Physiol. Res. 52: 147-157, 2003

Effect of Intermittent High Altitude Hypoxia on Gene Expression in Rat Heart and Lung

E. DEINDL, F. KOLÁŘ[1], E. NEUBAUER, S. VOGEL, W. SCHAPER, B. OŠŤÁDAL[1]

Department of Experimental Cardiology, Max-Planck-Institute for Physiological and Clinical Research, W. G. Kerckhoff Institute, Bad Nauheim, Germany and [1]Institute of Physiology, Academy of Sciences of the Czech Republic and Center for Experimental Cardiovascular Research, Prague, Czech Republic

Received March 8, 2002
Accepted June 6, 2002

Summary

Hypoxia has been identified as an important stimulus for gene expression during embryogenesis and in various pathological situations. Its influence under physiological conditions, however, has only been studied occasionally. We therefore investigated the effect of intermittent high altitude hypoxia on the mRNA expression of different cytokines and protooncogenes, but also of other genes described to be regulated by hypoxia, in the left ventricle (LV), the right ventricle (RV), atria and the lung of adult rats after simulation of hypoxia in a barochamber (5000 m, 4 hours to 10 days). Heme oxygenase-1 as well as transforming growth factor-β_1 showed an increased expression in all regions of the heart and the lung at different periods of hypoxia. For lactate dehydrogenase-A, we found a significant up-regulation in the RV and the lung, for lactate dehydrogenase-B up-regulation in the RV, but down-regulation in the LV and the atria. Vascular endothelial growth factor was up-regulated in the RV, the LV and the lung, but down-regulated in the atria. Its receptor Flk-1 mRNA was significantly increased in the atria and RV only. Expression of c-fos was found in the LV and RV only after 4 hours of hypoxia. The level of c-jun was significantly increased in the LV but decreased in the atria. Our data clearly demonstrate that intermittent hypoxia is a modulator of gene expression under physiological conditions. It differently regulates the expression of distinct genes not only in individual organs but even within one organ, i.e. in the heart.

Key words
Hypoxia regulated genes • Transforming growth factor-β • Vascular endothelial growth factor • c-jun • c-fos

Introduction

The most frequent diseases of modern times undoubtedly include hypoxic states of the cardiopulmonary system. They originate as a result of disproportion between the amount of oxygen supplied to the tissue and the amount actually required. The degree of hypoxic injury depends, however, not only on the intensity and duration of the hypoxic stimulus but also on the level of cardiac tolerance to oxygen deprivation. It has been repeatedly observed that cardiac tolerance to acute oxygen deprivation can be significantly increased by previous adaptation to permanent or intermittent hypoxia, both in populations living at high

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

altitude (Hurtado 1960) and in animals under experimental conditions (Poupa *et al.* 1966, Widimský *et al.* 1973, Asemu *et al.* 1999, Baker *et al.* 1999, Neckář *et al.* 2002). In addition to the protective effect, adaptation to chronic hypoxia may induce adverse influences on the cardiopulmonary system. It exerts opposite effects on the systemic and pulmonary vascular smooth muscle, bringing about vasodilation in the systemic but vasoconstriction and consequently structural remodeling in the pulmonary circulation. Resulting hypoxic pulmonary hypertension leads to right ventricular hypertrophy and eventually even to congestive heart failure. Both ventricles are therefore under the influence of chronic hypoxia, but the right ventricle has also to cope with an increased afterload. The most frequently used experimental model in research on chronic hypoxia is that of high altitude, either in a mountain environment or simulated under laboratory conditions in a normobaric or hypobaric chamber. This model permits to study the time-course of development of beneficial and adverse adaptive changes (Ošťádal *et al.* 1994).

Hypoxia has been demonstrated to stimulate the expression of several genes during embryogenesis or in various pathological situations. No data are, however, available regarding possible cardiac gene expression induced by intermittent high altitude (IHA) hypoxia, which represents a more physiological situation. Furthermore, to our knowledge, the influence of hypoxia on different regions of the heart has never been analyzed in detail. In order to contribute to the understanding of molecular mechanisms involved in the process of cardiac adaptation to chronic hypoxia, we studied the effect of simulated IHA hypoxia on the expression of different cytokines and protooncogenes in the heart as well as in the lung. We hypothesized that the gene expression pattern will differ in individual parts of the heart, which cope with a different hemodynamic load under hypoxic conditions. The selection of genes and time points is essentially based on our previous study dealing with a non-hypoxic model of pulmonary hypertension (Bauer *et al.* 1997). Thus, we analyzed the expression level of vascular endothelial growth factor (VEGF) and its signaling receptor Flk-1 (fetal liver kinase). VEGF (for a review see Neufeld *et al.* 1999) is an important angiogenic factor, which is likely to participate in the process of capillary sprouting as shown before in hypertrophied chronically hypoxic hearts (Partovian *et al.* 1998). We also investigated the expression level of transforming growth factor β_1 (TGF-β_1; for a review see Lawrence 1996) as this cytokine might contribute to the development of myocardial fibrosis. Furthermore, we examined the mRNA level of c-fos and c-jun, two protooncogenes, which might – in the form of activator protein-1 (AP-1) heterodimer – participate in the regulation of TGF-β_1 expression. Last but not least, we investigated the expression levels of lactate dehydrogenase-A (LDH-A), lactate dehydrogenase-B (LDH-B) and heme oxygenase-1 (HO-1) as markers of hypoxia. The expression level of the described genes was investigated in the left ventricle (LV), right ventricle (RV), the atria and the lung during early phase of adaptation of rats to IHA hypoxia.

