutants have the $Hif1a^{tm1jhu}$ mutant allele in which exon 2, encoding the bHLH domain of *Hif1a* gene, has been replaced by intragenic deletion with a neomycin resistance (neo^R) gene (13). The heterozygous *Hif1a* deficient mice showed a partial loss of HIF-1α protein expression levels (5, 28, 40). We obtained the *Hif1a* deficient mice on a mixed C57B6/129 genetic background from Dr. Gregg L. Semenza, Johns Hopkins University School of Medicine. To unify the genetic background, we backcrossed $Hif1a^{+/-}$ C57B6/129 mice onto the inbred FVB mouse strain (strain code 207, Charles River) for at least seven generations. The heterozygous *Hif1a* deficient mouse colony was bred and maintained in our laboratory. Offspring of $wt \times Hif1a^{+/-}$ mating were genotyped by PCR, using genomic DNA isolated from tails and amplifying neomycin (*Neo*) and *Hif1a* exon 2 sequences (13). Both *Neo* (463 bp) and *Hif1a* (317 bp) sequences were amplified from DNA of $Hif1a^{+/-}$ mice, whereas only *Hif1a* sequences were amplified from DNA of *wt* mice ($Hif1a^{+/+}$), respectively. The sequences of *Neo* primers were 5′-ACTGGCTGCTATTGGGCGAAGTG-3′ and 5′-GTAAAGCACGAGGAAGCGGTCAG-3′. Conditions for PCR were 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s, for 40 cycles. The sequences of *Hif1a* exon 2 primers were 5′-TGTAGTCTCCTGCTA-AAAG-3′ and 5′-TTATTCGAGTTAAGACAAAC-3′. Conditions for PCR were 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s, for 40 cycles.

Experimental protocol. Experimental mice were housed in a controlled environment (23°C; 12:12-h light-dark cycle) with free access to water and standard chow diet. Animals were adapted to moderate continuous normobaric hypoxia ($F_{I_{O_2}}$ 0.12) in a small room (5.6 m^3) supplied with three hypoxic generators (Everest Summit, Hypoxico, NY) for 4 wk. The concentration of oxygen was continuously monitored. An access compensatory hypoxic chamber prevented the occurrence of any reoxygenation during regular animal maintenance. Normoxic controls were kept under the same conditions, with the exception of having normal room air ($F_{I_{O_2}}$ 0.21). At the end of the adaptation period, hypoxic mice as well as normoxic controls were anesthetized with 2% isoflurane (Aerrane, Baxter SA) to measure pulmonary blood pressure. A fluid-filled catheter connected to an external pressure transducer (Bpr-02, Experimetria) was introduced into the RV via the right jugular vein. RV systolic pressure was averaged from three measurements within a 5-min interval, each comprising 10 cardiac cycles. Hematocrit was measured in the mixed venous blood taken from the RV catheter. Hearts were then rapidly excised, washed in sterile cold (0°C) saline, and dissected. The RV, the left ventricle (LV), the interventricular septum, and both atria were weighed.

Quantitative real-time PCR. RNA was isolated from the LV and RV of the individual hypoxic and normoxic adult mice (8 individual samples/each group) by Trizol (Invitrogen). The concentration of extracted RNA was quantified using NanoDrop. Quantitative real-time PCR (qRT-PCR) was performed using a LightCycler 480 Real-Time PCR system (Roche, Roche Applied Science, Mannheim, Germany) on cDNA samples. RNA samples (1 μg) were subjected to reverse transcription using Superscript II (Fermentas). cDNA was diluted 10×, and 4 μl was added to 6 μl of SyberGreen JumpStart Tag ReadyMix (Sigma). The PCR reactions were run with the initial AmpliTaq activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and for 60 s at 60°C. Values for detection above threshold level (Cq) for each gene were determined relative to measurements of a control gene in independent reactions with aliquots of the same sample. A proper reference gene was identified by running a panel of 12 potential endogenous control genes (TATAA Biocenter AB, Göteborg, Sweden) commonly used in gene expression studies, including *Gapdh, Tubb5, Actb, 18S rRNA*, etc. Hypoxanthine-guanine phosphoribosyltransferase1 (*Hprt1*) gene was selected as the best reference gene for our analyses due to its exhibiting the most constant expression among our representative test samples. Normalized Cq values ($\Delta Cq = Cq_{GENE} - Cq_{Hprt1}$) were compared between groups of hypoxia-exposed and control mice using an unpaired two-tailed *t*-test with an assumption of unequal variance (GraphPad Prism 4, GraphPad Software, San Diego, CA). The relative expression ratio of a target gene was computed, based on its real-time PCR efficiencies (*E*) and the crossing point (Cq) difference (Δ) of hypoxic sample vs. a control/normoxic sample. To compare the expression of each gene between its normoxic and hypoxic states, the ΔΔCq was determined: $\Delta\Delta Cq = \Delta Cq_{Hypoxia} - \Delta Cq_{Normoxia}$. The efficiency (E) for each reaction was derived from the slope of the linear portion of the amplification reaction (14). The relative amount of a target gene = $E^{-\Delta\Delta Cq}$. QRT-PCR data were analyzed using the GenEX5 program (http://www.multid.se/genex/). Primer sets were designed to exclude amplification of potentially contaminating genomic DNA by positioning the amplicons across exon-exon junctions. Primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3/). As much as possible, all primers were designed to have similar properties so that PCRs for different genes could be performed in the same run. Primers were selected according to the following parameters: length between 18 and 24 bases, melting temperature (Tm) between 58° and 60°C, and G + C content between 40 and 60% (optimal 50%). Primer sequences are presented in Table 1.

