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ORIGINAL ARTICLE

Chronic and intermittent hypoxia differentially regulate left ventricular inflammatory and extracellular matrix responses

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We evaluated the left ventricle (LV) response to hypoxia by comparing male Sprague Dawley rats exposed for 7 days to normoxia (control; $n=18$), chronic sustained hypoxia (CSH; $n=12$) and chronic intermittent hypoxia (CIH; $n=12$). Out of the 168 inflammatory, extracellular matrix and adhesion molecule genes evaluated, *Ltb*, *Cdh4*, *Col5a1*, *Ecm1*, *MMP-11* and *TIMP-2* increased in the LV (range: 87–138%), whereas *Tnfrsf1a* decreased 27%, indicating an increase in inflammatory status with CSH (all $P<0.05$). CIH decreased *Ltb*, *Spp1* and *Ccl5* levels, indicating reduced inflammatory status. While *Laminin β2* gene levels increased 123%, *MMP-9* and *fibronectin* gene levels both decreased 74% in CIH (all $P<0.05$). Right ventricle/body weight ratios increased in CSH ($1.1\pm0.1\,g\,g^{-1}$) compared with control ($0.7\pm0.1\,g\,g^{-1}$) and CIH ($0.8\pm0.1\,g\,g^{-1}$; both $P<0.05$). Lung to body weight increased in CSH, while LV/body weight ratios were similar among all three groups. With CIH, myocyte cross sectional areas increased 25% and perivascular fibrosis increased 100% (both $P<0.05$). Gene changes were independent of global changes and were validated by protein levels. MMP-9 protein levels decreased 94% and fibronectin protein levels decreased 42% in CIH (both $P<0.05$). Consistent with a decreased inflammatory status, HIF-2α and eNOS protein levels were 36% and 44% decreased, respectively, in CIH (both $P<0.05$). In conclusion, our results indicate that following 7 days of hypoxia, inflammation increases in response to CSH and decreases in response to CIH. This report is the first to demonstrate specific and differential changes seen in the LV during chronic sustained and CIH.
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INTRODUCTION

Hypoxia, exposure of the body to environments of low oxygen, can occur in response to variable patterns of low oxygen.[1] Hypoxia can be acute or chronic, depending on the length of exposure, and can be continuously sustained or intermittent, depending on the pattern of exposure.[2] The physiological and pathological responses to hypoxia differ depending on these characteristics, although the underlying mechanisms are not fully understood.

Sustained hypoxia occurs when oxygen levels fall from atmospheric levels of 21% to values that generally range from 8–12%. In humans, sustained hypoxia occurs in high altitude and in patients with chronic lung diseases such as chronic obstructive pulmonary disease and cystic fibrosis.[3] Estrada and Chesler[3] have previously shown that extracellular matrix (ECM) proteins, particularly collagen I, are elevated in the lungs of mice exposed to chronic sustained hypoxia (CSH). This increase was significant by 6 days after the hypoxic challenge was initiated, and collagen I was the earliest and largest gene changed in expression.

Intermittent hypoxia occurs when oxygen levels fall for brief episodes. In humans, intermittent hypoxia has been shown to occur during sleep apnea. Specifically, obstructive sleep apnea involves periods when breathing rates slow or cease.[4,5] The ensuing hypoxemia stimulates arterial chemoreceptors, which in turn activate the sympathetic nervous systems to restore breathing by arousing the individual. Patients with sleep apnea may repeat this pattern 30–50 times per hour during the sleep cycle. Several groups have previously shown that the rat model of chronic exposure to intermittent hypoxia (CIH) mimics many aspects of the arterial hypoxemia that accompanies sleep apnea.[6–8] Similar to humans with sleep apnea, rats exposed to CIH have elevated blood pressures and augmented sympathetic nervous system responses to acute exposures to hypoxia. Specifically, Sprague Dawley rats exposed to 7 days of intermittent hypoxia showed increased blood pressure, with an average increase in mean arterial pressure of 5.4 ± 1.0 mm Hg.[9] Further, blood pressure remained elevated throughout the day, even

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when the rats were not exposed to intermittent hypoxia. Knight *et al.*[10] previously showed an increase in mean arterial pressure in rats exposed to CIH during the light phase, as well as continued increase in mean arterial pressure during the normoxic dark phase, with no changes in heart rate. While the mechanisms for the persistence in high blood pressure is unknown, candidates include sympathetic nervous system stimulation and renin-angiotensin-aldosterone activation.[9]

Although hypoxia effects on lung and right ventricle have been well characterized, no one to date has used chronic sustained and chronic intermittent hypoxia (CIH) models to test the hypothesis that exposure to sustained versus intermittent hypoxia will yield a differential pattern of inflammatory and ECM gene changes in the left ventricle (LV). How changes in the lungs and right ventricle feed forward to alter LV structure and function are not well-described. In this study, we evaluated inflammatory and ECM changes that occur in the LV of Sprague Dawley rats exposed to chronic sustained *vs.* CIH of 7 days in duration.

MATERIALS AND METHODS

Rats

We used 42 male Sprague Dawley rats for this study, which was approved by the UTHSCSA Institutional Animal Care Program and conform to the Guide for the Care and Use of Laboratory Animals (National Research Council, Eighth Edition). Rats were divided into three groups; normoxic ($n=18$), CSH ($n=12$), and CIH ($n=12$). Normoxic rats were kept at 21% O_2 and CSH rats were kept at 10% O_2 for 7 days.[11,12] CIH rats were placed on an 8 h repeat of the following cycle, from 0800 to1600 hours each day for 7 days. Each cycle consisted of: decrease from 21 to 10% O_2 over 1 min; maintaining at 10% O_2 for 2 min; increase from 10 to 21% O_2 over 1 min and maintaining at 21% O_2 for 2 min. For the remaining 16 h per day, rats were kept at 21% O_2.[9] Following the last cycle on the 7th day, the rats were kept at 21% O_2 for 16 h before sacrifice.

Tissue collection/necropsy

The LV, right ventricle (RV) and lungs were collected and weighed individually. Each LV sample was cut transversely and divided into three sections. One section (the mid papillary section) was fixed in zinc-formalin for histology; one section was snap frozen for arrays (mRNA analysis) and one section was snap frozen for immunoblotting (protein analysis). With the exception of the array analyses, all samples were analyzed for each of the below described assays.

Histology

LV tissue from the mid-papillary region was embedded in paraffin and sectioned at 5 μm. One set of sections were stained with hematoxylin and eosin to evaluate myocyte cross-sectional areas and one set was stained with picrosirius red to measure the extent of collagen deposition.[13,14] For the picrosirius red staining, the slides were incubated with 0.05% Direct Red 80 (Sigma 365548, St Louis, MO, USA) in saturated picric acid for 10 min and washed in 0.5% acetic acid for 1 min.[15] Sections (5 random) were imaged and analyzed with Image-Pro Plus (Bethesda, MD, USA) as described previously.[16] Perivascular fibrosis was measured in the area surrounding the coronary artery and normalized to the cross sectional area of the artery to account for differences in artery diameters.