Methods

Experimental model

Adult male Wistar rats (220-330 g body weight) were exposed to IHA hypoxia simulated in a hypobaric chamber (5000 m) for 4 hours (n=5), 1 day (n=5), 3 days (n=4), 5 days (n=4) or 10 days (n=6) (8 hours of exposure per day). The control (normoxic) group (n=9) was kept for the corresponding period in an atmosphere equivalent to an altitude of 200 m. All animals had free access to water and a standard laboratory diet. The animals were sacrificed by decapitation within 30 min after the last exposure, the hearts and lungs (upper right lobe) were removed and the hearts were dissected into atria and free walls of the RV and LV. All tissue samples were frozen in liquid nitrogen and stored at –80 ^{o}C until further analysis. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

RNA isolation and Northern blot

Total RNA was isolated according to the method of Chomzynski and Sacchi (1987) from frozen tissue samples of the LV, RV, the atria and the lung of experimental and control animals. Fifteen micrograms of total RNA from control or experimental animals were size-fractionated per slot on a 1 % agarose gel containing 0.66 M formaldehyde. The integrity of the RNA was judged under UV light. After capillary transfer to a Hybond-N^+ membrane (Amersham) using 10 X SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) as transfer buffer, the fixation of the RNA to the filter followed by means of ultraviolet crosslinker (Stratagene). Northern hybridization was carried out according to a standard procedure (Sambrook *et al.* 1989).

Molecular probes

For Northern blot analysis, cDNA probes were randomly prime labeled to a specific activity of about 10^8 cpm.μg^{-1} using a *redi*prime labeling system (Amersham) and 40 µCi of (α-^{32}P)dCTP (3000 Ci·mmol^{-1}). cDNA probes used in our studies were as follows: LDH-A, human, 1700 bp, (ATCC); LDH-B, human, 1600 bp, (ATCC); VEGF, human, 520 bp (kindly provided by K. Weindel); Flk-1 (mouse, 2600 bp, kindly provided by G. Breier); TGF-β_1 (rat, 1700 bp, kindly provided by S.H. Hall), c-fos (mouse, 720 bp, kindly provided by H. Sharma); c-jun (mouse, 2600 bp, ATCC); 18 s (mouse, 770 bp, kindly provided by I. Oberbäumer). For HO-1 detection we used an oligonucleotide (20 mer, GCGTGCGCCACCAGCAGCTC, complementary to Mus musculus NM-010442.1 pos. 526 – 507) which was 5´ radiolabeled according to standard procedures (Sambrook *et al.* 1989).

Immune precipitation and immunoblotting

Frozen tissue samples were ground under liquid N_2, placed in protein extraction buffer (50 mM Tris/HCl at pH 7.4, 250 mM sucrose, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 % glycerol, 1 % Triton, 1 mM DTT, 0.5 mM PMSF) and homogenized by 50 strokes in an ice-cold Dounce homogenizer. The lysates were centrifuged at 15 000 x *g* at 4 °C for 30 min. Both 1 µg of a HIF-1α specific monoclonal antibody (Transduction Laboratories) and 30 µl of ProteinA-Sepharose (Santa Cruz Biotechnology) were added to 80 µg of protein extracts from either hypoxia-treated or control tissue and incubated on a rotating wheel at 4 °C overnight. The precipitates were washed. The beads were resuspended in 4x LDS sample buffer (Novex) and boiled for 10 min. Proteins were separated by using 3-8 % Tris-Acetate gels (Novex) and the gel electrophoresis was performed under non-reducing conditions. Following protein transfer to a nitrocellulose membrane (Novex), this was blocked for 2 h at RT with 5 % milk in TBS-T. The incubation with the primary antibody (anti-HIF-1α, dilution 1:500) occurred overnight at 4 °C, followed by incubation with the secondary antibody for 1 h at room temperature. Detection of immunoreactive bands was performed with the ECL-system (Amersham).

Quantification and statistical analysis

Signals were quantified with a PhosphorImager (Molecular Dynamics) using ImageQuant software. For normalization, the data of each hybridization signal were divided by the value of the matching 18S rRNA signal. The average of the data obtained from the control animals was used as 100 %. All data were presented as means ± S.E.M. The ANOVA and subsequent multiple comparisons by the Bonferroni test were used for statistical analysis and the results were considered as statistically significant at $p<0.05$.

Table 1. Heart weight parameters

Group	n	LV/BW [%]	RV/BW [%]	RV/LV
Controls	9	0.133±0.005	0.052±0.002	0.404±0.019
A4 h	5	0.133±0.008	0.052±0.001	0.393±0.016
A1	5	0.147±0.003	0.058±0.002	0.394±0.014
A3	4	0.142±0.003	0.061±0.003	0.427±0.014
A5	4	0.149±0.004	0.066±0.004*	0.447±0.030
A10	6	0.145±0.003	0.065±0.002*	0.450±0.008*

*Animals exposed to IHA hypoxia for 4 h (A4h), one day (A1), 3 days (A3), 5 days (A5) and 10 days (A10); RV/BW, right ventricular weight normalized to body weight; LV/BW, left ventricular weight normalized to body weight; RV/LV, right to left ventricular weight ratio. Data are mean ± S.E.M. *p<0.05 vs controls*.

Results

Weight parameters

Body weight of animals did not significantly differ between the groups. Exposure to IHA hypoxia led to RV hypertrophy: the RV/BW ratio increased significantly after 5 and 10 daily exposures for 8 h each. As the LV/BW ratio remained unchanged, the RV/LV index increased, reaching statistically significant difference from the controls after 10 exposures (Table 1).

