

Changes in the Expression and/or Activation of Regulatory Proteins in Rat Hearts Adapted to Chronic Hypoxia

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Abstract. Chronic intermittent high altitude (IHA) hypoxia results in long-term adaptation protecting the heart against acute ischemia/reperfusion injury; however, molecular mechanisms of this phenomenon are not completely elucidated so far. The present study was aimed at investigation of a modulating effect of IHA hypoxia on the expression and/or activation of selected regulatory proteins, with particular emphasis on differential responses in the right ventricle (RV) and left ventricle (LV). Adult male Wistar rats were exposed to IHA hypoxia of 7000 m simulated in a hypobaric chamber (8 h/day, 25 exposures), and protein contents and activities in myocardial fractions were determined by Western blot analysis. In markedly hypertrophic RV of hypoxic rats, gelatinolytic activity of MMP-2 and protein levels of carbonic anhydrase IX (a marker of hypoxia) were significantly enhanced. Study of mitogen-activated protein kinases (MAPKs) revealed no differences in the contents of total p38-MAPK in both ventricles between the IHA and normoxic control rats, whereas activation of p38-MAPK was decreased in the RV and moderately increased in the LV of IHA rats as compared to controls. Extracellular signal regulated kinase-2 (ERK-2) was partially up-regulated in the RV of IHA rats, and, in addition, expression of acidic fibroblast growth factor (aFGF), a potential activator of ERK cascade, was also significantly increased. In contrast, expression of ERKs in the LV as well as their activities in both ventricles, were not affected by IHA hypoxia. Differential effects of IHA hypoxia on c-Jun-N-terminal protein kinases (JNKs) in the RV and LV were also observed. As compared with the controls, total content of JNKs was increased in the RV of the IHA rats, while expression of JNKs in the LV was down-regulated. IHA hypoxia changed neither total levels of Akt kinase in both RV and LV, nor Akt kinase activity in the RV.

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However, increased levels of activated phospho-Akt kinase were found in the LV of IHA rats. The results demonstrate that adaptation of rat hearts to chronic IHA hypoxia is associated with distinct changes in the levels and/or activation of several regulatory proteins in two ventricles. The latter could be attributed to both myocardial remodeling and cardioprotection induced by chronic hypoxia.

Key words: Heart — Chronic hypoxia — Cell signaling — Regulatory proteins

Introduction

Long-term exposure of humans and animals to the hypoxic environment induces adaptive cardiopulmonary changes that enable to maintain circulatory demands and tissue homeostasis under conditions of limited oxygen availability. Not only continuous hypoxia but also intermittent hypoxia acting for only a relatively brief period of the day proved effective in eliciting characteristic adaptive responses (reviewed in Ostadal et al. 1994). The most distinctive feature of the chronically hypoxic heart is the right ventricular (RV) hypertrophy resulting from increased afterload due to hypoxic pulmonary hypertension. The left ventricle (LV) usually does not show hypertrophy because it essentially does not have to cope with increased load, unless there is rather severe and prolonged intermittent hypoxia (Kolar and Ostadal 1991; Pelouch et al. 1997). Chronic hypoxia stimulates the expression of a number of genes (Semenza 2001) although with different degree and temporal pattern in the two ventricles (Deindl et al. 2003). Subsequent complex remodeling involves all myocardial components including myocytes, coronary vessels, and extracellular matrix (Rakusan and Cicutti 1996; Pelouch et al. 1997; Novel-Chate et al. 1998; Reynafarje and Marticorena 2002; Hrbasova et al. 2003).

The important feature of chronically hypoxic hearts is their increased tolerance to an acute ischemia/reperfusion injury. This long-lasting cardioprotection is manifested by limitation of infarct size, improved recovery of the contractile function after ischemia, and reduced incidence and severity of ischemic and reperfusion ventricular arrhythmias. Although many factors have been proposed to play a potential role in this phenomenon, its detailed molecular mechanism is unknown. At present, limited evidence exists for the involvement of mitochondrial ATP-sensitive K^+ channels, reactive oxygen species, nitric oxide, protein kinase C, and opioids (reviewed in Kolar and Ostadal 2004).