RT^2-PCR arrays

We evaluated RT^2-PCR levels for inflammatory, ECM and adhesion molecule genes (control $n=6$, CSH $n=5$ and CIH $n=6$). LV tissue was homogenized in TRIzol reagent (Invitrogen 15596026, Grand Island, NY, USA) and RNA was extracted according to the manufacturer's protocol. cDNA was synthesized with the RT^2 First Strand Kit (Qiagen 330401, Valencia, CA, USA) and prepared for the arrays with the SYBR Green qPCR Mastermix (Qiagen 330522). The mRNA expression of 168 genes from the ECM and adhesion molecules and the inflammatory cytokines and receptors arrays (Qiagen PARN-013A and PARN-011A) were assessed. Results were analyzed based on the $2^{-\Delta Ct}$ values, with normalization to five housekeeping genes (*Rplp1*, *Hprt1*, *Rpl13a*, *Ldha* and *Actb*).[17]

Immunoblotting

The LV protein fractions were extracted with 1 × phosphate-buffered saline in 1 × protease inhibitor cocktail (Roche 11836153001, Indianapolis, IN, USA) to obtain the soluble fraction. The samples were centrifuged at 1000 *g* for 5 min. The insoluble protein pellet was homogenized in Reagent 4 (Sigma C0356, Sigma) with 1 × protease inhibitor cocktail. Protein concentration was determined with a Bradford assay, and 10–40 ug total protein was loaded onto 26-well 4–12% Criterion Bis-Tris gels (Bio-Rad, Hercules, CA, USA). Each sample set was run on a total of two gels. Equal protein loading and transfer was verified using the Pierce Memcode Reversible Staining kit (Thermo Scientific 25480, Rockford, IL, USA) for nitrocellulose membranes.

Soluble and insoluble protein levels were quantified by immunoblotting with the following antibodies: anti-β_1 adrenergic receptor (β_1 AR; Novus NB100-92439, 1:500, Littleton, CO, USA), anti-angiotensin II type 1 receptor (AT_1R; Millipore AB15552, 1:1000, Billerica, MA, USA), anti-endothelial nitric oxide synthase (eNOS; Abcam ab66127, 1:500, Cambridge, MA, USA), anti-fibronectin (Fn; Millipore AB1954, 1:10 000), anti-hypoxia-inducible factor 1α (HIF-1α; Santa Cruz sc-10790, 1:500, Santa Cruz, CA, USA), anti-hypoxia-inducible factor 2α (HIF-2α; Abcam ab73895, 1:500), anti-integrin β_2 (itgb2, Novus R-10110100, 1:1000), anti-laminin β_2 (Lamb2; Novus NBP1-00904, 1:500), anti-laminin γ1 or C1 (Lamc1; Novus NBP1-19643, 1:500), anti-matrix metalloproteinase-9 (MMP-9; Abcam, ab38898, 1:1000), anti-tissue inhibitor of metalloproteinase-1 (TIMP-1; Sigma T8322, 1:1000) and anti-tissue inhibitor of metalloproteinase-2 (TIMP-2; Millipore AB801, 1:1000).

Molecular imaging software (ImageJ) was used to measure densitometry, which was normalized to the total protein densitometries obtained from the reversible protein stained membranes. Results are shown as normalized arbitrary units (AU). Rat lung, mouse tumor or mouse kidney tissue were used as positive controls on each blot.

Statistical analysis

A $P<0.05$ was considered significant. Data are represented as mean ± s.e.m. Samples were analyzed by ANOVA with a Student Newman–Keuls post-test. Statistical analyses were performed using In Stat (GraphPad Software, La Jolla, CA, USA).

RESULTS

Necropsy

As shown in Table 1, LV/body weight was similar in all three groups, indicating that the period of hypoxia was not of sufficient duration to globally effect LV mass. Lung/body weight and RV/body weight ratios were both increased in the CSH group compared with the control and CIH groups, consistent with CSH inducing pulmonary hypertension.

Histology

The left ventricular myocyte cross sectional area increased in the CIH group compared with control (Figure 1a). Likewise, perivascular

Table 1 Necropsy data

	Control	*CSH*	*CIH*
Sample size	18	12	12
LV/BW ($mg\,g^{-1}$)	2.29 ± 0.03	2.35 ± 0.07	2.22 ± 0.04
RV/BW ($mg\,g^{-1}$)	0.72 ± 0.02	1.11 ± 0.03*	0.79 ± 0.02**
Lung/BW ($mg\,g^{-1}$)	4.72 ± 0.14	7.03 ± 0.92*	4.74 ± 0.16**
Lung wet/dry	84 ± 2	82 ± 3	75 ± 3

Abbreviations: BW, body weight; LV, left ventricle; RV, right ventricle.
Data are reported as mean ± s.e.m.
*$P<0.05$ *vs.* control.
**$P<0.05$ *vs.* CSH.