Statistical methods. The SAS system for Windows v. 9.2 was used to perform the statistical analysis. The proc GLM was employed to study the effect of the treatment condition (normoxia vs. hypoxia) as the main factor, also considering, however, the factors of gene deficiency ($Hif1a^{+/-}$ vs. *wt*), sex, and type of tissue (RV and LV). Eventually, all interactions of the main factor were also considered in the model, including interactions with sex, gene deficiency, and type of tissue. The type III sum of squares and its associated *P* value were considered for every factor and interaction within our study. The estimated least-squares means for all levels of factors and interactions were obtained and the associated *P* values calculated. To visualize the effects over all genes, for the factors of sex, genotype, and tissue type, we used a heat map (see Fig. 2).

RESULTS

Body and heart mass, pulmonary blood pressure, and hematocrit. After 4 wk of CCH, the *wt* males had a significantly lower body mass than the normoxic controls, whereas the body mass of hypoxic $Hif1a^{+/-}$ males remained unchanged compared with the $Hif1a^{+/-}$ males in normoxia (Table 2). The body mass of $Hif1a^{+/-}$ males was significantly different compared with *wt* males (28.7 ± 0.4 g vs. 24.9 ± 0.5 g, $P < 0.001$ by unpaired *t*-test). There was no difference in body mass between *wt* and $Hif1a^{+/-}$ females or between females under normoxic and hypoxic conditions. Hematocrit increased in both genotypes, $Hif1a^{+/-}$ and *wt,* and in both sex groups; however, the differences were more significant in males ($P \leq 0.001$) than in females ($P = 0.03$; Table 2). There was no significant effect of genotype on hematocrit levels after 4 wk of hypoxia. The development of RV hypertrophy in response to CCH was significant in *wt* males ($P = 0.03$). Surprisingly, change in the RV mass of *wt* females was not significant ($P = 0.08$). Although the mass ratio of RV to LV was increased in males ($P < 0.0009$) and females ($P < 0.03$), the effect of CCH was more evident in males (Table 2). These results suggest that basic adaptive physiological responses to chronic hypoxia are, to a certain extent, significantly different between the two sexes. CCH-induced RV hypertrophy was also detected in hypoxic $Hif1a^{+/-}$ males ($P = 0.007$) and females ($P = 0.002$).

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Table 1. *Primer sequences*

Gene Symbol	Gene Name	RefSeq ID	Forward Primer Sequence	Reverse Primer Sequence
Hif1a	Hypoxia inducible factor alpha	NM_010431	5′-CAGTACAGGATGCTTGCCAAAA-3′	5′-ATACCACTTACAACATAATTCACACACACA-3′
Pdgfra1	Alpha platelet-derived growth factor receptor	NM_011058	5′-GTCCCCATGCTTGAAAGGAA-3′	5′-CATCGTCCGAAAGGAGGTTTT-3′
Slc2a1	Solute carrier family 2, facilitated glucose transporter 1	NM_011400	5′-GGGCATGTGCTTCCAGTATGT-3′	5′-ACGAGGAGCACCGTGAAGAT-3′
Casp1	Caspase 1, apoptosis-related cysteine peptidase	NM_12362	5′-TGGTCTTGTGACTTGGAGGAC-3′	5′-AGAAACGTTTTGTCAGGGTCA-3′
Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	NM_001013367	5′-CCTTCGGGAAAGTGAAGGT-3′	5′-ATTTTTCCCACCACGTCAAG-3′
Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	NM_009761	5′-CCTCGTCTTCCATCCACAAT-3′	5′-TTCTTGTGGTGAAGGGCTGT-3′
Ldha	Lactate dehydrogenase A	NM_010699	5′-GCACTGACGCAGACAAGG-3′	5′-TGATCACCTCGTAGGCACTG-3′
Hprt1	Hypoxanthine guanine phosphoribosyl transferase	NM_013556	5′-GCTTGCTGGTGAAAAGGACCTCTCGAAG-3′	5′-CCCTGAAGTACTCATTATAGTCAAGGGCAT-3′
Gata2	GATA binding protein 2	NM_008090	5′-CCCAAGCTTCGATTCTGTGT-3′	5′-TTGACTCAGCACAATCGTCTC-3′
Igf2	Insulin-like growth factor 2	NM_001122736	5′-CGCGGCTTCTACTTCAGC-3′	5′-GGGGTGGCACAGTATGTCTC-3′
Flt1	FMS-like tyrosine kinase 1	NM_010228	5′-GAGGAGGATGAGGGTGTCTATAGGT-3′	5′-GTGATCAGCTCCAGGTTTGACTT-3′
Vegfa	Vascular endothelial growth factor A	NM_001025250	5′-ACTGGACCCTGGCTTTACTG-3′	5′-TGGGACTTCTGCTCTCCTTC-3′