These factors are also involved in the mechanism of ischemic preconditioning (Oldenburg et al. 2004) and in particular of its delayed phase (Kis et al. 2003) indicating that both, short-term and long-lasting cardioprotective phenomena may share at least some elements of the same signaling pathways (Kolar and Ostadal 2004). Studies of protective mechanisms of classical ischemic preconditioning also revealed the involvement of mitogen-activated protein kinase (MAPK) and PI3K/Akt kinase cascades, some of them being considered as components of survival pathways, especially those involved in the hypertrophic response (Strohm et al. 2000; Kis et al. 2003; Hausenloy et al. 2004). However, these mechanisms

have not been sufficiently elucidated so far in a setting of chronic intermittent high altitude (IHA) hypoxia, and, in particular, the role of protective cascades remains elusive. In order to identify potential factors involved in the process of cardiac adaptation to chronic hypoxia, we examined the effect of simulated IHA hypoxia on the expression and activation of different regulatory proteins in the heart. For this reason we investigated the effects of severe IHA hypoxia corresponding to 7000 m on the potentially protective kinase signaling systems (MAPKs, Akt kinase) and on the activities and levels of other regulatory proteins associated with cardiac remodeling, hypertrophy and oxidative load, such as matrix metalloproteinases (MMPs), acidic fibroblast growth factor (aFGF) and carbonic anhydrase IX (CA IX). CA IX is a transmembrane glycoprotein which catalyzes the reversible conversion of carbon dioxide to carbonic acid and contributes to regulation of intracellular and extracellular pH (Svastova et al. 2004). This enzyme is strongly hypoxia-inducible and is therefore considered to be an endogenous marker of hypoxia. FGFs are potential activators of some protein kinase pathways, especially of extracellular signal-regulated kinases (ERKs) cascade.

In line with the differences in gene expression induced by hypoxia in the LV and RV of the heart that have to deal with a different hemodynamic load under hypoxic conditions (Deindl et al. 2003), the pattern of expression and activation of regulatory proteins may also differ between the ventricles. Nevertheless, despite different degree of remodeling, both ventricles exhibit an increased tolerance to ischemic injury (Kolar and Ostadal 1991; Ostadal et al. 1994; Deindl et al. 2003). On this basis, we have hypothesized that examination of potential protective systems in each ventricle separately could be a useful strategy to identify the factors underlying the mechanisms of cardioprotective adaptive response in the hypoxic hearts.

Materials and Methods

Experimental model

Adult male Wistar rats were exposed to IHA of 7000 m (barometric pressure (P_B) = 308 mmHg, 41.1 kPa) in a hypobaric chamber for 8 h/day, 5 days/week. The total number of exposures was 25. The control group of animals was kept for the same period of time at P_B and oxygen pressure (PO_2) equivalent to an altitude of 200 m. All animals had free access to water and a standard laboratory diet. The animals were sacrificed by cervical dislocation the next day after the last hypoxic exposure. Tissue samples were taken from the RV and LV of their hearts, frozen in liquid nitrogen and stored at -80°C until further analysis. The studies were performed in accordance with Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Preparation of tissue fractions

The tissues from both, RV and LV, were wiped in liquid nitrogen, resuspended in ice-cold buffer A containing (in mmol/l): 20 Tris-HCl, 250 sucrose, 1.0 EGTA, 1.0

dithiothreitol, 1.0 phenylmethylsulfonylfluoride and 0.5 sodium orthovanadate (pH 7.4) and homogenized with a glas-teflon homogenizer. The homogenates were centrifuged at $700 \times g$ for 5 min at 4°C, pellets after this centrifugation were discarded and the supernatants were centrifuged again at $13,600 \times g$ for 30 min. The post-mitochondrial supernatants after this second centrifugation were termed as soluble fractions. To obtain the particulate fractions, the pellets from the second centrifugation were resuspended in buffer A containing 0.2% Triton X-100 and centrifuged at $5,000 \times g$ for 5 min. The Triton X-100 soluble supernatants represented the particulate fractions. The protein concentrations were estimated by the method of Bradford (1976).