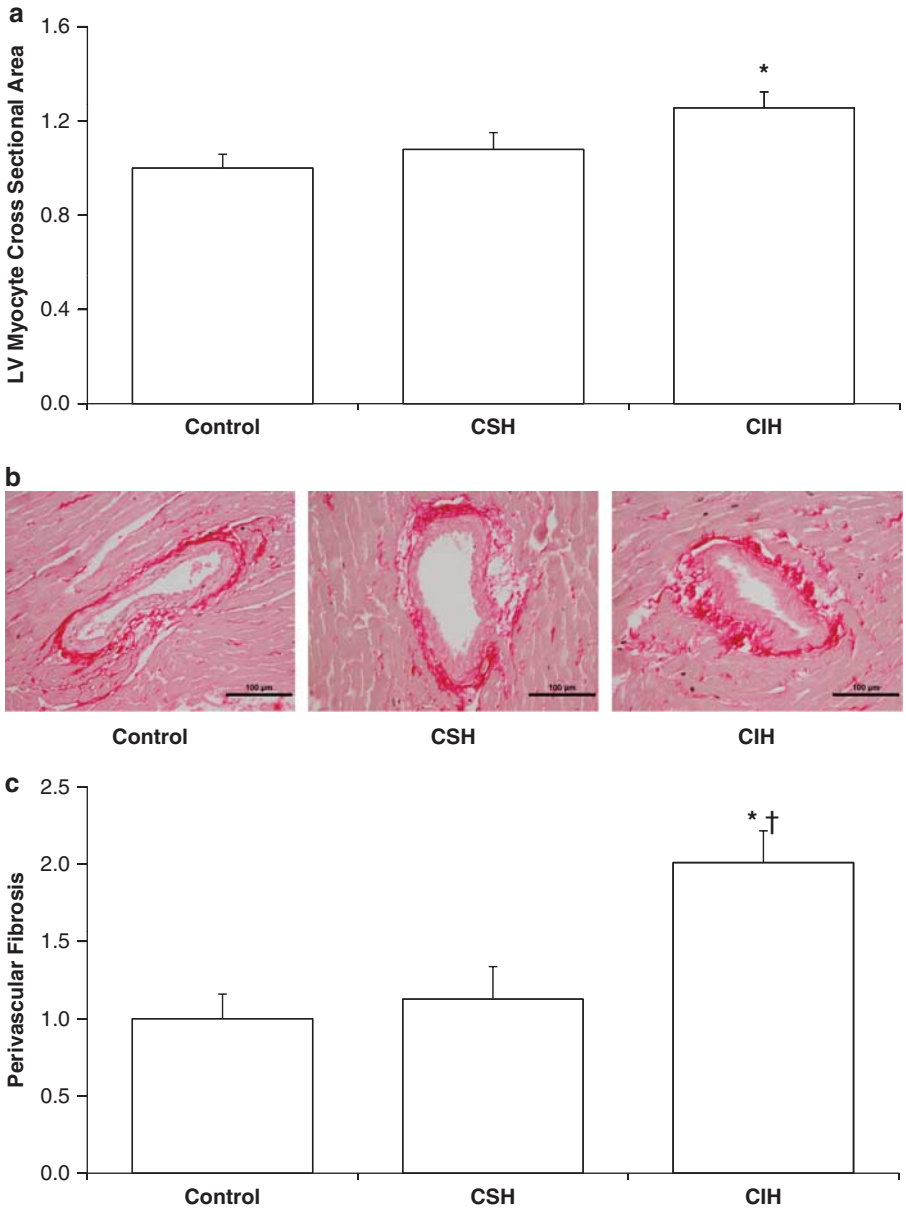

Figure 1. (**a**) Myocyte cross sectional area increases in CIH LV as compared with the control LV. (**b**) Sample images of Picrosirius Red stained LV tissue used to measure perivascular fibrosis that increased in CIH compared with the control and CSH LV (**c**). Data are mean ± s.e.m., with control set to 1.0. *$P<0.05$ *vs.* control, †$P<0.05$ *vs.* control and CSH.

collagen deposition (an indication of fibrosis) was increased in the LV of the CIH group (Figure 1b and c).

RT^2-PCR arrays

Out of 168 inflammatory, ECM and adhesion molecule genes measured through RT^2-PCR gene arrays, only 7 changed in CSH LV compared with control (all $P<0.05$). *Ltb*, *Cdh4*, *Col5a1*, *Ecm1*, *MMP-11* and *TIMP-2* all increased (range: 87–138%), whereas *Tnfrsf1a* decreased 27% from control values, indicating an overall increase in inflammatory status of the CSH LVs.

A total of 56 out of the 84 ECM genes and 68 out of the 84 inflammatory genes showed a statistically significant difference in mRNA expression among the three groups (statistical significance indicated by a $P<0.05$, Tables 2 and 3). The majority of genes was unaltered between control and CSH and was decreased in CIH. *Mif*, *Spp1*, *Fibronectin* and *MMP-9* all followed this trend. Two notable exceptions to the trend were *Laminin β2* and *Laminin γ1* (laminin C1). In both cases there was no significant difference in mRNA expression between the control and CSH groups, but there is a significant increase over both groups in the CIH group. The increase in perivascular fibrosis, with a decrease in ECM genes, in CIH seems contradictory and indicates that there was a dysregulation between ECM transcriptional and post-translational levels.

Immunoblotting

MMP-9, fibronectin, laminin β2 and laminin γ1 (laminin C1) were chosen for immunoblotting because of the gene level changes seen in the RT^2-PCR arrays. A significant decrease was seen in MMP-9 protein in the insoluble fraction in the CIH group (0.23 ± 0.01 AU)

Table 2 The 68 of the 84 inflammatory genes that showed significant changes among groups