The differences in RV mass between *wt* and *Hif1a*$^{+/-}$ genotypes, adapted to hypoxia, were not significant.

To determine whether the RV hypertrophy observed in hypoxic mice was associated with pulmonary hypertension, we measured pressure in the RV (Fig. 1). RV systolic pressure was significantly increased in hypoxic *wt* males (33 ± 3.0 mmHg) and females (32.6 ± 2.9 mmHg) compared with normoxic *wt* males (26.9 ± 5.0 mmHg) and females (26.5 ± 2.5 mmHg). In contrast, there were no significant differences in RV pressure between normoxic and hypoxic *Hif1a*$^{+/-}$ mice in either sex group. The differences between *Hif1a*$^{+/-}$ and *wt* genotypes in response to moderate CCH were highly significant in males ($P = 0.005$) and females ($P = 0.02$). Our results suggest that partial deficiency of *Hif1a* significantly alters the development of hypoxia-induced pulmonary hypertension. Since *Hif1a* partial deficiency reduces the increase in RV pressure associated with hypoxia, HIF1-regulated pathways may be directly involved in the development of pulmonary hypertension. Furthermore, our data suggest that RV hypertrophy is partially regulated by different molecular mechanisms and that pulmonary hypertension is not the sole determinant of RV hypertrophy in hypoxic animals, particularly in females.

Gene expression profiling. To explore the tissue-specific changes induced by adaptation to hypoxia in gene expression, we analyzed the expression of selected genes, coding proteins involved in the regulation of metabolism (AMP-activated pro-

Table 2. *Changes in body and heart mass, and hematocrit*

Group	*n*	BM, g	LV, mg	RV, mg	RV/LV	Hct, %
wt males						
Normoxia	10	27.5 ± 0.4	59.9 ± 1.0	21.1 ± 0.8	0.353 ± 0.012	44.7 ± 1.1
Hypoxia	10	24.9 ± 0.5*	51.9 ± 1.5*	24.6 ± 1.2*	0.479 ± 0.025*	52.9 ± 0.3*
wt females						
Normoxia	8	20.5 ± 0.5	49.2 ± 1.1	17.7 ± 0.4	0.363 ± 0.014	47.1 ± 0.6
Hypoxia	10	21.6 ± 0.5	45.0 ± 1.6	19.6 ± 0.9	0.439 ± 0.026*	49.8 ± 0.7*
Hif1a$^{+/-}$ males						
Normoxia	9	27.5 ± 1.0	58.8 ± 2.5	20.7 ± 1.3	0.353 ± 0.016	46.7 ± 0.8
Hypoxia	10	28.7 ± 0.4	54.7 ± 1.3	24.9 ± 0.6*	0.456 ± 0.012*	52.0 ± 0.6*
Hif1a$^{+/-}$ females						
Normoxia	10	22.2 ± 0.5	48.2 ± 1.3	16.9 ± 0.5	0.354 ± 0.014	46.2 ± 1.3
Hypoxia	11	22.4 ± 0.8	45.3 ± 2.0	20.6 ± 1.0*	0.460 ± 0.023*	50.3 ± 1.0*

Data are means ± SE from indicated number of animals (*n*) in each group. BM, body mass; LV, left ventricular mass; RV, right ventricular mass; RV/LV, right-to-left ventricular index; Hct, hematocrit; wt, wild type. Differences between hypoxia-exposed and controls were tested for statistical significance using an unpaired 2-tailed *t*-test (GraphPad Prism 4). *$P < 0.05$ vs. corresponding normoxic group.

