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Hypoxemia induces expression of heme oxygenase-1 and heme oxygenase-2 proteins in the mouse myocardium

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Heme oxygenase (HO) catalyzes oxidative breakdown of heme, and constitutes two isozymes, HO-1 and HO-2. Here, we explored the tissue-specific regulation of expression of HO-1 and HO-2 under hypoxemia. There was no significant change in the overall expression levels of HO-1 and HO-2 mRNAs and proteins in the lung during adaptation of C57BL/6 mice to normobaric hypoxia (10% O_2). However, immunohistochemical analysis revealed the increased expression of HO-1 and HO-2 proteins after 28 days of normobaric hypoxia in the pulmonary venous myocardium that is the extension of the left atrial myocardium into pulmonary venous walls. Moreover, the expression of HO-2 protein was increased in the sub-endocardial myocardium of ventricles under hypoxia, while HO-1 protein level was increased in the full-thickness walls. Thus, hypoxemia induces expression of both HO-1 and HO-2 proteins in the myocardium. Using C57BL/6 mice lacking HO-2 (HO-2$^{-/-}$), which manifest chronic hypoxemia, we also showed that the HO-1 protein level in the lung was similar between HO-2$^{-/-}$ mice and wild-type mice. Unexpectedly, HO-1 protein level was lower by 35% in the HO-2$^{-/-}$ mouse liver than the wild-type liver. These results indicate that the expression of HO-1 protein is regulated in a tissue-specific manner under hypoxemia.

Keywords: bilirubin/heart/heme oxygenase/hypoxemia/hypoxia/liver/lung/myocardium/oxygen sensor.

Abbreviations: HO, heme oxygenase; L-PGDS, lipocalin-type prostaglandin D synthase; PG, prostaglandin.

Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism and constitutes two structurally related isozymes, HO-1 and HO-2 (*1*–*3*). Both isozymes share ~43% overall amino acid identity (*2*, *4*, *5*) and cleave heme to form biliverdin IXα, carbon monoxide (CO) and iron at the expense of molecular oxygen and NADPH (*6*, *7*). Biliverdin IXα is rapidly reduced to bilirubin IXα. However, HO-1 and HO-2 show the distinct features in their structures and expression profiles (*8*). Human HO-1 of 288 amino acids lacks a cysteine residue (*4*), whereas human HO-2 of 316 amino acids contains three cysteine residues (*5*), each of which is present as a dipeptide of cysteine and proline (CP motif) and may function as the heme-binding site (*9*). HO-2 has been therefore postulated to play a regulatory role by sequestering heme to maintain the intracellular heme level (*9*). Recently, it has been proposed that HO-2 functions as an oxygen sensor (*10*, *11*). It is, therefore, of physiological significance to study the regulation of HO-2 expression under hypoxia in the cardio-pulmonary tissues.

Expression of HO-1 mRNA is induced by various environmental factors in human cell lines, despite that expression level of HO-2 mRNA is maintained within narrow ranges (*12*–*14*). On the other hand, HO-1 expression is reduced by various environmental factors (*15*–*19*), which also represents an important response for maintaining cellular homeostasis (*8*). Likewise, the expression level of HO-2 is decreased under certain conditions, such as the placental tissues of abnormal pregnancies (*20*) and cultured human cell lines (*21*,*22*). In this connection, the expression levels of both HO-1 and HO-2 proteins are significantly reduced in the mouse liver after 7 days of normobaric hypoxia (10% O_2 at the sea-level barometric pressure), and concomitantly returned to the basal levels after 14 days of hypoxia, as judged by western blot analysis (*23*). Thereafter, the expression levels of HO-1 and HO-2 proteins remained at the basal level in the liver for up to 28 days of hypoxia (*23*). In contrast, the expression levels of both HO-1 and HO-2 proteins are increased in the heart after 28 days of normobaric hypoxia (*23*). These results indicate the difference in the regulation of HO-1 and HO-2 expression between the liver and the heart.

Hypoxemia is a common manifestation of various diseases, such as chronic obstructive pulmonary disease and sleep apnea syndrome (*24*). Hypoxemia is a hemodynamic stress and may cause pulmonary hypertension that generates pressure overload to the right ventricle. Among the mechanisms that cause hypoxemia, ventilation–perfusion mismatch is the most common cause of hypoxemia (*24*). We have shown

that HO-2 deficient (HO-2$^{-/-}$) mice exhibit mild hypoxemia and attenuated hypoxic ventilatory responses with normal hypercapnic ventilatory responses (*10*). Considering the normal morphology of the alveolar structure of HO-2$^{-/-}$ mice (*10*), we have proposed that the ventilation–perfusion mismatch is responsible for the hypoxemia in HO-2$^{-/-}$ mice (*10*, *24*). On the other hand, HO-2$^{-/-}$ mice exhibit the hypertrophy of the pulmonary venous myocardium, which may reflect adaptation to persistent hypoxemia (*25–27*). The pulmonary venous myocardium represents the extension of atrial myocardium into the vascular walls of the pulmonary veins (*26*, *27*). Moreover, overall expression of HO-1 protein is increased in the heart and testis of HO-2$^{-/-}$ mice (*28*). These results suggest that HO-2$^{-/-}$ mice may compensate for the loss of HO-2 by increasing the expression of HO-1. Indeed, HO-2$^{-/-}$ mice are fertile and show relatively mild phenotypes (*29*).