	Control (n = 6)	*CSH* (n = 6)	*Fold change*	*CIH* (n = 5)	*Fold change*
Casp1	0.5983 ± 0.0934	0.4020 ± 0.0499	−1.49	0.1639 ± 0.0388*,**	−3.65
Ccl2	0.4180 ± 0.1052	0.2371 ± 0.0423	1.15	0.0530 ± 0.0127*	−2.90
Ccl3	2.8323 ± 0.2654	3.8188 ± 0.6559	−1.02	1.7044 ± 0.3870**	−2.95
Ccl5	0.4591 ± 0.0719	0.3082 ± 0.0522	1.06	0.0757 ± 0.0474*,**	−3.04
Ccl6	1.7565 ± 0.1615	1.5791 ± 0.2856	1.27	0.7749 ± 0.2006*,**	−3.27
Ccl7	0.5418 ± 0.1534	0.3979 ± 0.0682	−1.76	0.0340 ± 0.0179*,**	−7.89
Ccl9	0.5157 ± 0.1067	0.3664 ± 0.0507	−1.05	0.0710 ± 0.0455*,**	−3.38
Ccl11	3.1125 ± 0.4240	3.5892 ± 0.6352	1.25	1.0725 ± 0.2680*,**	−3.01
Ccl12	1.3934 ± 0.1205	1.3653 ± 0.3146	−1.39	0.4722 ± 0.2631*,**	−9.62
Ccl17	1.2715 ± 0.1099	1.3462 ± 0.2587	1.35	0.4176 ± 0.2066*,**	−1.66
Ccl19	2.5450 ± 0.1522	3.2367 ± 0.5000	−1.49	0.7786 ± 0.3960*,**	−6.07
Ccl21b	1.9782 ± 0.2417	1.8919 ± 0.3323	−1.11	0.5853 ± 0.2332*,**	−2.27
Ccl22	2.6259 ± 0.2531	3.2870 ± 0.6227	−1.36	0.8730 ± 0.3834*,**	−15.94
Ccl24	0.7506 ± 0.1242	0.5412 ± 0.0876	−1.41	0.0780 ± 0.0502*,**	−7.27
Ccr1	0.7984 ± 0.1388	0.6658 ± 0.0808	−1.20	0.1525 ± 0.0913*,**	−5.23
Ccr2	0.7729 ± 0.1649	0.5990 ± 0.0773	−1.28	0.1041 ± 0.0567*,**	−9.62
Ccr3	0.9325 ± 0.2008	0.5780 ± 0.1048	−1.29	0.1428 ± 0.0645*,**	−7.42
Ccr4	0.6223 ± 0.1432	0.3856 ± 0.0595	−1.61	0.0862 ± 0.0542*	−6.53
Ccr5	0.5455 ± 0.1569	0.2846 ± 0.0322	−1.61	0.0415 ± 0.0237*	−7.22
Ccr6	1.0990 ± 0.1741	0.7267 ± 0.1210	−1.92	0.2231 ± 0.1340*,**	−13.16
Ccr7	0.3455 ± 0.0700	0.2983 ± 0.0570	−1.51	0.0352 ± 0.0158*,**	−4.93
Ccr8	0.9507 ± 0.1591	0.7131 ± 0.1098	−1.16	0.1682 ± 0.0831*,**	−9.80
Ccr9	0.8092 ± 0.1499	0.5719 ± 0.0901	−1.33	0.1304 ± 0.0993*,**	−5.65
Ccr10	0.2785 ± 0.0652	0.2180 ± 0.0309	−1.41	0.0289 ± 0.0145*,**	−6.21
Cd40lg	0.5350 ± 0.0292	0.3806 ± 0.0556	−1.41	0.1359 ± 0.0843*,**	−3.94
Crp	0.6293 ± 0.1188	0.5198 ± 0.1005	−1.21	0.1021 ± 0.0658*,**	−6.16
Cx3cl1	0.1249 ± 0.0245	0.1525 ± 0.0283	1.22	0.0306 ± 0.0136*,**	−4.08
Cx3cr1	0.5497 ± 0.0502	0.4449 ± 0.0628	−1.24	0.1402 ± 0.0710*,**	−3.92
Cxcl1	1.1406 ± 0.1840	1.3472 ± 0.1821	1.18	0.2908 ± 0.1533*,**	−3.92
Cxcl6	2.1287 ± 0.2497	2.5186 ± 0.4235	−1.01	0.8528 ± 0.3439*,**	−3.87
Cxcl9	1.9066 ± 0.2229	1.9531 ± 0.2852	−1.26	0.7591 ± 0.2122*,**	−6.16
Cxcl10	1.8937 ± 0.2857	1.8711 ± 0.2524	1.18	0.4889 ± 0.2480*,**	−2.50
Cxcl11	0.3047 ± 0.0688	0.2414 ± 0.0370	1.02	0.0495 ± 0.0303*,**	−2.51
Cxcr3	0.4016 ± 0.0388	0.4212 ± 0.0500	1.05	0.0843 ± 0.0323*,**	−4.77
Cxcr5	0.4742 ± 0.1503	0.2924 ± 0.0699	−1.62	0.0221 ± 0.0101*	−21.48
Il1a	0.7347 ± 0.1992	0.4468 ± 0.0926	1.02	0.0709 ± 0.0346*	−2.93
Il1b	0.3242 ± 0.0870	0.1606 ± 0.0230	−1.02	0.0385 ± 0.0152*	−2.91
Il1f5	0.9238 ± 0.0799	0.8435 ± 0.1176	1.46	0.1569 ± 0.1166*,**	−1.74
Il1f6	1.5892 ± 0.0805	1.4520 ± 0.1737	1.09	0.4541 ± 0.2886*,**	−3.66
Il1r1	0.8995 ± 0.1281	0.8631 ± 0.0880	1.04	0.1722 ± 0.0856*,**	−2.85
Il1r2	0.8744 ± 0.1154	0.8825 ± 0.1615	−1.06	0.1759 ± 0.0898*,**	−4.06
Il2rb	1.9173 ± 0.2154	2.4160 ± 0.3655	−1.64	1.0587 ± 0.4031**	−10.36
Il2rg	0.8826 ± 0.1088	1.0361 ± 0.1821	−2.02	0.3028 ± 0.1483*,**	−8.42
Il3	0.9266 ± 0.1687	0.7029 ± 0.0692	−1.10	0.1685 ± 0.0677*,**	−5.89
Il5	1.7305 ± 0.1439	1.5127 ± 0.3478	−1.09	0.6404 ± 0.3450*	−3.50
Il6ra	1.0027 ± 0.1487	0.8995 ± 0.0846	−1.04	0.2577 ± 0.1321*,**	−5.22
Il6st	0.5689 ± 0.0815	0.4527 ± 0.0633	1.01	0.0981 ± 0.0666*,**	−4.97
Il8ra	0.6146 ± 0.2060	0.3079 ± 0.0482	1.26	0.0462 ± 0.0178*	−1.81
Il8rb	2.4375 ± 0.2197	2.7154 ± 0.3332	1.17	1.2485 ± 0.3658*,**	−2.91
Il10	1.4787 ± 0.2065	1.5089 ± 0.2547	−1.32	0.5043 ± 0.2173*,**	−5.50
Il10ra	0.6239 ± 0.0723	0.6109 ± 0.1087	−1.14	0.2148 ± 0.0833*,**	−2.70
Il11	1.4252 ± 0.1758	2.0872 ± 0.2599	−1.11	0.8172 ± 0.2446**	−3.89
Il13	0.3455 ± 0.0405	0.3768 ± 0.0671	−1.26	0.0943 ± 0.0661*,**	−5.80
Il16	1.1499 ± 0.0714	1.1944 ± 0.1978	−2.00	0.4031 ± 0.2324*,**	−13.31
Il17b	0.3332 ± 0.0664	0.3151 ± 0.0425	1.11	0.0821 ± 0.0500*,**	−1.95
Itgam	0.5470 ± 0.0435	0.4563 ± 0.1040	−1.20	0.1979 ± 0.1157*	−2.76
Itgb2	1.2784 ± 0.0728	1.3206 ± 0.3440	1.03	0.3609 ± 0.2281*,**	−3.54
Lta	0.5109 ± 0.0891	0.7173 ± 0.1461	1.40	0.0860 ± 0.0437*,**	−5.94
Ltb	0.6569 ± 0.0285	1.4203 ± 0.3237*	2.16	0.1365 ± 0.0623*,**	−4.81
Mif	17.9616 ± 1.9580	16.5233 ± 2.2920	−1.09	7.0926 ± 1.5193*,**	−2.53

Table 2 (Continued)

	Control (n = 6)	*CSH (n = 6)*	*Fold change*	*CIH (n = 5)*	*Fold change*
Pf4	4.2233 ± 0.4362	5.1480 ± 0.8168	1.22	2.2032 ± 0.5955*,**	−1.92
RDG	1.0036 ± 0.1439	0.8366 ± 0.1506	−1.20	0.1861 ± 0.1286*,**	−5.39
Spp1	2.2627 ± 0.2777	1.7058 ± 0.2635	−1.33	0.6914 ± 0.3227*,**	−3.27
Tgfb1	1.1531 ± 0.1451	1.3656 ± 0.1668	1.18	0.2231 ± 0.1348*,**	−5.17
Tnfrsf1a	0.4346 ± 0.0250	0.3167 ± 0.0415*	−1.37	0.0574 ± 0.0398*,**	−7.57
Tnfrsf1b	0.1760 ± 0.0448	0.1181 ± 0.0376	−1.49	0.0229 ± 0.0123*	−7.69
Tollip	0.2783 ± 0.0607	0.2401 ± 0.0623	−1.16	0.0259 ± 0.0123*,**	−10.76
Xcr1	0.5216 ± 0.1407	0.3038 ± 0.0710	−1.72	0.0368 ± 0.0245*	−14.18

Data are $2^{-\Delta CT}$ values and are presented as mean ± s.e.m. Fold changes relative to control levels.
*$P<0.05$ compared to control.
**$P<0.05$ compared to CSH.

Table 3 The 56 of the 84 ECM and adhesion molecule genes that showed significant changes among groups