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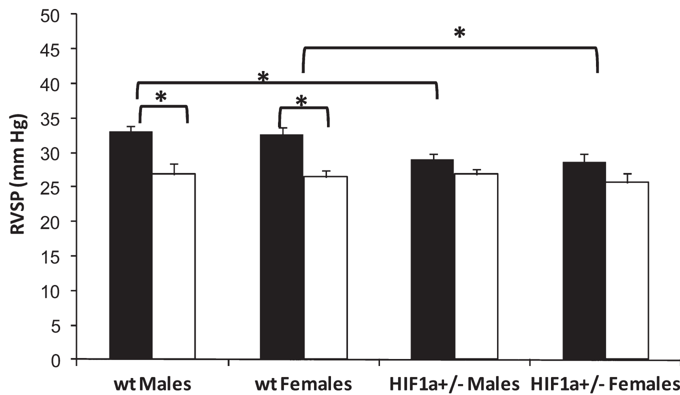

Fig. 1. Changes in right ventricular systolic pressure (RVSP) after 4 wk of continuous hypoxia. The pressure was significantly increased in *wt* males ($P < 0.0064$) and *wt* females ($P < 0.0003$) exposed to hypoxia (solid bars) compared with normoxic controls (open bars). The pressure of *Hif1a*$^{+/-}$ males and females exposed to 4 wk of hypoxia was not significantly affected compared with normoxic *Hif1a*$^{+/-}$ groups. The differences between hypoxic *Hif1a*$^{+/-}$ and *wt* males were statistically significant ($P < 0.005$). Similarly, hypoxic *Hif1a*$^{+/-}$ and *wt* females responses were significantly different ($P < 0.02$). *$P \leq 0.05$, using an unpaired 2-tailed *t*-test (GraphPad Prism 4).

tein kinase, *Prkaa*), apoptosis (caspase-1, *Casp1*), growth factor receptors (platelet-derived growth factor receptor alpha, *Pdgfra*), and transcription factors (*Hif1a*; GATA binding protein 2, *Gata2*) in the LV and RV. We also analyzed the gene expression of six HIF1 target genes, involved in glucose metabolism (lactate dehydrogenase A, *Ldha*; glucose transporter 1, *Slc2a1*), vasculogenesis and angiogenesis (vascular endothelial growth factor A, *Vegfa*; Vegf receptor-1, *Flt1*), apoptosis (BCL2/adenovirus E1B interacting protein 3-like, *Bnip3l*), and cell proliferation (insulin-like growth factor 2, *Igf2*) to directly evaluate HIF1-pathway responses to CCH. To further investigate the functional role of HIF1a, we analyzed hypoxia-induced gene expression changes in *Hif1a*$^{+/-}$ males and females. The gene expression was differentially regulated between the LV and RV of the heart (Fig. 2). In the RV, *wt* males and females responded to moderate CCH with the increased expression of the majority of the measured genes, while *Hif1a*$^{+/-}$ female and male responses were limited to one and two genes, respectively. In contrast to the RV expression patterns, in the LV, the largest increase in RNA levels was detected in *Hif1a*$^{+/-}$ males and the smallest expression changes were detected in *wt* males adapted to CCH. The significant interaction of sex and gene deficiency under hypoxic conditions was detected, with $P < 0.001$ and $P < 0.0001$, respectively (SAS system, proc GLM). Our results show that gene expression regulation in chronically hypoxic hearts was tissue specific and was significantly influenced by sex and *Hif1a* partial deficiency.

Sex differences. To test whether gene expression in the heart is regulated by CCH in a sex-dependent manner, the gene expression profiles in the RV and LV of female and male mice, adapted to CCH, were compared. On average, the relative expression of the measured genes was higher in males than in females, after 4 wk of hypoxia (Fig. 3). Of 11 measured genes, the expression of seven genes was significantly altered in hypoxic *wt* males, while the expression of four genes was significantly changed by hypoxia in the RV of *wt* females. The CCH transcriptional adaptive responses in the RV of *Hif1a* deficient females and males were detected in one and two genes, respectively (Fig. 3). Unexpectedly, *Hif1a*$^{+/-}$ male and female mutants responded to hypoxia with major transcription changes in the LV. The expression of nine genes was significantly altered in the *Hif1a*$^{+/-}$ male LV, whereas four genes were affected in the LV of *Hif1a*$^{+/-}$ females. The main difference between male and female responses to CCH was the expression of *Vegfa* mRNA. We designed the *Vegfa* primers for our qRT-PCR experiments to detect all primary *Vegfa* isoforms: VEGF 120, VEGF 164, and VEGF 188. Our results show that males responded to CCH with a significant downregulation of *Vegfa* mRNA in the RV, after 4 wk of adaptation. However, none of the hypoxic female groups showed any significant changes in *Vegfa* mRNA levels compared with the normoxic groups. Our results suggest that the expression of *Vegfa* in CCH exposure is also differently regulated based on sex.