In the present study, we analysed the expression levels of HO-1 and HO-2 in the lung and heart of wild-type mice kept under normobaric hypoxia (10% O_2). In addition, using HO-2$^{-/-}$ mice, we explored the potential role of HO-2 for the expression of HO-1 in the lung and liver.

Materials and Methods

Animal treatment

Male C57BL/6 mice (5-week old) were obtained from the Animal Experimental Center of Tohoku University School of Medicine (Sendai, Japan) and were housed for 1 week before the beginning of the study. All animal experiments were performed based on the institutionally approved protocol of Tohoku University School of Medicine. Mice were maintained under normoxia or normobaric hypoxia (10% O_2) for 28 days, as detailed previously (*23*, *30*, *31*). The O_2 concentration chosen is equivalent to the value at an altitude of ~5,000 m, but with normobaric pressure at the sea level. Normobaric hypoxia was used as a means to generate hypoxemia. The hypoxic chamber was opened twice a week for 15 min for feeding and cleaning up cages. At the end of the exposure, the animals were anesthetized with diethyl ether and killed. The lungs were isolated from the same mice that were used to isolate the hearts and livers in our earlier report (*23*).

Morphological examination of the lung

The lungs were isolated from C57BL/6 mice maintained under normoxia or hypoxia for 28 days, fixed in 4% paraformaldehyde for 24 h at room temperature, and paraffin-embedded. Deparaffinized sections of the lung were stained by elastica Masson straining method to evaluate muscularization (medial thickening) of pulmonary arterioles. Pathological changes of the pulmonary arterioles (<50 μm in diameter) were assessed by the microscopic examination, as previously reported (*32*, *33*).

Western blot analysis

Tissue extracts were prepared from the hearts and lungs of mice exposed to normoxia or hypoxia for up to 28 days, as detailed previously (*23*). Each sample (20 μg of protein) was analysed on a SDS-polyacrylamide gel (10%) and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA). The membranes were treated overnight at 4°C in Tris–buffered saline with 0.1% Tween 20 (TBS-T), containing 5% non-fat dry milk and were washed three times each for 5 min in TBS-T at room temperature. The membranes (western blots) were then incubated with a rabbit polyclonal antibody against rat HO-1 (*34*) or mouse HO-2 (SPA-897, StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) in TBS-T for 1 h at room temperature or overnight at 4°C. The reaction mixture contained 5% non-fat milk for HO-1 antibody (a dilution of 1 : 1000) and 1% non-fat milk for HO-2 antibody (a dilution of 1 : 2000). The specific immunocomplexes were detected with a western blot kit (ECL Plus, Amersham Biosciences, Little Chalfont, UK). Expression of α-tubulin was determined as an internal control with α-tubulin antibody (Neo Markers, Fremont, CA, USA).

Northern blot analysis

Total RNA was extracted from the lung and subjected to Northern blot analysis, as detailed previously (*23*). The expression of β-actin mRNA was examined as an internal control (*23*). The cDNA probes for HO-1, HO-2 and β-actin mRNAs were labelled with [α-^{32}P] dCTP (Amersham Biosciences, UK) by the random priming method and were used as hybridization probes, as described previously (*23*).

Immunohistochemical analysis

The hearts and lungs were isolated from wild-type mice maintained under normoxia or hypoxia for 28 days. Paraffin-embedded tissues of wild-type mice were sectioned at 2.5 μm in thickness. Immunohistochemical study was performed on the deparaffinized sections using a standard labelled streptavidin–biotin method (Nichirei, Tokyo, Japan), as described previously (*28*). Rabbit polyclonal antibody to rat HO-1 (*34*) was used at a 1 : 400 dilution, and rabbit polyclonal antibody to mouse HO-2 (SPA-897, StressGen, Biotechnologies, Victoria, British Columbia, Canada) was used at a 1 : 200 dilution. Nuclei on the tissue sections were visualized with hematoxylin.

HO-2$^{-/-}$ mice

HO-2$^{-/-}$ mice with C57BL/6J5129/Sv mixed genetic background were generated as previously described (*29*). Male homozygous HO-2$^{-/-}$ mice were backcrossed with female C57BL/6J mice. After the backcross for six generations, heterozygous HO-2 mice were intercrossed and their littermates were used for this study (*10*). Tissue extracts were prepared from the lung and liver of HO-2$^{-/-}$ mice (10-week old) and age-matched wild-type mice, as described previously (*28*). These tissues were isolated from the same HO-2$^{-/-}$ mice and wild-type mice that were used to isolate the hearts and testes in our earlier report (*28*). This series of animal experiments was also performed based on the institutionally approved protocols of Tohoku University School of Medicine and Fukushima Medical University.