	Control (n = 6)	*CSH (n = 6)*	*Fold change*	*CIH (n = 5)*	*Fold change*
Adamts1	0.5087 ± 0.0787	0.6817 ± 0.0193	1.34	0.2379 ± 0.1441**	−2.14
Adamts2	0.1385 ± 0.0392	0.1567 ± 0.0115	1.13	0.0253 ± 0.0152*,**	−5.48
Adamts5	0.1686 ± 0.0406	0.2360 ± 0.0150	1.40	0.1033 ± 0.0193**	−1.63
Adamts8	0.1231 ± 0.0284	0.1396 ± 0.0103	1.13	0.0369 ± 0.0262*,**	−3.34
Cdh1	0.2286 ± 0.0408	0.2874 ± 0.0114	1.12	0.0708 ± 0.0438*,**	−4.21
Cdh3	0.5370 ± 0.1309	0.8487 ± 0.0766	1.26	0.2381 ± 0.1591**	−3.23
Cdh4	0.2356 ± 0.0650	0.5414 ± 0.0295*	1.58	0.1059 ± 0.0668**	−2.25
Cntn1	0.4683 ± 0.0546	0.5916 ± 0.0496	2.30	0.1679 ± 0.0912*,**	−2.23
Col2a1	0.3168 ± 0.1116	0.5722 ± 0.0363	1.26	0.1380 ± 0.1035**	−2.79
Col4a2	0.2470 ± 0.0597	0.3573 ± 0.0125	1.81	0.0758 ± 0.0408*,**	−2.30
Col4a3	0.2003 ± 0.0399	0.2276 ± 0.0063	1.45	0.0676 ± 0.0300*,**	−3.26
Col5a1	0.6144 ± 0.1541	1.1491 ± 0.0393*	1.14	0.2732 ± 0.1476**	−2.96
Ctnna2	0.5629 ± 0.0847	0.6763 ± 0.0345	1.87	0.2105 ± 0.1243*,**	−2.25
Ctnnb1	0.7670 ± 0.1923	1.2016 ± 0.0375	1.24	0.5756 ± 0.1667**	−3.22
Ecm1	0.2381 ± 0.0469	0.4572 ± 0.0426*	1.31	0.0625 ± 0.0417**	−2.58
Emilin1	0.0774 ± 0.0249	0.1210 ± 0.0124	1.20	0.0206 ± 0.0130**	−2.67
Entpd1	0.3241 ± 0.0586	0.4085 ± 0.0285	1.57	0.1260 ± 0.0783**	−1.33
Fbln1	0.4557 ± 0.0831	0.5957 ± 0.0535	1.92	0.1665 ± 0.1032**	−3.81
Fn1	0.1930 ± 0.0501	0.2611 ± 0.0119	1.56	0.0510 ± 0.0389*,**	−3.75
Hapln1	0.3227 ± 0.0838	0.3056 ± 0.0079	1.26	0.0915 ± 0.0491*,**	−2.57
Icam1	0.7025 ± 0.1473	0.9534 ± 0.0257	1.31	0.2254 ± 0.1291*,**	−2.74
Itga2	0.6929 ± 0.1116	0.9175 ± 0.0708	1.35	0.2467 ± 0.1145*,**	−3.78
Itga3	0.2198 ± 0.0682	0.3647 ± 0.0087	0.95	0.0695 ± 0.0462**	−3.53
Itga4	0.5210 ± 0.0513	0.4574 ± 0.0347	1.36	0.2636 ± 0.0299*,**	−3.12
Itgav	0.4317 ± 0.0772	0.6634 ± 0.0127	1.32	0.1797 ± 0.0924*,**	−2.81
Itgb1	0.1426 ± 0.0380	0.1084 ± 0.0063	1.66	0.0373 ± 0.0092*	−3.16
Itgb2	0.3667 ± 0.1041	0.5443 ± 0.0364	−1.14	0.1650 ± 0.1047**	−1.98
Itgb3	0.4782 ± 0.1158	0.8860 ± 0.0707	1.54	0.2555 ± 0.1540**	−2.40
Itgb4	0.3582 ± 0.0865	0.5503 ± 0.0173	−1.31	0.1129 ± 0.0757*,**	−3.82
Lama1	0.3297 ± 0.0855	0.4965 ± 0.0331	1.48	0.1717 ± 0.0765**	−2.22
Lama2	0.7372 ± 0.1555	0.9234 ± 0.0088	1.85	0.2812 ± 0.1476*,**	−1.87
Lamb2	1.2805 ± 0.2769	1.2126 ± 0.1102	1.54	2.8610 ± 0.6099*,**	−3.17
Lamb3	0.2979 ± 0.0905	0.3765 ± 0.0249	1.51	0.0893 ± 0.0673**	−1.92
Lamc1	1.0829 ± 0.3779	0.3982 ± 0.0196	1.25	2.4866 ± 0.4716*,**	−2.62
MMP2	0.1871 ± 0.0382	0.2517 ± 0.0159	−1.06	0.0625 ± 0.0260*,**	2.23
MMP3	0.2979 ± 0.0645	0.3721 ± 0.0036	1.26	0.0866 ± 0.0597*,**	−3.34
MMP7	0.4265 ± 0.0805	0.6121 ± 0.0200	−2.72	0.1642 ± 0.1156**	2.30
MMP8	0.2302 ± 0.0511	0.2321 ± 0.0113	1.61	0.0662 ± 0.0380*,**	−3.78
MMP9	0.2737 ± 0.0693	0.3364 ± 0.0085	1.81	0.0702 ± 0.0497*,**	−2.00
MMP11	0.7803 ± 0.1612	1.2582 ± 0.0531*	1.19	0.2065 ± 0.1314*,**	−5.97
MMP13	0.1900 ± 0.0623	0.3435 ± 0.0169	1.09	0.0949 ± 0.0673**	−3.06
MMP14	0.1061 ± 0.0276	0.1262 ± 0.0107	1.21	0.0178 ± 0.0105*,**	−2.32
MMP15	0.0542 ± 0.0138	0.0590 ± 0.0052	1.35	0.0177 ± 0.0019*,**	−3.00

Table 3 (Continued)

	Control (n = 6)	*CSH* (n = 6)	*Fold change*	*CIH* (n = 5)	*Fold change*
MMP16	0.3084 ± 0.0545	0.3717 ± 0.0184	1.25	0.1329 ± 0.0727**	−3.44
Ncam1	0.4444 ± 0.0810	0.5104 ± 0.0156	1.44	0.1521 ± 0.0701*,**	−2.60
Ncam2	0.2035 ± 0.0440	0.1883 ± 0.0159	1.01	0.0591 ± 0.0376*,**	−3.48
Sell	0.1456 ± 0.0314	0.2093 ± 0.0063	1.23	0.0408 ± 0.0241*,**	−3.90
Spock1	0.5378 ± 0.0818	0.5924 ± 0.0317	1.15	0.1217 ± 0.0608*,**	−2.92
Spp1	0.6756 ± 0.1066	0.7817 ± 0.0249	−1.08	0.2713 ± 0.1729*,**	−3.44
Syt1	0.1027 ± 0.0313	0.0750 ± 0.0032	1.44	0.0182 ± 0.0105*	−3.57
Tgfbi	0.6631 ± 0.1243	0.8022 ± 0.0300	1.10	0.3045 ± 0.0918*,**	−2.49
Thbs2	0.5894 ± 0.1138	0.7216 ± 0.0232	1.10	0.2352 ± 0.1204*,**	−4.42
TIMP-1	0.2855 ± 0.0604	0.2568 ± 0.0112	1.16	0.0569 ± 0.0387*,**	−2.49
TIMP-2	0.1793 ± 0.0400	0.3372 ± 0.0204*	−1.37	0.0449 ± 0.0306*,**	−5.64
Vcam1	0.1894 ± 0.0465	0.1483 ± 0.0122	1.21	0.0336 ± 0.0205*,**	−2.18
Vtn	0.0920 ± 0.0253	0.1050 ± 0.0061	1.22	0.0249 ± 0.0157*,**	−2.51

Data are $2^{-\Delta CT}$ values and are presented as mean ± s.e.m. Fold changes relative to control levels.
*$P<0.05$ compared to control.
**$P<0.05$ compared to CSH.