Effect of genotype on gene expression changes induced by hypoxia. Similar to the smaller physiological changes, the transcriptional responses of *Hif1a* heterozygous mutants in the RV were less significant than in *wt* males, after 4-wk adaptation to CCH. Of 11 measured genes, the expression of seven genes was significantly altered by hypoxia in *wt* males, while the expression of two genes was significantly altered in hypoxic *Hif1a*$^{+/-}$ males. Of seven affected genes in the RV of *wt* hypoxic males, five genes were HIF1-mediated target genes (*Ldha, Flt1, Slc2a1, Igf2,* and *Vegfa*). In contrast, only one HIF1 target gene (*Vegfa*) was altered by 4-wk hypoxia in the RV of *Hif1a*$^{+/-}$ males (Fig. 3). Interestingly, the largest hypoxia-induced expression changes were detected in the LV of *Hif1a*$^{+/-}$ males. The mRNA expression of nine genes was significantly altered by hypoxia in the LV of *Hif1a*$^{+/-}$ males, whereas significantly altered response was detected in only one gene of 11 analyzed genes in the of *wt* male LV. The *Hif1a*$^{+/-}$ females responded to hypoxia with an increased expression of mRNA of just one gene, *Casp1*, in the RV, whereas *wt* females showed significant increase in the expression of four genes in the RV after CCH adaptation. Intriguingly, the *Hif1a*$^{+/-}$ males and females responded to hypoxia with increased *Casp1* expression, although the levels of *Casp1* mRNA were not significantly affected in hypoxic *wt* mice. The expression of *Casp1* was significantly increased, 1.5-fold on average, in the two heart ventricles of *Hif1a*$^{+/-}$ by CCH compared with normoxic *Hif1a*$^{+/-}$ controls. These results show that the regulation of gene expression is significantly affected by *Hif1a* deficiency and that the hypoxic responses are differently regulated in the two heart ventricles.

DISCUSSION

Gene expression profiling of adaptive responses to hypoxia. In the present study, we found that an adaptation to moderate CCH was associated with a substantially differential response between the RV and LV myocardium. Although we analyzed relatively a small number of genes, we established that the expression of the majority of investigated genes (with a specific focus on HIF1 pathways) increased only in the RV compared with the LV of *wt* males and females. This differential expression can be attributed to the fact that both heart ventricles are exposed to different changes of workload under conditions of chronic hypoxia, while only the RV is challenged

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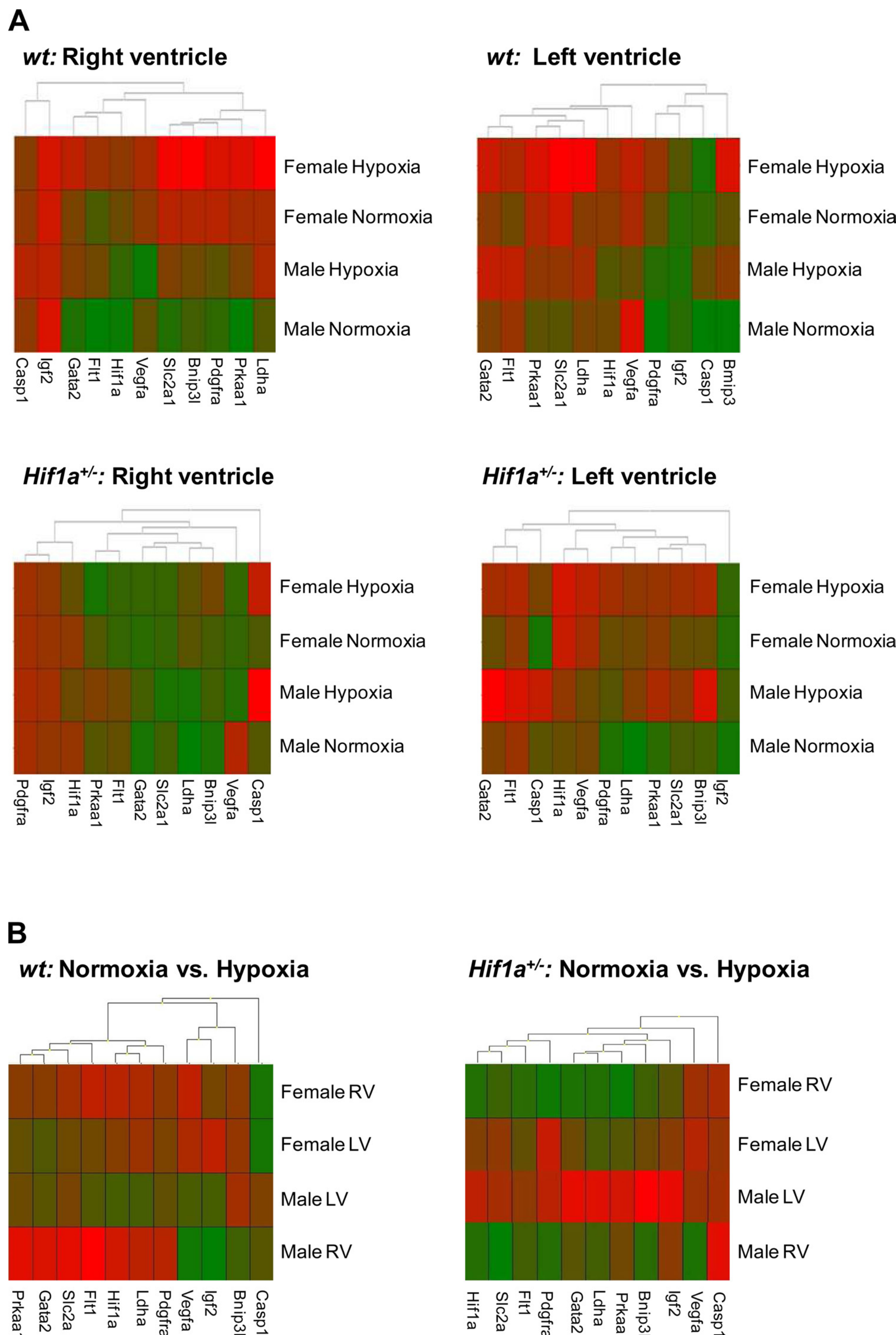