Statistical analysis

All data were derived from 3 to 5 animals per each treatment, and are expressed as means ± SEM. Statistical analyses were performed with two-way analysis of variance (factorial design) with a *post hoc* comparison test (Fisher's Protected Least Significant Difference exact test) with commercially available software (Statview 4.0, Carabasas, CA). Two-tailed Student's *t*-test was also used for comparison between the two groups. Differences between mean values were considered significant when $P < 0.05$.

Results

Effects of hypoxia on the pulmonary artery and expression of HO-1 and HO-2 in the lung

We have reported that C57BL/6 mice respond to normobaric hypoxia (10% O_2) by enhancing CO production, as judged by the increased CO level in the arterial blood (*23*). The arterial CO content reflects the overall heme degradation (*35*). Importantly, the increased CO level was returned to the basal level (normoxia level) after 28 days of hypoxia (*23*). In contrast, the time-course study revealed the increase in the expression levels of HO-1 and HO-2 proteins in the heart at 28 days of normobaric hypoxia, as judged by western blot analysis (*23*). To confirm whether these changes reflect the adaptation to the pressure overload, which was generated by pulmonary hypertension, we analysed the alveolar architecture and the

pulmonary arterioles (<50 μm in diameter) after 28 days of hypoxia (Fig. 1A and B). The vascular wall of the small pulmonary artery was noticeably thickened in the hypoxia-exposed mice, which is consistent with the so-called muscularized artery, associated with pulmonary hypertension (*32*, *33*). In contrast, no noticeable changes were detected in the alveolar architecture. These results support the notion that the pressure overload was indeed generated under the hypoxic conditions employed, thereby confirming the validity of the hypoxic model used.

Accordingly, using the same model of chronic hypoxemia, we analysed the expression levels of HO-1 and HO-2 proteins in the lung of the mice kept under hypoxia (Fig. 1C). Unexpectedly, no significant changes were detected in the overall expression levels of HO-1 and HO-2 proteins in the lung after 28 days of hypoxia, whereas the expression levels of HO-1 and HO-2 proteins were increased in the heart by 2.8- and 1.5-folds (Fig. 1C), respectively, as reported previously (*23*). It is also noteworthy that the mice did not show noticeable cardiac hypertrophy after 28 days of hypoxia; namely, there was no significant difference in the heart weights between normoxia (160 ± 10 mg/heart, $n = 3$) and hypoxia (180 ± 50 mg/heart, $n = 3$).

Expression levels of HO-1 and HO-2 in the lung during adaptation to hypoxia

There was no noticeable change in the expression levels of HO-1 and HO-2 proteins in the lung after 28 days of hypoxia (Fig. 1C), which prompted us to perform the time-course study to detect the possible changes during earlier phase of hypoxia. Using the same model of chronic hypoxemia, we analysed the expression profiles of HO-1 and HO-2 mRNAs and proteins in the lung of the mice kept under hypoxia (Fig. 2). However, no significant change was detected in the overall expression levels of HO-1 and HO-2 mRNAs and proteins in the lung during adaptation to normobaric hypoxia. There may be the tendency that expression levels of HO-1 and HO-2 are increased during acute phase of hypoxia (within 1 day), but the difference was not statistically significant.

Increased expression of HO-1 and HO-2 proteins in the pulmonary venous myocardium

We next analysed the expression profiles of HO-1 and HO-2 in the lung by immunohistochemistry. The expression of HO-1 and HO-2 proteins was detected in the alveolar architecture under normoxia and hypoxia (Fig. 3) and the pulmonary artery (data not shown). Moreover, HO-1 and HO-2 proteins are expressed in the pulmonary venous myocardium (Fig. 3A and C, compared with E), and their expression levels were marginally increased under hypoxia (Fig. 3B and D). We then analysed the tissue sections at higher magnification, showing that hypoxia increased the expression levels of HO-1 and HO-2 proteins in the pulmonary venous myocardium (four small panels shown in Fig. 3). Consistent in part with such a small increase, there was no significant change