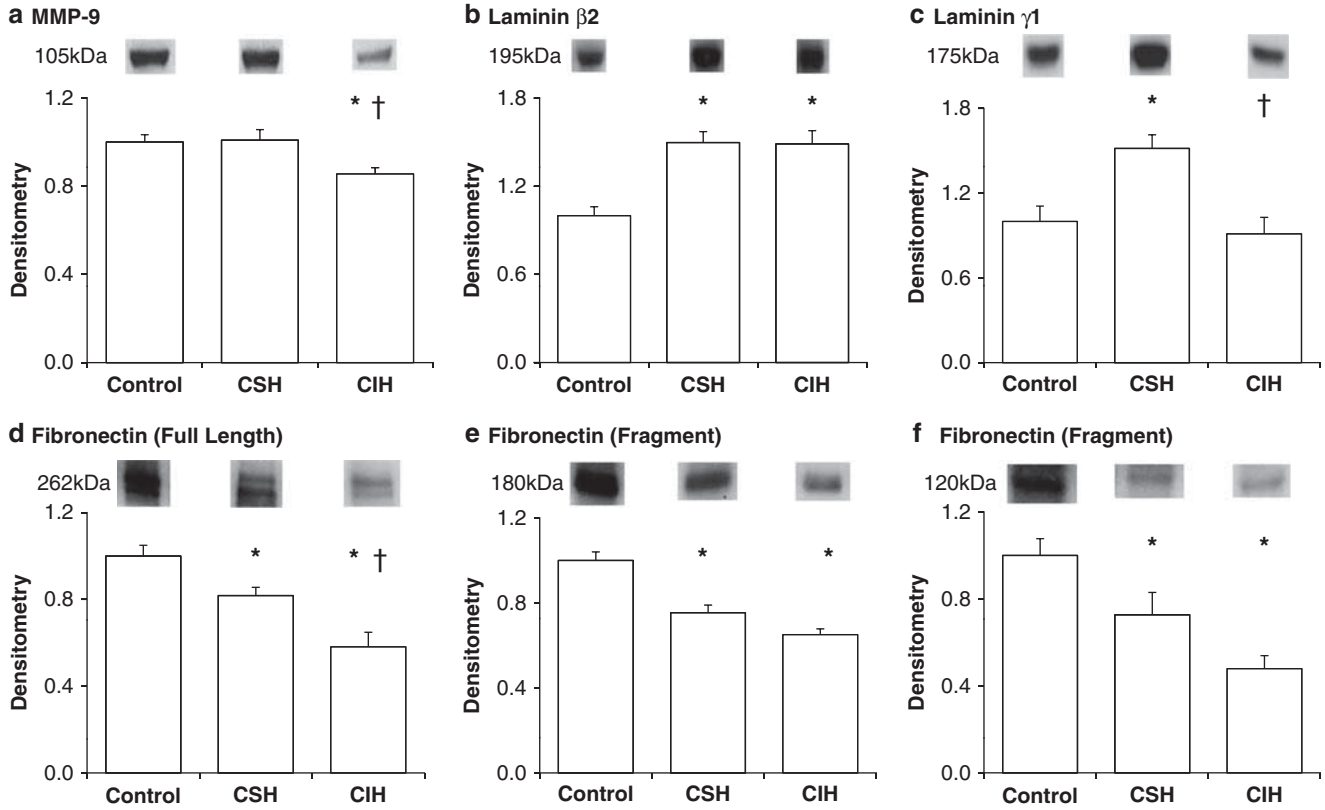

Figure 2 LV immunoblotting of MMP-9, Laminin β2, Laminin γ1 (C1) and Fibronectin. (**a**) MMP-9 protein levels showed a significant decrease in the CIH rats compared with the control and CSH rats. (**b**) Laminin β2 increased in the CSH and CIH rats compared with the control. (**c**) Laminin γ1 (laminin C1) also increased in CSH compared with the control, but decreased in the CIH LV. (**d**–**f**) Fibronectin protein (full length and the 120 and 180 kDa fragments) decreased from control to CSH and CIH, and full length fibronectin was further decreased from CSH to CIH LV. Data are mean ± s.e.m. arbitrary densitometry units, which were normalized to total protein densitometry values. Control $n=18$, CSH $n=12$ and CIH $n=12$. *$P<0.05$ *vs.* control, †$P<0.05$ *vs.* CSH.

compared to control (0.26 ± 0.01 AU) and CSH (0.27 ± 0.01 AU), supporting the array data (Figure 2a). Soluble MMP-9 also showed a 37% decrease from CSH to CIH. Levels of the full length, as well as the 180 and 120 kDa fragments of fibronectin, decreased in CIH compared with control and CSH, consistent with the decrease in gene levels (Figure 2d–f). Full length fibronectin was significantly lower in CSH compared to control, which also mirrors the gene changes.

Laminin β2 protein levels increased from the control group to the CSH and CIH groups (Figure 2b). Laminin γ1 showed a significant

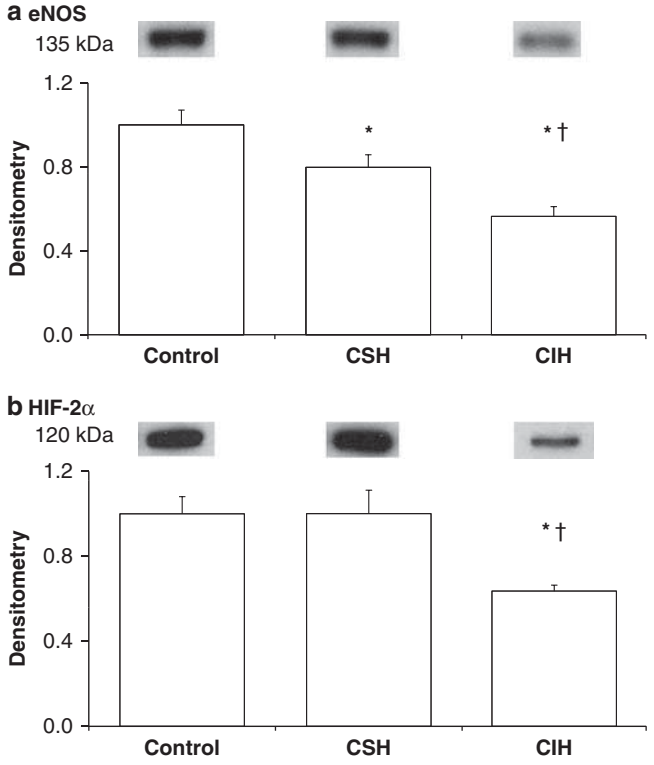

Figure 3 LV immunoblotting of eNOS and HIF-2α. (**a**) eNOS protein levels showed a significant change in all three groups compared to each other, with levels decreasing from control to CSH to CIH. (**b**) HIF-2α levels decreased in the CIH group compared with the control and CSH. Data are mean ± s.e.m. arbitrary densitometry units, which were normalized to total protein densitometry values. Control $n=18$, CSH $n=12$ and CIH $n=12$. $^*P<0.05$ *vs.* control; $\dagger P<0.05$ *vs.* CSH.

increase in CSH levels, compared with control and CIH (Figure 2c). These results demonstrated that laminin β2 protein level differed from mRNA level in the CSH group, whereas laminin γ1 protein levels differed from mRNA expression in both the CSH and CIH groups. This indicates that both laminin β2 and γ1 are post-translationally regulated in the setting of hypoxia.