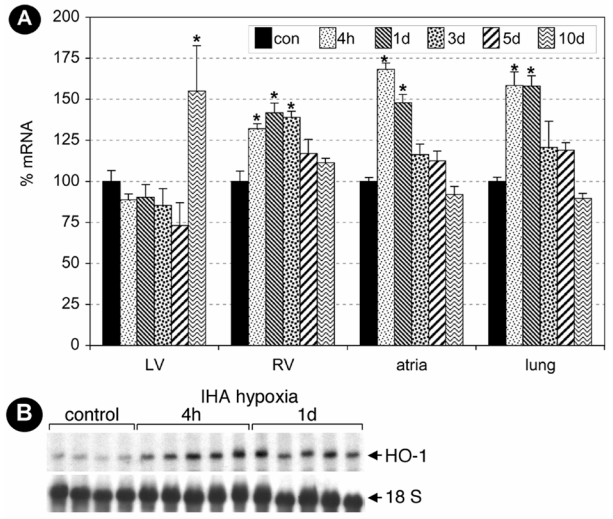

Fig. 1. A. *Bar graphs representing the time course of the HO-1 transcript level in the LV, the RV, the atria and the lung in control (con) and experimental tissue samples (from 4 hours up to 10 days of IHA hypoxia).* ***B.*** *Representative Northern blot showing the mRNA level of HO-1 in the atria of control animals and after 4 hours and 1 day of IHA hypoxia (top). For normalization, the hybridization signal of HO-1 was divided by the value of the matching 18S rRNA signal (bottom). * $p<0.05$ vs controls.*

IHA hypoxia divergently regulates the expression of LDH-A, LDH-B and HO-1

Repetitive cycles of hypoxia resulted in a significant up-regulation of the HO-1 mRNA in all regions of the heart and the lung (Fig. 1). In the LV, we found 1.5-fold values of the controls on day 10, in the RV about 1.3- to 1.4-fold values at hour 4, day one and day 3, and in the atria and the lung about 1.5- to 1.7-fold values of controls at hour 4 and day 1. For LDH-A, our results displayed in the RV a significant mRNA induction of about 1.4- to 1.6-fold values of controls at all intervals analyzed (Fig. 2A). In the LV and atria, however, IHA hypoxia did not lead to an altered LDH-A expression (Fig. 2A, 2B). In the lung, the transcript was up-regulated to values of about 1.75- to 2-fold of controls at hour 4 and day one (Fig. 2A). For LDH-B, our results displayed a minor but significant up-regulation of the mRNA (about 1.25-fold of control) exclusively in the RV from hour 4 up to day 5 (Fig. 2C). In the LV and atria, we found a significant down-regulation to about 70 % of the control on day one (LV) and from day 3 to day 10 (atria, Fig. 2C). In the lung, however, the expression level of LDH-B was not influenced by IHA hypoxia (Fig. 2C).

The protein level of hypoxia-inducible factor-1α (HIF-1α)

To define the influence of IHA hypoxia on the protein level of HIF-1α we performed Western blot analysis on tissue samples of the RV and the lung of control and experimental animals (IHA hypoxia for 3 days). The results displayed no change of the protein level of HIF-1α in the RV and the lung of experimental compared to control animals (Fig. 3, data shown for the RV).

The influence of increased work load and/or hypoxia on the expression of VEGF

Analyzing the expression level of VEGF (VEGF-A, Fig. 4A, 4B) by means of the Northern blot, we found a significant up-regulation (3.5-fold) of the corresponding mRNA on day 3 and a minor one (2-fold) on day 5 in the LV compared to the controls. For the same periods of time, an up-regulation in the RV was found, however, the induction was smaller (although significant on day 3). For the atria, our results showed a significant down-regulation of the transcript to about 50 % of controls on day 10. The most prominent alteration in the expression level of VEGF was detected in the lung. Northern blot results displayed a significant up-regulation of about 3.7-fold of controls on day 3, of about 3.4-fold on day 5 and of about 1.5-fold on day 10. For VEGF-B, a transcript which shows cross-hybridization with our cDNA probe for VEGF-A, we found values comparable to the controls in all experimental tissue samples (Fig. 4B).

IHA hypoxia is not sufficient to induce Flk-1 expression in the lung

For Flk-1, the VEGF-receptor 2, we found an up-regulation of the mRNA on days 3 and 5 in the LV similar to VEGF, but this was not significant (Fig. 5). In the RV, we saw a continuous up-regulation of the Flk-1

mRNA culminating on day 3 in a significant 2.8-fold value of the controls. On day 5, the value decreased to a 2.5-fold value and on day 10 to a 1.9-fold value (Fig. 5). A significant up-regulation of the Flk-1 mRNA was also detectable in the atria at hour 4 and day one (2-fold of controls, Fig. 5). However, in the lung we could not show a significant change in Flk-1 mRNA expression in response to IHA hypoxia (Fig. 5).

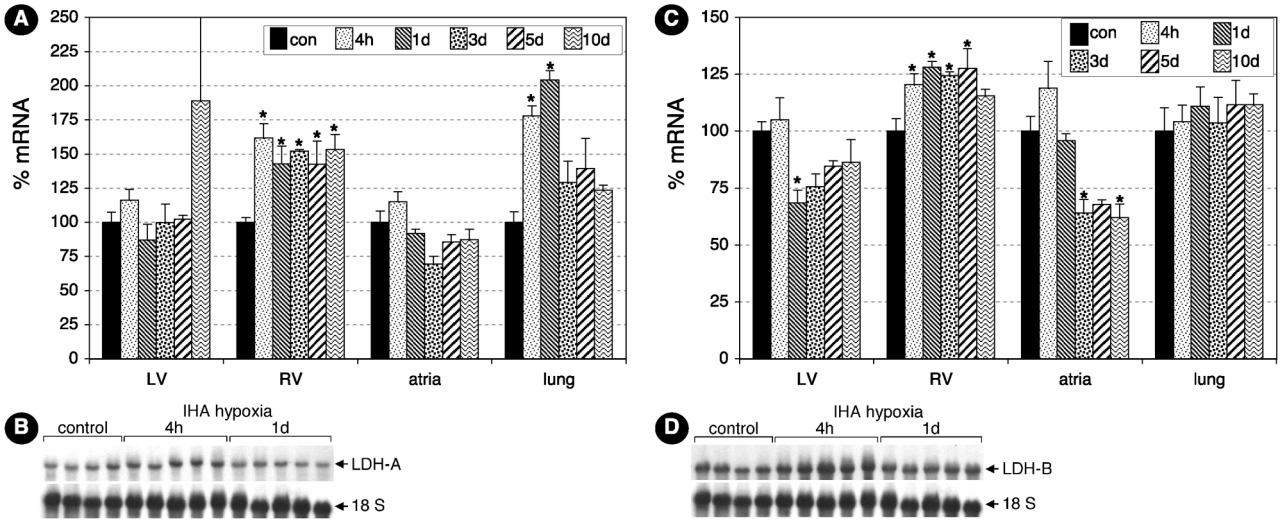

Fig. 2. *The expression level of LDH-A (***A***) and LDH-B (***C***) in the distinct regions of the heart and the lung in control (con) and experimental tissue samples (from 4 hours up to 10 days of IHA hypoxia).* **B, D.** *Representative Northern blots showing the transcript level of LDH-A (B, top) and LDH-B (D, top) in the atria of control and experimental tissue samples (4 hours and 1 day of IHA hypoxia). To control rRNA loading, the blots were rehybridized with a probe specific for 18S rRNA (bottom). * p<0.05 vs controls.*