Fig. 2. Gene expression profiles/heat map. An expression profile of 11 individual genes (columns) across different groups of samples (rows) was visualized through hierarchical agglomerate clustering (GenEx5). The expression levels of each gene were measured in 8 different individual samples for each group, and their averages were displayed as a single cell in the heat map. *A*: the colors used in the *A* heat maps correspond to the ΔCq, which is the value for detection above threshold level (Cq) for each gene normalized to the levels of a reference gene (*Hprt1*) in the same sample. Boxes in bright red represent samples expressing genes at the highest levels; bright green represents genes with the lowest expression. *B*: the colors used in the *B* heat maps correspond to changes in gene expression between normoxic and hypoxic conditions. Boxes in bright red represent samples with the largest increase in gene expression; bright green represents genes with the largest decrease in gene expression between normoxia and hypoxia. These graphic representations show that expression profiles differ considerably between the right (RV) and left ventricles (LV) and are significantly affected by sex and *Hif1a* partial deficiency. Abbreviations: lactate dehydrogenase A (*Ldha*), BCL2/adenovirus E1B interacting protein 3-like (*Bnip3l*), hypoxia-inducible factor 1a (*Hif1a*), insulin-like growth factor 2 (*Igf2*), Vegf receptor-1 (*Flt1*), platelet derived growth factor receptor alpha (Pdgfra), protein kinase AMP-activated (*Prkaa*), glucose transporter 1 (*Slc2a1*), Caspase-1 (*Casp1*), GATA binding protein 2 (*Gata2*), vascular endothelial growth factor A (*Vegfa*).

by pulmonary hypertension. Therefore, our data suggest that the majority of analyzed genes were regulated by pressure load. In our model, the adaptation to moderate CCH was associated with the transcriptional upregulation of metabolic genes in the RV myocardium of *wt* males and females, including glycolytic enzyme *Ldha*, glucose transporter *Slc2a1*, and cardiac metabolism regulator *Prkaa* (Fig. 3). The reprogramming of cardiac energy metabolism to facilitate glucose utilization has been associated with the development of myocardial hypertrophy (1, 18, 32). We also identified adaptive changes in the transcriptional profiles of nonmetabolic genes involved in the regulation of cell proliferation (*Igf2*), apoptosis (*Bnip3l*), vasculogenesis and angiogenesis (*Vegfa* and *Flt1*), and transcription (*Gata2*). The transcription factor GATA2 is one of the regulatory elements required for a functional hypoxia-responsive element. The complementing roles of GATA2 and HIF1a in transcriptional regulation and their functional and physical relationship have been shown in previous reports (34, 37). In our experiments, *Gata2* mRNA was upregulated in the RV of hypoxic *wt* males and females, suggesting an increased