As inflammation and ECM responses were suppressed in the CIH group, we evaluated eNOS, HIF-1α, HIF-2α, β2 integrin, TIMP-1, TIMP-2, AT_1 receptor and β_1AR protein levels. HIF-1α, β2 integrin, TIMP-1, TIMP-2, AT_1 receptor and β_1AR levels showed no differences among groups. Levels of eNOS decreased in CSH from control and were decreased further in the CIH group (Figure 3a). Levels of HIF-2α were significantly decreased in the CIH group, compared with both the control and CSH groups (Figure 3b).

DISCUSSION

The goal of this study was to evaluate the response of the LV to CSH and CIH, focusing on inflammatory and ECM responses. The key findings were (a) lung and right ventricle mass increases with chronic sustained but not CIH, while LV mass was not increased in either group; and (b) CIH induces far more gene changes in the LV than CSH following 7 days exposure to hypoxia. Our results reveal the early LV inflammatory and ECM gene changes that occur before LV dysfunction develops.

Although we saw an increase in the myocyte cross sectional area and perivascular fibrosis in the CIH, we did not observe a change in LV mass in either of the hypoxic conditions. The early increase in RV and lung masses is due to the direct effect of hypoxia on the lungs and pulmonary artery. Our results are consistent with the LV having a later pathological response to hypoxia than the lung and right ventricle.[18] While LV mass was not changed, hypertrophy of the individual myocytes and perivascular fibrosis occurred, suggesting that these two parameters are early indicators of response.

There were multiple inflammatory and ECM gene changes, all of which occurred in the absence of an increase in LV mass. These changes, therefore, provide indicators of LV response that precede alterations at the tissue level. While CSH showed an increase in a small number of genes associated with inflammation, CIH exhibited a dramatic increase in two and decrease in 122 inflammatory, ECM and adhesion molecule genes. Many of the genes that were upregulated in CSH are ones connected to cell growth. For example, Ecm1 inhibits the proteolytic activity of MMP-9, thereby stimulating endothelial cell proliferation.[19] MMP-11 and TIMP-2 have also both been shown to exert potent growth promoting activity.[20,21] The only gene that was significantly downregulated was the transcription factor Tnfrsf1a, which is a negative regulator of inflammation.[22,23] Overall, the initial response to CSH is an increase in inflammatory factors.

CIH has been previously associated with increased HIF-1, c-fos, activator protein-1, nuclear factor κB, cAMP-response element-binding protein and reactive oxygen species in cell culture models.[2] These stimuli are known inducers of inflammatory and ECM accumulation. In our study, CIH had the opposite effect on the expression of the inflammation, ECM and adhesion molecule genes, downregulating a larger number of them. For the changes in CIH, only two genes increased (laminins β2 and γ1). Laminins are essential for cell adhesion, migration, signaling and differentiation.[24,25] Protein levels of MMP-9, MMP-13, fibronectin, eNOS and HIF-2α were all decreased in CIH, showing an overall reduction in the inflammatory and angiogenic responses. Lu *et al.*[26] showed that hypoxia induced MMP-13 expression in astrocytes, which enhanced the permeability of the brain endothelial cells. ECM responses in general and MMP responses in particular are dependent on location, timing and stimulus. This gene response may be indicative of an initial cardiac protection provided by this particular hypoxic pattern.

While hypoxia is a proliferative stimulus for pulmonary artery adventitial fibroblasts,[27] our results suggest a decrease in LV fibroblast numbers or downregulation of fibroblast activity in the setting of intermittent hypoxia. Hypoxia in the rat has also been shown to increase osteopontin (spp1) expression in the lung.[28] The fact that our study showed reduced, and not increased, inflammatory and ECM responses indicates that the above listed factors may be more relevant to periods of CIH beyond the 7-day time point evaluated in this study. Chen *et al.* have shown that short-term intermittent hypoxia for 1–3 days is protective on the LV, while long-term intermittent hypoxia for 4–8 weeks was deleterious.[29] They observed increased LV mass at the 4 and 8 week time points, which was associated with eccentric cardiac remodeling and increased inflammation. Our study is consistent with this concept—that there is a reversal in response over time, with short-term exposure showing a beneficial effect, while long-term exposure has a deleterious effect.

The rat strain used is also an important variable in the response to hypoxia, as Kraiczi *et al.*[30] have shown that spontaneously hypertensive rats, but not the Wistar-Kyoto control rats, developed increased LV mass in response to long-term intermittent hypoxia. This variation in response also suggests that hypoxia superimposed on an underlying cardiac disease such as hypertension or heart failure would show an exacerbated response. In humans, pulmonary hypertension occurs in 20–40% of patients with obstructive sleep

apnea, although right ventricular failure is not common unless the apnea is accompanied by left heart disease or chronic respiratory disease.[5] As the initial responses to intermittent and sustained hypoxia differed dramatically, it will be important to fully understand these differences over the long term, in order to better develop clinical treatments specific to conditions that exhibit each type of hypoxia.

There were several limitations to this study. One limitation is that while intermittent hypoxia is widely used as a model of arterial hypoxemia that accompanies sleep apnea, it does not fully recapitulate the syndrome. In particular, patients with sleep apnea have hypercapnia, while intermittent hypoxia is associated with hypocapnia. While this disparity suggests a limitation, there are data suggesting that the presence or absence of changes in CO_2 levels do not alter blood pressure responses to CIH, which indicates that this may not be a significant limitation.[6,31] Another limitation is that only one time point was evaluated. As the output list for this study was so extensive, we evaluated only one time point in order to provide a more in-depth study with mechanistic insight. We selected the 7-day time point to determine the early changes in gene and protein expression. Future studies will build on this report to add in both shorter and longer time points, to more fully understand the transitions in responses. In addition, cardiac function changes along the time continuum of response will need to be monitored. The use of agents that modify the inflammatory and ECM responses is also warranted.

In summary, this is the first study to show the specific and differential responses of the LV to CIH and CSH.