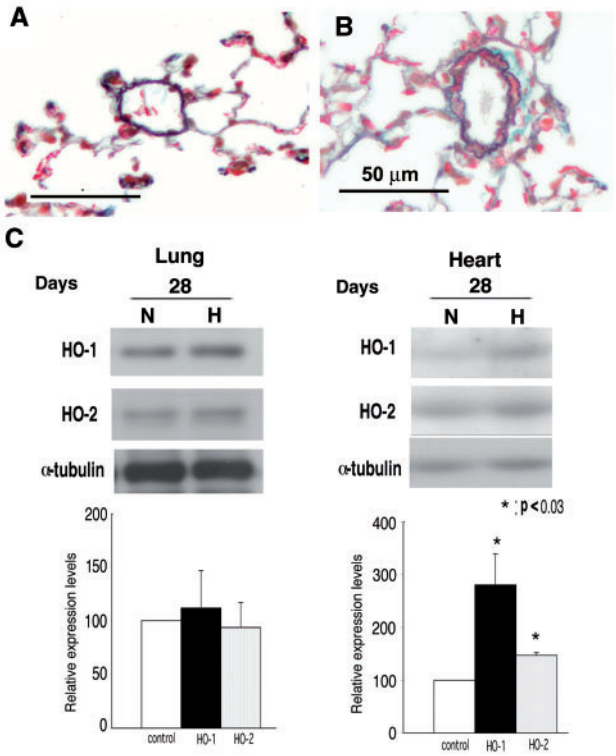

Fig. 1 Effects of hypoxia on the pulmonary artery and expression levels of HO-1 and HO-2 proteins in the lung and heart. (A) and (B) Remodeling of the pulmonary artery after adaptation to normobaric hypoxia. Wild-type C57BL/6 mice were exposed to hypoxia or kept under normoxia for 28 days. The lungs were isolated from mice exposed to normoxia (A) or hypoxia (B). Deparaffinized sections of the lung were stained with Elastica–Masson trichrome to evaluate muscularization of pulmonary arterioles: elastic lumina, stained in black-purple, collagen (green) and smooth muscle (red). A scale bar of each panel indicates 50 μm. (C) Effect of hypoxia on expression levels of HO-1 and HO-2 proteins. The hearts and lungs were isolated from wild-type mice after exposure to normobaric hypoxia (H) for 28 days or age-matched control kept under normoxia (N). The data shown are one representative of four or five independent experiments with similar results ($n = 5$ for lungs and $n = 4$ for hearts). To normalize the expression levels of HO-1 and HO-2, the western blot was reused for α-tubulin. At bottom, relative expression levels of HO-1 and HO-2 proteins are shown. The intensities of the signals in the western blots were normalized with respect to the intensity for α-tubulin. The relative expression level of HO-1 or HO-2 protein indicates the ratio of each normalized value to that of the control kept under normoxia and is shown as percentage (%). For easy comparison, the level of HO-1 or HO-2 under normoxia is shown as an open column. A symbol represents statistically significant difference compared to the respective control ($^*P < 0.03$).

in the expression levels of HO-1 and HO-2 proteins in the lungs excised from mice exposed to hypoxia by western blot analysis (Fig. 2). The undetectable change in the expression levels may also reflect the fact that the lung consists of many cell types, in which the pulmonary venous myocardium represents a small population. In contrast, the heart consists of a single predominant population of myocardium.

It is noteworthy that the pulmonary venous myocardium is thickened in HO-$2^{-/-}$ mice that manifest chronic hypoxemia (*10*). However, unlike the HO-$2^{-/-}$ mice (*10*), the pulmonary venous myocardium was not noticeably thickened in the wild-type