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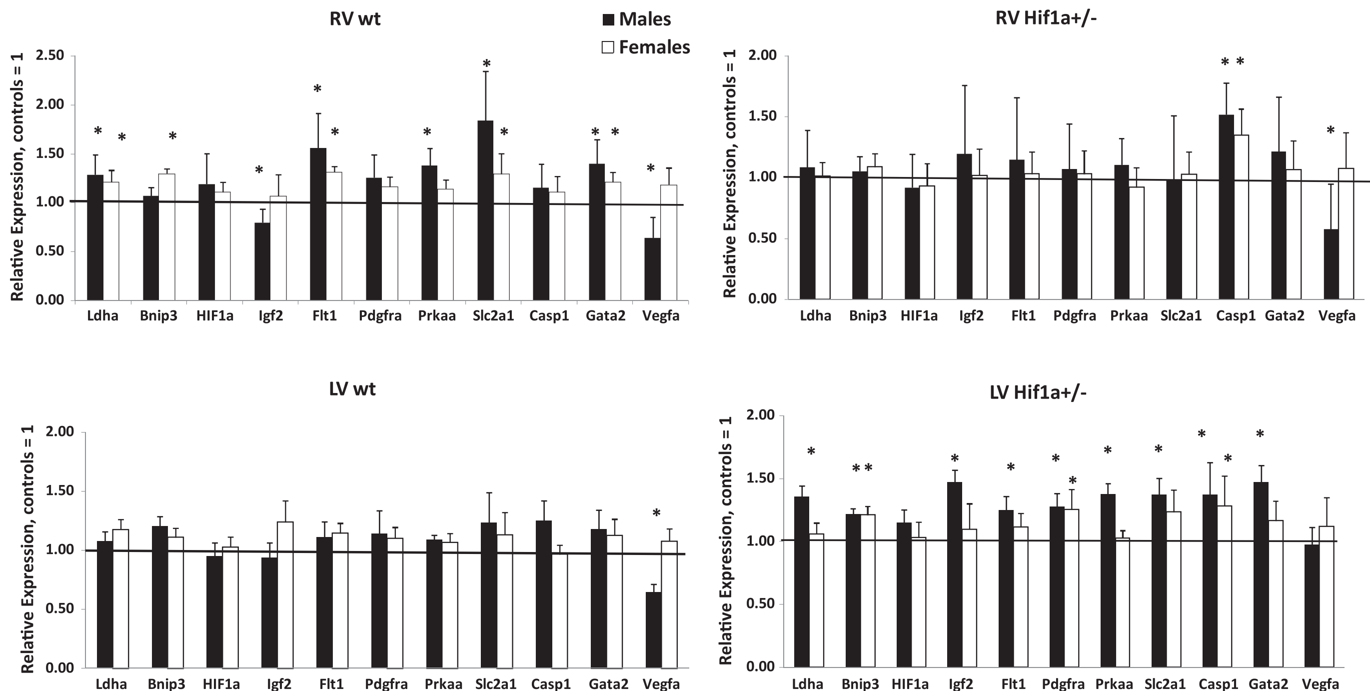

Fig. 3. Quantitative real-time PCR analysis of expression profiles in chronically hypoxic hearts of males and females. Total RNA was extracted from the LV and RV of *wt* and $Hif1a^{+/-}$ males and females after 4 wk of continuous hypoxia. Specific mRNAs were quantified, as described in MATERIALS AND METHODS. The data represent an expression of mRNA relative to normoxic controls, normalized by the housekeeping mRNA of *Hprt1*. Results are expressed as means ± SE (each experiment in duplicate; $n = 8$ per group). Differences in normalized Ct values between hypoxia-exposed and controls were tested for statistical significance using an unpaired 2-tailed *t*-test (GraphPad Prism 4). *Significant difference between normoxic and hypoxic groups at $P < 0.05$.

level of activation of HIF pathways via hypoxia-responsive element. Although the expression of *Hif1a* mRNA was not significantly increased in the analyzed hypoxic hearts, increased HIF1 transcriptional activity was evidenced through the altered mRNA expression of HIF1 transcriptional targets (*Flt1, Bnip3l, Ldha, Slc2a1, Igf2, Vegfa*). Our data are supported by several in vivo studies which show that *Hif1a* mRNA levels are only transiently increased in response to hypoxia, returning to basal levels within 24 h, and that the oxygen-sensitive α-subunit of HIF1 is predominantly regulated at the level of protein stability (3, 4, 36, 38). Furthermore, our findings suggest that persistent pulmonary hypertension primarily affects gene expression adaptive profile in the RV and that each ventricle can respond independently. Our data underscore the crucial importance of analyzing changes in gene expression separately from different regions of the heart because analyses of changes in the whole heart lead, by necessity, to inaccurate results and conclusions.

Vegfa mRNA expression changes. In our studies, significant changes were observed in the gene expression of *Vegfa*, a key target gene of local adaptation to hypoxia. Systemic hypoxia is one of the stimuli for in vivo induction of *Vegfa* expression, which affects vasculogenesis and angiogenesis in many organs. The expression of *Vegfa* is differentially regulated, based on the specific cell type, specific organ, severity of hypoxia, time of hypoxic exposure, and species reaction (22, 29). In addition, our results show that the expression of *Vegfa* in CCH exposure is also differently regulated based on sex. The time kinetics of *Vegfa* expression during hypoxic exposure is based on the formation of new capillaries, which cause an increase in oxygenation with a correlated downregulation of *Vegfa* expression, thus limiting the hypoxic stimulus (22). Previous studies have shown that initial increase in the expression of *Vegfa* mRNA in hypoxia is followed by the downregulation of *Vegfa* expression (7, 22), which corresponds with our current data. Furthermore, of 11 analyzed genes, *Vegfa* was the only gene with significant expression changes detected in both heart ventricles. These data suggest that the reduction of *Vegfa* mRNA in *wt* males after 4 wk of CCH was not regulated through an increased pressure load, but through other compensatory mechanisms, which were affected by sex.