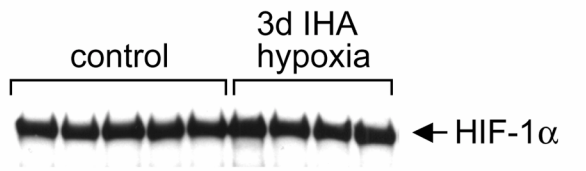

Fig. 3. *Western blot showing the protein level of HIF-1α in the RV of control and experimental tissue samples (3 days IHA hypoxia).*

Up-regulation of TGF-β_1 in individual regions of the heart and the lung

Northern blot results displayed for TGF-β_1 (Fig. 6) a slight but significant up-regulation of the corresponding transcript in the LV on day 3 and in the RV on day one. After a continuous down-regulation of the mRNA to control levels on day 5 in the RV, the expression level of TGF-β_1 showed a second greater peak on day 10 with a value of about 2.3-fold of controls. In the atria, we again found a slight but significant up-regulation at a very early period, i.e. at 4 hours. For the lung, our results demonstrated a slight but significant up-regulation of the TGF-β_1 mRNA on day one.

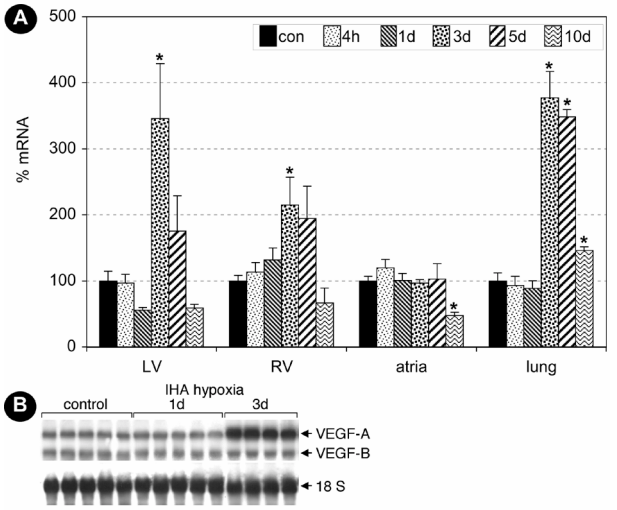

Fig. 4. A. *The mRNA level of VEGF (VEGF-A) in the LV, the RV, the atria and the lung in control (con) and experimental tissue samples (from 4 hours up to 10 days of IHA hypoxia).* **B.** *Representative Northern blot showing the mRNA level of VEGF-A and VEGF-B in the lung of experimental animals after 1 day and 3 days of IHA hypoxia and in the lung of control animals (top). Bottom, the expression level of the 18S rRNA. * p<0.05 vs controls.*

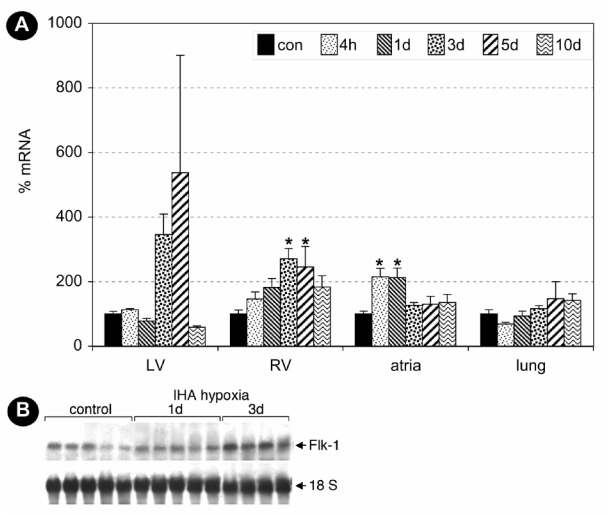

Fig. 5. A. *the expression level of Flk-1 in the distinct regions of the heart and the lung in control (con) and experimental tissue samples (from 4 hours up to 10 days of IHA hypoxia).* **B.** *Representative Northern blot showing the mRNA level of Flk-1 in the lung of control and experimental animals after 1 day and 3 days of IHA hypoxia (top). For normalization, the blot was rehybridized with a probe specific for 18S rRNA (bottom). * p<0.05 vs controls.*

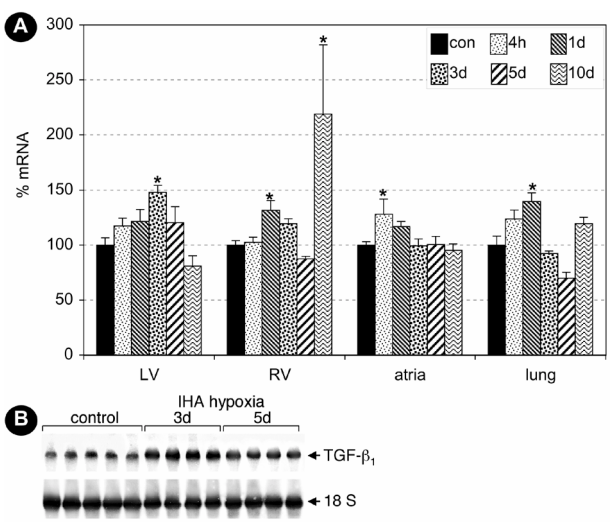

Fig. 6. A. *Bar graphs showing the mRNA level of TGF-β_1 in the LV, the RV, the atria and the lung in control (con) and experimental tissue samples (from 4 hours up to 10 days of IHA hypoxia).* **B.** *Representative Northern blot showing the transcript level of TGF-β_1 in the LV of control and experimental animals after 3 days and 5 days of IHA hypoxia (top). To control rRNA loading, the blots were rehybridized with a probe specific for 18S rRNA (bottom). * p<0.05 vs controls.*