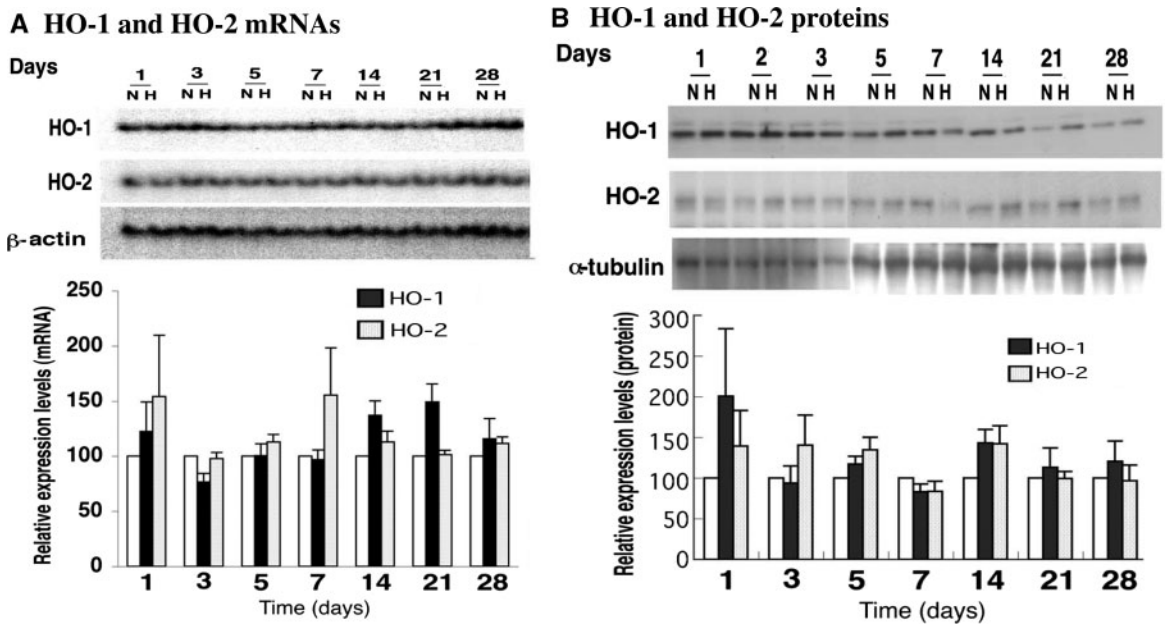

Fig. 2 Expression profiles of HO-1 and HO-2 in the lung during normobaric hypoxia. (A) Northern blot analysis of HO-1 and HO-2 mRNAs. The lungs were isolated from C57BL/6 mice after exposure to hypoxia (H) for the indicated time (day) or age-matched control kept under normoxia (N). Total RNAs were extracted from each lung tissue, and subjected to Northern blot analysis. Each lane contains 20 μg of total RNA. The bottom blot shows the expression of β-actin mRNA as an internal control. Shown is one of the three independent experiments. The bottom panel shows the relative expression levels of HO-1 and HO-2 mRNAs, representing the ratio of each normalized value under hypoxia to that of the age-matched control mice, kept under normoxia. The intensities of the signals in the northern blots were normalized with respect to the intensity for β-actin. The level of HO-1 or HO-2 mRNA under normoxia at each time point is shown as open column for easy comparison. (B) Western blot analysis of HO-1 and HO-2 proteins. The tissue extracts were prepared from the lungs, and subjected to western blot analysis. Each lane contains 20 μg protein. The data shown are one of three independent experiments with similar results. To normalize the expression levels of HO-1 and HO-2, the same western blot was reused for α-tubulin. The intensities of the signals in the western blots were quantified, and the intensity of HO-1 or HO-2 protein was normalized with respect to the intensity for α-tubulin. The relative expression level of HO-1 or HO-2 protein indicates the ratio of each normalized value to that of the respective age-matched control kept under normoxia and is shown as percentage (%) (bottom panel). For easy comparison, the level of HO-1 or HO-2 under normoxia at each time point is shown as open column. No significant difference was detected at any time points. The relative expression on day 2 was not presented (bottom panel), because no change was detected.

mice exposed to normobaric hypoxia for 28 days (Fig. 3).

Increased expression of HO-1 and HO-2 proteins in the ventricular walls

Immunohistochemical analysis revealed that HO-1 and HO-2 proteins are expressed in the entire walls of both right and left ventricles under normoxia (Fig. 4A and C, compared with E). Hypoxia increased the expression levels of HO-1 protein in the full-thickness walls of the ventricles (Fig. 4A and B). In contrast, the expression of HO-2 protein was increased mainly in the sub-endocardial regions (Fig. 4C and D). We then analysed the tissue sections at higher magnification (Fig. 4, small panels), confirming that hypoxia increased the expression levels of HO-2 protein in the sub-endocardial myocardium of the right ventricle (small panel at bottom) and the left ventricle (not shown).

Distinct expression profiles of HO-1 protein in the HO-2$^{-/-}$ mouse lung and liver

To explore the regulation of HO-1 expression in the lung under hypoxemia, we measured the expression level of HO-1 protein in the lungs of HO-2$^{-/-}$ mice, another model of chronic hypoxemia (*10*). HO-2$^{-/-}$ mice exhibit the thickening of the pulmonary venous myocardium with over-expression of HO-1 protein (*10*), which may reflect adaptation to persistent hypoxemia. However, western blot analysis revealed no noticeable difference in the overall expression level of HO-1 protein in the lung between HO-2$^{-/-}$ mice and wild-type mice (Fig. 5A), despite the increase in the expression of HO-1 protein in the pulmonary venous myocardium (*10*). These results are consistent in part with the expression level of HO-1 protein in the lung of wild-type mice during acclimatization to hypoxia (Fig. 1C). The identity of HO-2$^{-/-}$ mice was confirmed by western blot analysis of the testis; namely, HO-2 protein was undetectable in the tissue extracts from the testes of HO-2$^{-/-}$ mice (*28*). We also measured the expression level of HO-1 protein in the liver, because the expression levels of HO-1 and HO-2 proteins were transiently decreased in the liver during adaptation of wild-type C57BL/6 mice to normobaric hypoxia (*23*), which may reflect the liver-specific regulation of heme catabolism. Unexpectedly, the expression level of HO-1 protein was lower by 35% in the liver of HO-2$^{-/-}$ mice, compared to the wild-type mouse liver (Fig. 5B). These results suggest that HO-2$^{-/-}$ mice compensate for the loss of HO-2 by modulating HO-1 expression in the tissue-specific manner.