PERSPECTIVES

The responses of the LV to chronic sustained *vs.* intermittent hypoxia are very distinct, and understanding how these processes are similar and distinct will provide mechanistic insight to develop treatment strategies. Our data indicate that the pattern of hypoxia (sustained *vs.* intermittent) yields LV responses that are very distinct.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Brown ST, Buttigieg J, Nurse CA. Divergent roles of reactive oxygen species in the responses of perinatal adrenal chromaffin cells to hypoxic challenges. *Respir Physiol Neurobiol* 2010; **174**: 252–258.
2 Nanduri J, Nanduri RP. Cellular mechanisms associated with intermittent hypoxia. *Essays Biochem* 2007; **43**: 91–104.
3 Estrada KD, Chesler NC. Collagen-related gene and protein expression changes in the lung in response to chronic hypoxia. *Biomech Model Mechanobiol* 2009; **8**: 263–272.
4 Gottlieb DJ, Yenokyan G, Newman AB, O'Connor GT, Punjabi NM, Quan SF, Redline S, Resnick HE, Tong EK, Diener-West M, Shahar E. Prospective study of obstructive sleep apnea and incident coronary heart disease and heart failure: the Sleep Heart Health Study. *Circulation* 2010; **122**: 352–360.
5 Sajkov D, McEvoy RD. Obstructive sleep apnea and pulmonary hypertension. *Prog Cardiovasc Dis* 2009; **51**: 363–370.
6 Bao G, Randhawa PM, Fletcher EC. Acute blood pressure elevation during repetitive hypocapnic and eucapnic hypoxia in rats. *J Appl Physiol* 1997; **82**: 1071–1078.
7 Omenn GS, States DJ, Adamski M, Blackwell TW, Menon R, Hermjakob H, Apweiler R, Haab BB, Simpson RJ, Eddes JS, Kapp EA, Moritz RL, Chan DW, Rai AJ, Admon A, Aebersold R, Eng J, Hancock WS, Hefta SA, Meyer H, Paik YK, Yoo JS, Ping P, Pounds J, Adkins J, Qian X, Wang R, Wasinger V, Wu CY, Zhao X, Zeng R, Archakov A, Tsugita A, Beer I, Pandey A, Pisano M, Andrews P, Tammen H, Speicher DW, Hanash SM. Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics* 2005; **5**: 3226–3245.
8 de Paula PM, Tolstykh G, Mifflin S. Chronic intermittent hypoxia alters NMDA and AMPA-evoked currents in NTS neurons receiving carotid body chemoreceptor inputs. *Am J Physiol Regul Integr Comp Physiol* 2007; **292**: R2259–R2265.
9 Hinojosa-Laborde C, Mifflin SW. Sex differences in blood pressure response to intermittent hypoxia in rats. *Hypertension* 2005; **46**: 1016–1021.
10 Knight WDLJ, Carreno FR, Toney GM, Mifflin SW, Cunningham JT. Chronic intermittent hypoxia increases blood pressure and expression of FosB/ΔFosB in central autonomic regions. *Am J Physiol Regul Integr Comp Physiol* 2011; **301**: 10.
11 Zhang W, Carreno FR, Cunningham JT, Mifflin SW. Chronic sustained and intermittent hypoxia reduce function of ATP-sensitive potassium channels in nucleus of the solitary tract. *Am J Physiol Regul Integr Comp Physiol* 2008; **295**: R1555–R1562.
12 Ilyinsky O, Tolstykh G, Mifflin S. Chronic hypoxia abolishes posthypoxia frequency decline in the anesthetized rat. *Am J Physiol Regul Integr Comp Physiol* 2003; **285**: R1322–R1330.
13 Lindsey ML, Yoshioka J, MacGillivray C, Muangman S, Gannon J, Verghese A, Aikawa M, Libby P, Krane SM, Lee RT. Effect of a cleavage-resistant collagen mutation on left ventricular remodeling. *Circ Res* 2003; **93**: 238–245.
14 Zamilpa R, Kanakia R, Cigarroa Jt, Dai Q, Escobar GP, Martinez HM, Jimenez F, Ahuja S, Lindsey ML. CC chemokine receptor 5 deletion impairs macrophage activation and induces adverse remodeling following myocardial infarction. *Am J Physiol. Heart Circ Physiol* 2011; **300**: H1418–H1426.
15 Dolber PC, Spach MS. Picrosirius red staining of cardiac muscle following phosphomolybdic acid treatment. *Stain Technol* 1987; **62**: 23–26.
16 Lin J, Lopez EF, Jin Y, Van Remmen H, Bauch T, Han HC, Lindsey ML. Age-related cardiac muscle sarcopenia: combining experimental and mathematical modeling to identify mechanisms. *Exp Gerontol* 2008; **43**: 296–306.
17 McCurdy SM, Dai Q, Zhang J, Zamilpa R, Ramirez TA, Dayah T, Nguyen N, Jin YF, Bradshaw AD, Lindsey ML. SPARC mediates early extracellular matrix remodeling following myocardial infarction. *Am J Physiol Heart Circ Physiol* 2011; **301**: H497–505.
18 Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol* 2009; **297**: L1013–L1032.
19 Fujimoto N, Terlizzi J, Aho S, Brittingham R, Fertala A, Oyama N, McGrath JA, Uitto J. Extracellular matrix protein 1 inhibits the activity of matrix metalloproteinase 9 through high-affinity protein/protein interactions. *Exp Dermatol* 2006; **15**: 300–307.
20 Kwon YJ, Hurst DR, Steg AD, Yuan K, Vaidya KS, Welch DR, Frost AR. Gli1 enhances migration and invasion via up-regulation of MMP-11 and promotes metastasis in ERalpha negative breast cancer cell lines. *Clin Exp Metastasis* 2011; **28**: 437–449.
21 Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J Cell Sci* 1994; **107**(Pt 9): 2373–2379.
22 Mohammed FF, Smookler DS, Taylor SE, Fingleton B, Kassiri Z, Sanchez OH, English JL, Matrisian LM, Au B, Yeh WC, Khokha R. Abnormal TNF activity in Timp3-/- mice leads to chronic hepatic inflammation and failure of liver regeneration. *Nat Genet* 2004; **36**: 969–977.
23 Smookler DS, Mohammed FF, Kassiri Z, Duncan GS, Mak TW, Khokha R. Tissue inhibitor of metalloproteinase 3 regulates TNF-dependent systemic inflammation. *J Immunol* 2006; **176**: 721–725.
24 Adair-Kirk TL, Senior RM. Fragments of extracellular matrix as mediators of inflammation. *The Int J Biochem Cell Biol* 2008; **40**: 1101–1110.
25 Suzuki N, Yokoyama F, Nomizu M. Functional sites in the laminin alpha chains. *Connect Tissue Res* 2005; **46**: 142–152.
26 Lu DY, Yu WH, Yeh WL, Tang CH, Leung YM, Wong KL, Chen YF, Lai CH Fu WM. Hypoxia-induced matrix metalloproteinase-13 expression in astrocytes enhances permeability of brain endothelial cells. *J Cell Physiol* 2009; **220**: 163–173.
27 Stenmark KR, Davie N, Frid M, Gerasimovskaya E, Das M. Role of the adventitia in pulmonary vascular remodeling. *Physiology* 2006; **21**: 134–145.
28 Bull TM, Coldren CD, Geraci MW, Voelkel NF. Gene expression profiling in pulmonary hypertension. *Proc Am Thorac Soc* 2007; **4**: 117–120.
29 Chen L-M, Kuo W-W, Yang J-J, Wang S-GP, Yeh Y-L, Tsai F-J, Ho Y-J, Chang M-H, Huang C-Y, Lee S-D. Eccentric cardiac hypertrophy was induced by long-term intermittent hypoxia in rats. *Exp Physiol* 2007; **92**: 409–416.
30 Kraiczi H, Magga J, Sun XY, Ruskoaho H, Zhao X, Hedner J. Hypoxic pressor response, cardiac size, and natriuretic peptides are modified by long-term intermittent hypoxia. *J Appl Physiol* 1999; **87**: 2025–2031.
31 Baatar D, Patel K, Taub DD. The effects of ghrelin on inflammation and the immune system. *Mol Cell Endocrinol* 2011; **340**: 44–58.