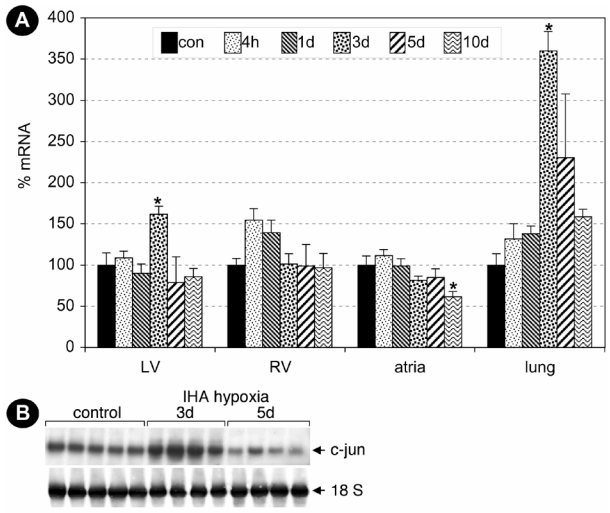

Fig. 7. A. *The time course of the mRNA level of c-jun in the LV, the RV, the atria and the lung in control (con) and experimental tissue samples (from 4 hours up to 10 days of IHA hypoxia).* **B.** *Representative Northern blot showing the transcript level of c-jun in the LV after 3 days and 5 days of IHA hypoxia and in control animals (top). For normalization, the hybridization signal of c-jun was divided by the value of the matching 18S rRNA signal (bottom). * p<0.05 vs controls.*

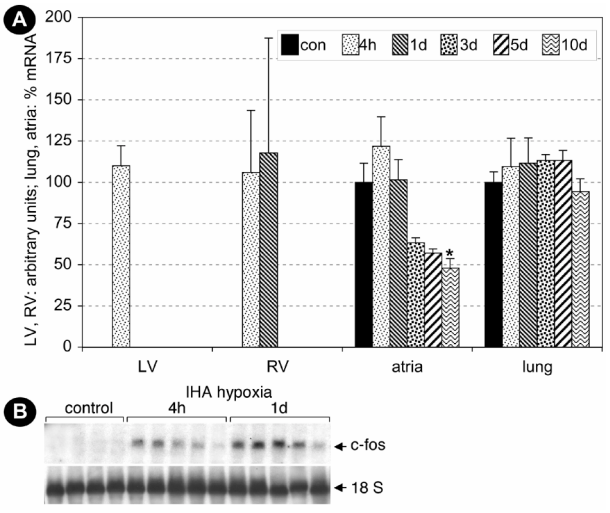

Fig. 8. A. *The mRNA level of c-fos in the distinct regions of the heart and the lung in control (con) and experimental tissue samples (from 4 hours up to 10 days of IHA hypoxia).* **B.** *Representative Northern blot showing the mRNA level of c-fos in the RV of control animals and after 4 hours and 1 day of IHA hypoxia (top). To control rRNA loading, the blots were rehybridized with a probe specific for 18S rRNA (bottom).* * p<0.05 vs controls.*

The protooncogene c-jun is up-regulated in the lung

To characterize the influence of IHA hypoxia on the expression of c-jun, we analyzed the level of the corresponding mRNA by means of the Northern blot in different regions of the heart and the lung (Fig. 7). Our data revealed no appreciable alteration of the level of c-jun in the ventricular myocardium, except for an increase on day 3. For the atria, our results showed a significant down-regulation on day 10. However, in the lung the mRNA-level of c-jun was significantly increased on day 3.

Involvement of c-fos

Analyzing the mRNA abundance of c-fos (Fig. 8), we were not able to detect a basal expression of the transcript in the LV or RV. However, we could prove the transcript after 4 h of hypoxia in the LV and after 4 h and one day in the RV. In the atria and in the lung, we found a basal expression level of c-fos in all tissue samples analyzed. Except for the significant down-regulation of c-fos in the atria on day 10, we observed no significant change in the mRNA level under hypoxic conditions.

Discussion

In the present study we analyzed the influence of IHA hypoxia on the expression of distinct hypoxia regulated genes, cytokines and protooncogenes in the heart and the lung of an *in vivo* rat model. Our data clearly show that hypoxia is a direct or indirect modulator of gene expression under these physiological conditions. The two organs and even individual regions of a single organ (the heart) react differently to hypoxia in gene expression. Significant regional differences in the time course and degree of gene expression in the myocardium are probably due to different workload imposed on the various hypoxic regions.