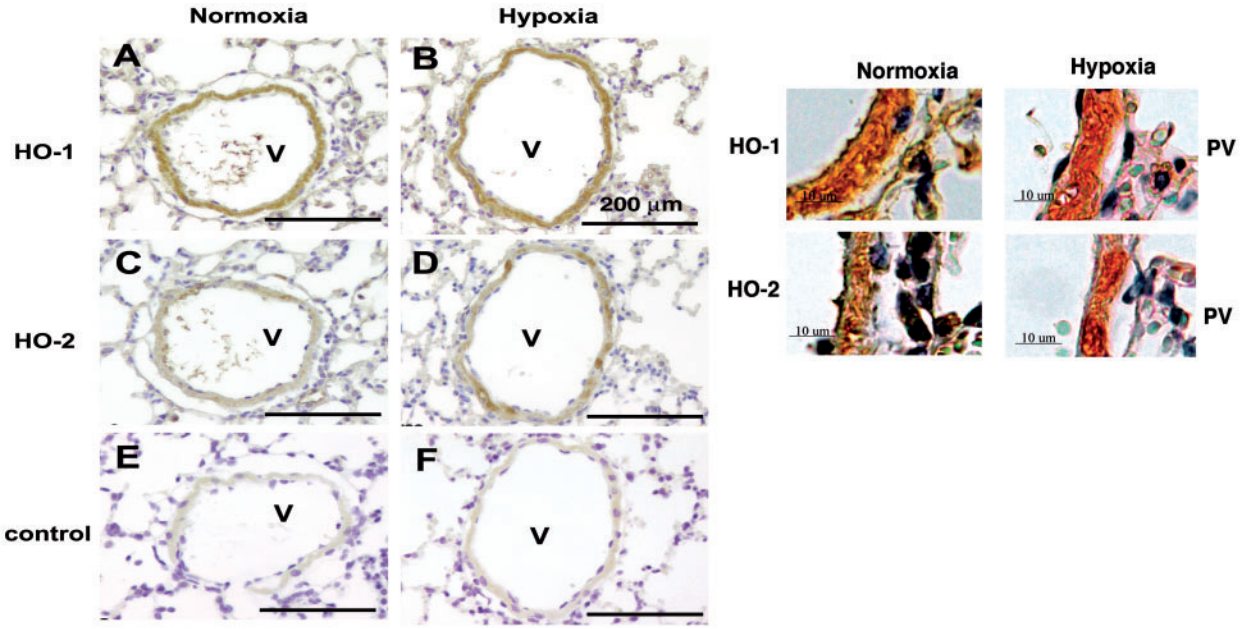

Fig. 3 Increased expression of HO-1 and HO-2 proteins in the pulmonary venous myocardium. Immuno-histochemical analysis was performed in the lungs, isolated from wild-type mice exposed to normoxia (A, C and E) or hypoxia (B, D and F) for 28 days. Tissue sections were stained with anti-HO-1 (A and B) or anti-HO-2 antibody (C and D), except for panels stained with control serum (E and F). Letter V indicates the lumen of the pulmonary vein. A scale bar of each panel indicates 200 μm. Four small panels at right side show the portion of the pulmonary venous myocardium at higher magnification (scale bar, 10 μm), stained with anti-HO-1 antibody (top panels) or anti-HO-2 antibody (bottom panels). PV indicates the pulmonary venous myocardium.