Differences between male and female responses to hypoxia. On average, the relative expression of measured genes was smaller in *wt* females than in *wt* males. These smaller changes in gene expression may be correlated with a smaller effect of CCH on the body mass and the development of RV hypertrophy in *wt* females. These significant sex differences in adaptation to hypoxia may reflect the different sensitivity of males and females to oxygen deprivation and other stresses. Sex dependence in the development of cardiac hypertrophy and the reduced risk for cardiovascular diseases in females have also been reported in both epidemiological and experimental studies (reviewed in 24, 26). An additional possibility is that the temporal gene expression profile of hypoxic females may be different from the temporal profile of hypoxic males. This assumption is supported by recent studies, using neonatal mice (12). In this model, the fold change of the measured genes was also higher in males than females after 4-wk hypoxia; however, the relative gene expression was higher in females after 1-wk hypoxia (12). Taken together, our data reveal significant differences between males and females in cardiac adaptive responses to hypoxia and lend support to the necessity of opti-

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mizing diagnostic and therapeutic procedures in clinical practice with respect to sex.

Changes associated with Hif1a deficiency. Several in vitro and animal studies have shown that HIF1 is crucial for the maintenance of normal cardiac functions and that activation of HIF1-regulated pathways plays a cardiovascular protective role (2, 6, 8, 11, 16). In contrast, the chronic activation of these pathways may contribute to cardiac degeneration and progression toward heart failure, as reported for mice with cardiac myocyte-specific deletion of the von Hippel-Lindau protein (21). The chronicity and intensity of HIF1 pathways activation are major determinants of whether the responses are pathological or beneficial. In this study, *Hif1a*$^{+/-}$ males and females adapted well to relatively moderate CCH with significantly altered gene expression profiles compared with hypoxic *wt* mice. In contrast to hypoxic *wt*, the transcriptional responses were significantly increased in the majority of analyzed genes in the *Hif1a*$^{+/-}$ LV, whereas the expression changes induced by hypoxia were only detected in two genes in the RV of *Hif1a*$^{+/-}$ males and in one gene in the RV of *Hif1a*$^{+/-}$ females. This striking difference between gene expression of *wt* and *Hif1a* deficient mutant mice exposed to moderate CCH was an unexpected finding. Based on our data, sustained hypoxia activates predominantly adaptive transcriptional responses in the RV of *wt*, whereas gene expression in the LV is essentially maintained at the normoxic levels. Thus the significantly smaller pressure load on the RV of *Hif1a*$^{+/-}$ and/or the limited availability of *Hif1a* may result in normoxic mRNA levels in the RV of *Hif1a*$^{+/-}$ as a means of adaptation to CCH. However, we can only speculate about the conditions that primarily affect gene expression in the LV of hypoxic *Hif1a*$^{+/-}$ mice. It is unlikely that the expression changes in the partially *Hif1a* deficient LV are related to pressure load conditions as CCH may decrease rather than increase systemic peripheral resistance and blood pressure. This indication is in agreement with the unchanged LV mass in hypoxic *Hif1a*$^{+/-}$. Therefore, the potential compensatory mechanisms associated with dysfunction of HIF1 pathways, inducing the CCH-adaptive expression pattern in the hypoxic *Hif1a*$^{+/-}$ LV, remain to be identified.

Along with small changes in the adaptive expression profiles of analyzed genes in the RV, the changes in body mass and RV systolic pressure were significantly smaller in hypoxic *Hif1a*$^{+/-}$ than in hypoxic *wt*. Using *Hif1a* deficient mice, other studies have also shown that CCH induces a significantly smaller degree of pulmonary hypertension (33, 39) and impaired ventilatory responses (17) compared with *wt* mice. Heterozygous deficient *Hif1a* mice were protected against hypoxia-induced pulmonary vascular remodeling, resulting in the decreased muscularization of pulmonary arterioles (39). An important signal in eliciting cardiorespiratory responses to hypoxia, including pulmonary remodeling, is the release of reactive oxygen species (19, 20). Interestingly, the production of reactive oxygen species is impaired in *Hif1a*$^{+/-}$ mice exposed to hypoxia (28), which may also contribute to the observed phenotype.

Taken together with other studies, our results indicate that HIF1 plays an important role in the development of hypoxic pulmonary hypertension. Since the development of severe pulmonary hypertension is linked to eventual heart failure, the downregulation of *Hif1a* expression could play an important beneficial role and it could influence existing therapies. We have shown that dysfunction of HIF1 pathways massively affects transcriptional profiles of analyzed genes in hypoxic hearts of *Hif1a*$^{+/-}$ mutants. With an increasing knowledge of HIF1 function, HIF1 therapeutical potential is evident and, therefore, the modulation of HIF1 activity is a high-priority target for cardiac treatments. Thus a detailed understanding of HIF1 regulation in the heart is of clinical importance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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