Our results have shown that HO-1, a microsomal membrane enzyme that catalyzes the first and rate-limiting reaction in heme catabolism, was up-regulated in all regions of the heart and the lung. The observed regional differences in the time course and the degree of up-regulation point to a complex regulation of gene expression. For LDH-A (which is like LDH-B a subunit of the active LDH enzyme), we found an up-regulation exclusively in the RV and the lung. HO-1 and LDH-A contain like VEGF a hypoxia-inducible factor-1 (HIF-1) binding site (Levy *et al.* 1995, Liu *et al.* 1995, Semenza *et al.* 1996, Lee *et al.* 1997). HIF-1 (α/β heterodimer) is a basic helix-loop-helix transcription factor (for review see Semenza 2000) that transactivates genes encoding proteins that participate in the homeostatic response to hypoxia (Wang *et al.* 1995). Except for the atria, LDH-A and HO-1 were up-regulated at the same periods of IHA hypoxia in the heart and the lung. VEGF, however, was up-regulated at quite different time points. The increased transcript levels of VEGF observed in our model might therefore be not only due to an increased transcription mediated by the binding of HIF-1 to its binding site on the VEGF promoter, but may also be due to mRNA stabilization mediated by proteins that bind to sequences located in the 3‘ untranslated region of VEGF (Claffey *et al.* 1998). The expression of HIF-1 itself is also promoted by both permanent and intermittent hypoxia (Prabhakar *et al.* 2001). Although increased mRNA levels of the components of HIF-1 have been reported, the majority of studies suggested that the regulation must occur by either translational or posttranslational mechanisms (Gleadle and Ratcliffe 1998). Aimee *et al.* (1988) have recently described that HIF-1 is up-regulated in a hypoxia-dependent manner on the protein level in ferret lungs. On reoxygenation, however, HIF-1 was rapidly degraded, with a half-life of less than one minute. Using Western blot analysis we found no change in the protein level of HIF-1α in the distinct regions of the heart and the lung in experimental as compared to control animals. This was probably due to HIF-1α ubiquity and proteosomal degradation induced during periods of normoxia in our model of IHA hypoxia.

Buono and Lang (1999) demonstrated that LDH-B mRNA decreased in primary rat and chicken cells in response to hypoxia but it increased to control values after reoxygenation. Our results displayed a significant down-regulation of LDH-B in the LV and the atria, i.e. in those tissue samples where we found unchanged mRNA levels for LDH-A. However, in the RV and the lung, where we found a significant up-regulation of LDH-A, our results displayed an unchanged expression level for LDH-B. An exception occurred in the RV where a minor, although significant up-regulation of LDH-B was observed, which might be due to an overshoot reaction after reoxygenation. Since LDH-B preferentially converts lactate to pyruvate under aerobic conditions and LDH-A preferentially converts pyruvate to lactate under anaerobic conditions, the two isoenzymes have to be regulated in an opposite way, which is supported by our data.

For TGF-β_1, we found a significant up-regulation of the mRNA in the LV on day 3 of IHA hypoxia; in the RV the initial increase on day one was

followed by an even stronger induction of the gene after 10 days. TGF-β_1 has been shown to increase the biosynthesis of collagen types I and III in various *in vivo* and *in vitro* systems (Eghbali 1989, Eghbali *et al.* 1991). The myocardial extracellular matrix is mainly composed of fibrillar collagens (Medugorac and Jacob 1983), where collagen I and III represent more than 80 % of all collagen types (McClain 1974, Medugorac and Jacob 1983, Pelouch *et al.* 1985). Our results on the weight parameters showed a moderate but significant increase of the RV/BW ratio on days 5 and 10 of IHA hypoxia and of the RV/LV ratio on day 10; these effects are more pronounced after the extension of hypoxic exposure to 5 weeks (Widimský *et al.* 1973). We have previously shown that the RV hypertrophy resulting from hypoxic pulmonary hypertension is associated with abnormal accumulation of interstitial collagen, in particular of collagen type I. Moreover, fibrosis is also present in the non-hypertrophied LV myocardium of chronically hypoxic rats (Ošťádal *et al.* 1978). It is possible to speculate, therefore, that increased expression of TGF-β_1 might contribute to the development of fibrosis under these conditions.

Besides its effect on collagen expression, TGF-β_1 has also the capability to induce VEGF expression (Pertovaara *et al.* 1994). Thus, it is possible that the increased mRNA level of VEGF found on day 3 of IHA hypoxia in the LV and RV was induced in response to TGF-β_1. VEGF and its signal transducing receptor Flk-1 are the major inducers of angiogenesis under hypoxic conditions. It is therefore likely that the increased expression of VEGF, proven in both LV and RV, may give rise to an increased proliferation of capillaries as shown before in the hypertrophied RV of rats exposed to chronic hypoxia (Partovian *et al.* 1998). Moreover, VEGF exerts a long-lasting stimulatory effect on endothelial nitric oxide generation by increasing constitutive nitric oxide synthase expression (van der Zee *et al.* 1997). Nitric oxide has been proposed to play a role as a mediator of increased tolerance to ischemia in the chronically hypoxic hearts (Baker *et al.* 1999). Increased VEGF expression might therefore contribute by these two mechanisms to the protective effect of chronic hypoxia on the cardiac muscle, manifested as a decrease of injury due to acute oxygen deprivation.

For Flk-1, we found a strong up-regulation on day 3 and 5 of IHA hypoxia in the LV and RV; however, the effect in the LV was not statistically significant. Previous reports on the influence of hypoxia on the expression of Flk-1 are contradictory. Although it was shown in several *in vitro* studies that hypoxia induces Flk-1 expression (Tudor *et al.* 1995, Brogi *et al.* 1996, Waltenberg *et al.* 1996), Marti and Risau (1998) reported no up-regulation of the Flk-1 mRNA in an *in vivo* model. Our results are difficult to explain at present because the promoter of Flk-1 does not contain a hypoxia-inducible element such as VEGF or Flt-1 (Gerber *et al.* 1997). The hypoxia-induced changes of the expression of Flk-1 might be indirect since VEGF has the capability to potentiate the expression of this receptor (Waltenberg *et al.* 1996).

Concerning the expression of the protooncogenes c-fos and c-jun, we found no measurable amounts of c-fos by Northern blot except after 4 h of hypoxia in the LV and after 4 h and one day in the RV. On the other hand, c-jun expression was detectable in all tissue samples from the LV and RV, and a significant up-regulation of c-jun was observed on day 3 in the LV. These results contradict the relevance of the expression of c-fos and c-jun for the formation of an AP-1 heterodimer. Furthermore, the time course of c-jun up-regulation in the LV coincides with the up-regulation of the TGF-β_1 mRNA, making it unlikely that an AP-1 homodimer formed by c-jun plays a role in the induction of TGF-β_1. However, both c-jun in the LV and RV and c-fos in the RV might have some other important roles in their function as protooncogenes.