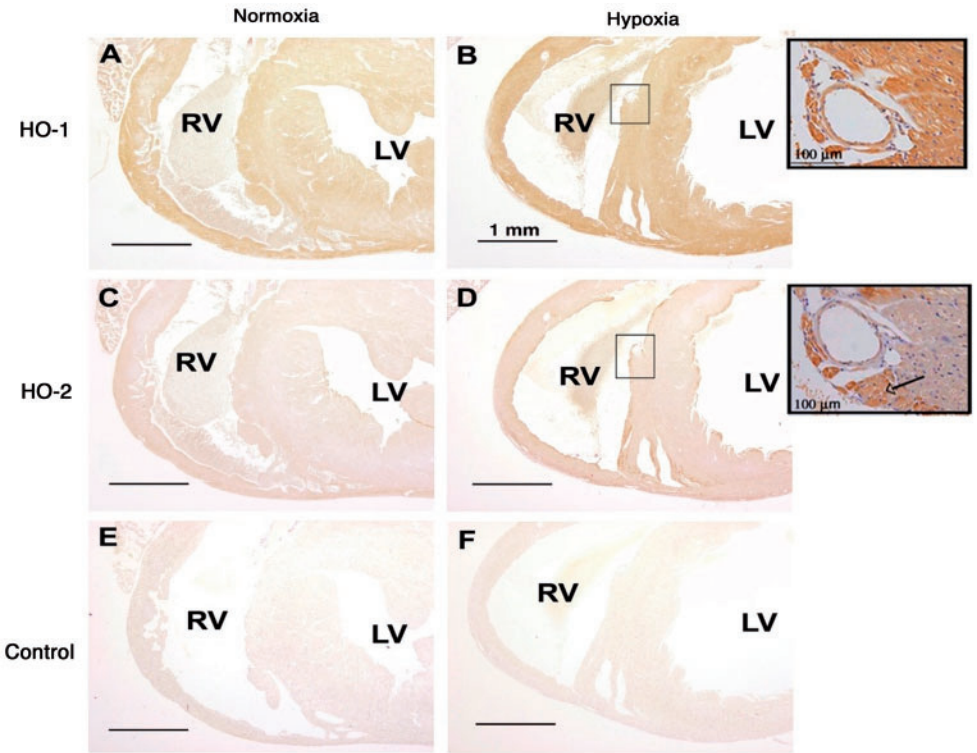

Fig. 4 Hypoxia increases expression of HO-1 and HO-2 proteins in the ventricular walls. Immuno-histochemical analysis was performed in the hearts, isolated from mice exposed to normoxia or hypoxia for 28 days. Tissue sections of both ventricles were stained with anti-HO-1 (A and B), anti-HO-2 antibody (C and D) or control serum (E and F). A scale bar of each panel indicates 1 mm. Right ventricle, RV and left ventricle, LV. A selected region of the RV, marked with a square, is enlarged and shown at the right side of LV in B and D (a scale bar, 100 μm). Note that the induction of HO-2 is localized in the sub-endocardial regions (arrow).

Discussion

In this study, using normobaric hypoxia as a means to generate hypoxemia in mice, we show for the first time that the expression of HO-1 and HO-2 proteins is increased in the pulmonary venous myocardium after acclimatization to hypoxemia. In addition, the expression of HO-2 protein is increased in the sub-endocardial myocardium of the right and left ventricles, whereas the expression of HO-1 protein is increased in the entire walls of both ventricles. The increase in the expression of HO-1 and HO-2 proteins in the myocardium may reflect the adaptation to hemodynamic stress. In contrast, there is no significant change in the expression of HO-1 and HO-2 mRNAs and proteins in the lung during acclimatization to hypoxia. Likewise, the expression level of HO-1 protein in the lung is similar between wild-type mice and HO-$2^{-/-}$ mice, another model of mild hypoxemia (*10*).

The unexpected finding of the present study is the increased expression of HO-2 protein restricted to the sub-endocardial myocardium. Importantly, the sub-endocardial myocardium is linked to the Purkinje conducting system located beneath the endocardial endothelial cells (*36*), and is responsible for synchronized contraction of the right and left ventricles. Because HO-2 is involved in Ca signaling (*37*), it is conceivable that HO-2 may play a regulatory role in Purkinje cells, the main function of which depends on Ca dynamics (*36*). In this context, it has been reported that HO-2 interacts with α-subunit of a large conductance, calcium-sensitive potassium channel (the BK channel) (*11*). Considering the regulatory role of HO-2 for the ventilation–perfusion matching (*10*,*25*), we speculate that HO-2 may also contribute to matching coronary blood flow to myocardial oxygen consumption.

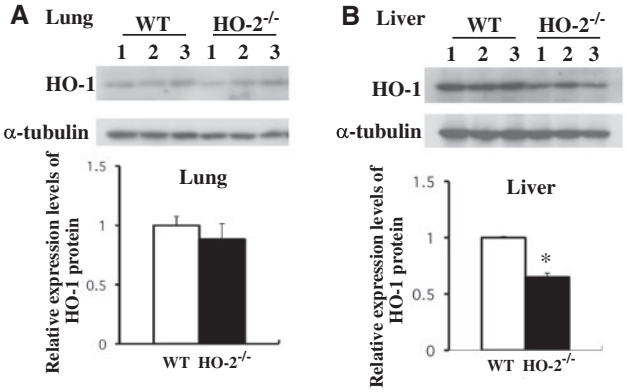

Fig. 5 Expression levels of HO-1 protein in the lung and liver of HO-$2^{-/-}$ mouse. The tissue extracts were prepared from the lungs (A) and livers (B) of age-matched wild-type mice (10 weeks) and HO-$2^{-/-}$ C57BL/6 mice ($n = 3$ for each group of mice), and subjected to western blot analysis. Top panels show western blot analysis of HO-1 and α-tubulin proteins. Bottom panels show the relative expression levels of HO-1 protein in each organ. To normalize the expression levels of HO-1, the same western blot was reused for α-tubulin. The intensities of the signals in the western blots were quantified, and the intensity of HO-1 protein was normalized with respect to the intensity for α-tubulin. The relative expression level of HO-1 protein indicates the ratio of each normalized value to that of the respective control and is shown as ratio. The data shown are one of two independent experiments with similar results. The data shown are derived from three animals. An asterisk represents statistically significant differences compared to the wild-type mouse liver ($^*P < 0.001$). No significant difference was detected in the lung.