The structure, metabolism and function of the normal and hypoxic atrial tissue differ significantly from the ventricular myocardium (Bass *et al.* 1989). This is reflected by the different influence of IHA hypoxia on gene expression. For TGF-β_1, we found a slight but significant up-regulation in atria that was already obvious after 4 h of hypoxia. The expression of the proto-oncogenes c-fos and c-jun was demonstrable in all atrial samples, but IHA hypoxia did not result in an up-regulation of the corresponding mRNAs. On the contrary, a significant down-regulation was observed on day 10; a similar result was obtained for VEGF. For the mRNA of Flk-1, we found a significant up-regulation in the atria after 4 h and 1 day of hypoxia. Our data indicate that the effect of IHA hypoxia on the expression of the distinct genes in the atrial tissue was significantly less pronounced than in the ventricular myocardium. We cannot exclude that gene expression also differed in the right and left atria, which were exposed to different loading conditions under hypoxia.

The lung responded to IHA hypoxia with a significant increase of TGF-β_1 on day one followed by significant up-regulation of the VEGF mRNA on days 3,

5 and 10. These data may suggest that, in the lung, hypoxia as well as TGF-β_1 are responsible for the increased expression of the VEGF transcript. An up-regulation of VEGF due to hypoxia in the lung was described earlier (Tudor *et al.* 1995, Marti and Risau 1998), but its role is unclear and it is unlikely to be associated with angiogenesis (Fehrenbach *et al.* 1999). Since VEGF can exert its function in this organ as a vascular permeability factor, its increased expression might be the basis for high altitude pulmonary edema as was previously shown for the ischemia/reperfusion-related edema formation in the brain (van Bruggen *et al.* 1999). The possible role of two VEGF receptors in the lungs is not yet well defined. Although it was shown earlier that hypoxia results in an increased expression of Flk-1 (Tudor *et al.* 1995), we and others (Marti and Risau 1998) could not confirm that hypoxia leads to an up-regulation of Flk-1 in the lung. However, we were able to detect the mRNA of both c-fos and c-jun in the lung. For c-jun, we found a strong up-regulation only after 3 days of IHA hypoxia. Since c-jun is an immediate early gene, the late up-regulation of its mRNA might indicate that hypoxia was not severe enough to induce the up-regulation of the gene before day 3.

To our knowledge, this study represents the first attempt to address early changes in gene expression in various cardiac regions under conditions of intermittent high altitude hypoxia in the *in vivo* model. Obviously, it would be desirable to know whether the observed alterations in transcript levels are reflected at the level of respective proteins for better understanding the roles of these factors in cardiopulmonary effects of chronic hypoxia. These results may contribute to a basis for achieving the capacity to reduce the negative effects and preserve or induce the positive effects of chronic hypoxia *via* modulating gene expression.

Acknowledgements

We thank G. Stämmler for the statistical analysis. This work was supported by GA CR 305/01/0279 and 305/00/1659.

Abbreviations:

IHA: intermittent high altitude
VEGF: vascular endothelial growth factor
Flk: fetal liver kinase (VEGF receptor)
TGF-β: transforming growth factor-β
HO-1: heme oxygenase-1
LDH: lactate dehydrogenase
HIF: hypoxia-inducible factor

References

AIMEE Y, YU AY, FRID MG, SHIMODA LA, WIENER CM, STENMARK K, SEMENZA GL: Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. *Am J Physiol* **275**: 818-826, 1988.

ASEMU G, PAPOUŠEK F, OŠŤÁDAL B, KOLÁŘ F: Adaption to high altitude hypoxia protects the rat heart against ischemia-induced arrythmias. Involvement of mitochondrial K_{ATP} channel. *J Mol Cell Cardiol* **31**: 1821-1831, 1999.

BAKER JE, HOLMAN P, KALYARANAMAN B, GRIFFITH OW, PRITCHARD KA: Adaptation to chronic hypoxia confers tolerance to subsequent myocardial ischemia by increased nitric oxide production. *Ann NY Acad Sci* **874**: 236-253, 1999.

BASS A, ŠAMÁNEK M, OŠŤÁDAL B, HUČÍN B, STEJSKALOVÁ M, PELOUCH V: Differences between atrial and ventricular energy supplying enzymes in children. *J Appl Cardiol* **3**: 397-405, 1989.

BAUER EP, KUKI S, ARRAS M, ZIMMERMAN R, SCHAPER W: Increased growth factor transcription after pulmonary artery banding. *Eur J Cardiothorac Surg* **11**: 818-823, 1997.

BROGI E, SCHATTEMAN G, WU T, KIM EA, VARTICOVSKI L, KEYT B, ISNER JM: Hypoxia-induced paracrine regulation of vascular endothelial growth factor receptor expression. *J Clin Invest* **97**: 469-476, 1996.

BUONO RJ, LANG RK: Hypoxic repression of lactate dehydrogenase-B in retina. *Exp Eye Res* **69**: 685-693, 1999.

CHOMCZYNSKI P, SACCHI N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156-159, 1987.

CLAFFEY KP, SHIH SC, MULLEN A, DZINNIS S, CUSICK JL, ABRAMS KR, LEE SW, DETMAR M: Identification of a human VPF/VEGF 3´ untranslated region mediating hypoxia-induced mRNA stability. *Mol Biol Cell* **9**: 469-481, 1998.

EGHBALI M: Cellular origin and distribution of transforming growth factor-ß1 in the normal rat myocardium. *Cell Tissue Res* **256**: 553-558, 1989.

EGHBALI M, TOMEK R, SUKHAMTE VP, WOODS C, BHAMBI B: Differential factors of transforming growth factor-ß1 and phorbol myristate acetate on cardiac fibroblasts. *Circ Res* **69**: 483-490, 1991.

FEHRENBACH H, KASPER M, HAASE M, SCHUH D, MULLER M: Differential immunolocalization of VEGF in rat and human adult lung, and in experimental rat lung fibrosis: light, fluorescence, and electron microscopy. *Anat Rec* **254**: 61-73, 1999.