The pulmonary veins have been largely ignored until recently, as they are regarded as passive conduits. Recently, the pulmonary venous myocardium attracts much attention, because atrial fibrillation frequently originates from the pulmonary venous myocardium (*38*,*39*). We have reported that expression of lipocalin-type prostaglandin (PG) D synthase (L-PGDS) is induced in the pulmonary venous myocardium as well as in the myocardium of both ventricles of C57BL/6 mice kept under normobaric hypoxia (*28*). L-PGDS catalyzes the isomerization of PGH_2 to produce PGD_2 (*40*), and PGD_2 exerts various functions (*40*), including induction of HO-1 expression in cultured human cell lines (*41*,*42*). We have, therefore, proposed the regulatory network involving L-PGDS/PGD_2 and HO-1 (*43*). In this context, expression of L-PGDS mRNA and protein was not detectable in the same preparations of the lung total RNAs and protein extracts used in the present study, as judged by Northern blot and western blot analyses (data not shown). It is also noteworthy that carboxyhemoglobin level is significantly higher in the arterial blood than that in the central venous blood, taken from the right atrium, in critically ill patients and healthy individuals prior to orthopedic surgery (*44*). Considering the results of the present study, we suggest that a substantial amount of CO may be produced and released from the pulmonary vasculatures. In fact, the increased expression of HO-1 protein is associated with bilirubin IXα accumulation within the aortic walls in a rabbit model of atherosclerosis, suggesting that heme is actually degraded in the vascular wall by the induced HO-1 (*45*). It is, therefore, conceivable that HO-1 and HO-2 in the pulmonary venous myocardium may be responsible for the production of CO, thereby causing the arterio–venous CO difference.

Unlike the HO-$2^{-/-}$ mice, the pulmonary venous myocardium was not thickened in the wild-type mice exposed to normobaric hypoxia for 28 days, suggesting that the normobaric hypoxic condition employed is not sufficient to cause hypertrophy of the pulmonary venous myocardium. In fact, the thickening of the pulmonary venous myocardium has been shown to represent the adaptation to high-altitude hypoxia with low-barometric pressure in mice (*27*) and humans (*26*).

The over-expression of HO-1 protein was reported in the lung of HO-$2^{-/-}$ mice by other investigators (*46*), which is, however, different from the present study. We do not know the reason that could account for the discordant result. In this connection, we have shown that the expression levels of HO-1 protein are higher by 1.7-fold in the testis and by 2.5-fold in the heart of HO-$2^{-/-}$ mice than those in the respective tissue of wild-type mice (*28*). It is also noteworthy that the expression level of HO-1 protein is significantly lower in the liver of HO-$2^{-/-}$ mice than that

in the wild-type liver. Importantly, we isolated testes, hearts, lungs and livers from the same HO-2$^{-/-}$ mice and wild-type mice. Considering the facts that the CO contents in the arterial blood are similar between the HO-2$^{-/-}$ mice and the wild-type mice (*10*, *29*) and that heme level remained unchanged in various tissues of HO-2$^{-/-}$ mice (*47*), we suggest that HO-2$^{-/-}$ mice maintain the overall heme catabolism by increasing, keeping or decreasing the expression level of HO-1, depending on the organs. In this connection, the mutant mouse with the hepatocyte-specific deletion of HO-1 gene, generated by the conditional knockout method, are viable and exhibit no severe phenotype under basal conditions, despite that only 30% levels of HO-1 are expressed in the mutant mouse liver (*48*). It is, therefore, conceivable that the amount of heme catabolism may be maintained in the liver of HO-2$^{-/-}$ mice, despite the 35% decrease in HO-1 protein.

The decrease in the HO-1 expression in the HO-2$^{-/-}$ liver is of particular interest, with respect to the liver-specific regulation of heme catabolism. In case of the human liver, expression of HO-1 protein appears to increase or decrease, depending on the local hemodynamic status. HO-1 is expressed mainly in a subpopulation of Kupffer cells in the liver of control subjects (*49*). However, in cirrhotic livers with portal hypertension, which is associated with the increase in intrasinusoidal resistance and regenerative changes in the liver parenchyma, HO-1 protein is expressed in Kupffer cells and hepatocytes. Likewise, in acute hepatic failure, HO-1 expression is increased in hepatocytes (*50*). In contrast, there is a significant decrease in the HO-1 protein expression in Kupffer cells and hepatocytes in idiopathic portal hypertension that are characterized by an increase in pre-sinusoidal resistance (*49*). These results indicate that the expression level of HO-1 protein is dynamically changed in the liver, depending on the cellular microenvironments.

In summary, we have provided the *in vivo* evidence for the inter-tissue and inter-cell differences in the regulation of expression of HO-1 and HO-2 proteins under hemodynamic stress. Moreover, HO-2$^{-/-}$ mice maintain the overall heme catabolism by resetting the expression level of HO-1 in a tissue-specific manner, suggesting that HO-2 may be involved in the regulation of HO-1 expression under certain conditions.

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Conflict of Interest

None declared.

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