GERBER HP, CONDRELLI F, PARK J, FERRARA N: Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is upregulated by hypoxia. *J Biol Chem* **272**: 23659-23667, 1997.

GLEADLE MJ, RATCLIFFE PJ: Hypoxia and the regulation of gene expression. *Mol Med Today* **4**: 122-129, 1998.

HURTADO A: Some clinical aspects of life at high altitudes. *Ann Intern Med* **53**: 247-258, 1960.

LAWRENCE DA: Transforming growth factor-ß: a general review. *Eur Cytokine Netw* **7**: 363-374, 1996.

LEE PJ, JIANG BH, CHIN BY, IYER NV, ALAM J, SEMENZA GL, CHOI AM: Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J Biol Chem* **272**: 5375-5381, 1997.

LEVY AP, LEVY NS, WEGNER S, GOLDBERG MA: Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* **270**: 13333-13340, 1995.

LIU YX, COX SR, MORITA T, KOUREMBANAS S: Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells - identification of a 5´ enhancer. *Circ Res* **77**: 638-643, 1995.

MARTI HH, RISAU W: Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors. *Proc Natl Acad Sci USA* **95**: 15809-15814, 1998.

MCCLAIN PE: Characterization of cardiac muscle collagen. Molecular heterogeneity. *J Biol Chem* **249**: 2303-2311, 1974.

MEDUGORAC I, JACOB R: Characterisation of left ventricular collagen in the rat. *Cardiovasc Res* **17**: 15-21, 1983.

NECKÁŘ J, PAPOUŠEK F, NOVÁKOVÁ O, OŠŤÁDAL B, KOLÁŘ F: Cardioprotective effects of chronic hypoxia and ischaemic preconditioning are not additive. *Basic Res Cardiol* **97**: 161-167, 2002.

NEUFELD G, COHEN T, GENGRINOVITCH S, POLTORAK Z: Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* **13**: 9-22, 1999.

OŠŤÁDAL B, MIŘEJOVSKÁ E, HURYCH J, PELOUCH V, PROCHÁZKA J: Effect of intermittent high altitude hypoxia on the synthesis of collagenous and non-collagenous proteins of the right and left ventricular myocardium. *Cardiovasc Res* **12**: 303-308, 1978.

OŠŤÁDAL B, KOLÁŘ F, PELOUCH V, PROCHÁZKA J, WIDIMSKÝ J: Intermittent high altitude and the cardiopulmonary system. In: *The Adapted Heart*. M NAGANO, N TAKEDA, NS DHALLA (eds), Raven Press, New York, 1994, pp 173-182.

PARTOVIAN C, ADNOT S, EDDAHIBI S, TEIGER E, LEVAME M, DREYFUS M, RAFFESTIN B, FRELIN C: Heart and lung VEGF mRNA expression in rats with monocrataline- or hypoxia-induced pulmonary hypertension. *Am J Physiol* **275**: H1948-H1956, 1998.

PELOUCH V, OŠŤÁDAL B, PROCHÁZKA J, URBANOVÁ D, WIDIMSKÝ J: Effect of high altitude hypoxia on the protein composition of the right ventricular myocardium. *Prog Resp Res* **20**: 41-48, 1985.

PERTOVAARA L, KAIPAINEN A, MUSTONEN T, ORPANA A, FERRARA N, SAKSELA O, ALITALO K: Vascular endothelial growth factor is induced in response to transforming growth factor-ß in fibroblastic and epithelial cells. *J Biol Chem* **269**: 6721-6274, 1994.

POUPA O, KROFTA K, PROCHÁZKA J, TUREK Z: Acclimatization to simulated high altitude and acute cardiac necrosis. *Fed Proc* **25**: 1243-1246, 1966.

PRABHAKAR NR, FIELDS RD, BAKER T, FLETCHER EC: Intermittent hypoxia: cell to system. *Am J Physiol* **281**: L524-L528, 2001.

SAMBROOK J, FRITSCH EF, MANIATIS T: *Molecular Cloning: A Laboratory Manual.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

SEMENZA G: Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit Rev Biochem Mol Biol* **35**: 71-103, 2000.

SEMENZA GL, JIANG BH, LEUNG SW, PASSANTINO R, CONCORDET JP, GIALLONGA A: Hypoxia response elements in aldolase A, enolase and lactate dehydrogenase A gene promoter contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* **271**: 32529-32537, 1996.

TUDER RM, FLOOK BE, VOELKEL NF: Increased gene expression of VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic ischemia. *J Clin Invest* **95**: 1789-1807, 1995.

VAN BRUGGEN N, THIBODEAUX H, PALMER JT, LEE WP, FU L, CAIRNS B, TUMAS D, GERLAI R, WILLIAMS SP, VAN LOOKEREN, CAMPAGNE M, FERRERA N: VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion injury in the mouse brain. *J Clin Invest* **104**: 1613-1620, 1999.

VAN DER ZEE R, MUROHARA T, LUO Z, ZOLLMANN F, PASSERI J, LEKUTAT C, ISNER JM: Vascular endothelial growth factor / vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* **95**: 1030-1037, 1997.

WALTENBERG J, MAYR U, PENTZ S, HOMBACH V: Functional upregulation of vascular endothelial growth factor receptor KDR by hypoxia. *Circulation* **94**: 1647-1654, 1996.

WANG G, JIANG BH, RUE EA, SEMENZA GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci USA* **92**: 5510-5514, 1995.

WIDIMSKÝ J, URBANOVÁ D, RESSL J, OŠŤÁDAL B, PELOUCH V, PROCHÁZKA J: Effect of intermittent altitude hypoxia on the myocardium and lesser circulation in the heart. *Cardiovasc Res* **7**: 798-808, 1973.

Reprint requests

Elisabeth Deindl, Ph.D., Max-Planck-Institute, Department of Experimental Cardiology, Benekestrasse 2, D-61231 Bad Nauheim, Germany. FAX: +49 603 2705419. E-mail e.deindl@kerckhoff.mpg.de