Electrophoresis and immunochemical Western blot analysis

Samples of soluble or particulate fractions containing equivalent amounts of ventricular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Total contents or activities of some regulatory proteins were determined by Western blot analysis using specific antibodies. For Western blot assays, proteins after electrophoretic separation were transferred to nitrocellulose membrane. Specific anti-p38-MAPK, anti-ERK, anti-JNK, anti-Akt kinase (all from Santa Cruz Biotechnology), anti-phospho-p38-MAPK, anti-phospho-ERK, anti-phospho-JNK, anti-phospho-Akt kinase (all from Cell Signaling), anti-FGF-1 (Austral Biologicals) antibodies, and mouse monoclonal anti-CA IX antibody (MAb M75, prepared in laboratory of Dr. Pastorekova, Pastorekova et al. 1992) were used for primary immunodetection. Peroxidase-labelled anti-rabbit or anti-mouse immunoglobulins (Amersham Biosciences) were used as the secondary antibodies. Bound antibodies were detected by the enhanced chemiluminescent (ECL) method. Quantitative gel analysis was performed using Phosphorimager SF (Molecular Dynamics, Krefeld, Germany).

Measurement of MMP activities by gelatin zymography

The gelatinolytic activities of MMPs were determined by the modified method of Schonbeck et al. (1997). Laemmli buffer without 2-mercaptoethanol in proportion 1:1 was added to protein samples isolated from the RV and LV of control and IHA rats. Non-heated samples were subjected to electrophoresis on 10% SDS-polyacrylamide gels co-polymerized with gelatin (2 mg/ml). After electrophoresis, gels were washed twice for 20 min each with 50 mmol/l Tris-HCl (pH 7.4), containing 2.5% Triton X-100, at 25°C. After washing, the gels were incubated overnight at 37°C in substrate buffer containing 50 mmol/l Tris-HCl, 10 mmol/l CaCl_2 and 1.25% Triton X-100, pH 7.4. After this incubation, the gels were stained with 1% Coomassie Brilliant Blue G-250, resolved in mixture of methanol:acetic acid:water (4:1:5) for 2 h at room temperature and then destained with 40% methanol and 10% acetic acid. Gelatinolytic activities of MMPs (MMP-2) were detected as transparent bands against dark blue background.

Statistical evaluation

Data were expressed as means \pm S.E.M. Statistical significance of differences between the groups was analysed by the unpaired Student's *t*-test. Differences were considered as significant at $p < 0.05$.

Results*Characteristics of rats adapted to IHA hypoxia**Weight parameters and hematocrit*

Adaptation of rats to chronic hypoxia resulted in increased hematocrit and led to a marked delay in body weight growth when compared with the normoxic controls. The heart weight increased significantly, and the latter effect was to a major extent due to hypertrophy of the RV. Thus, the RV relative weight (RV/body weight) represented 172% of that in the normoxic control group, whereas the relative weight of the LV increased to 122% of the control value (Table 1).

Table 1. Body weight, heart weight parameters and hematocrit of rats adapted to chronic hypoxia and of normoxic controls

Parameter	Normoxic	Hypoxic
<i>n</i>	11	9
BW (g)	343 \pm 8	293 \pm 4*
RVW (mg)	162 \pm 5	236 \pm 8*
LVW (mg)	394 \pm 18	410 \pm 19
SW (mg)	189 \pm 8	212 \pm 12
RVW/BW (mg/g)	0.472 \pm 0.007	0.808 \pm 0.028*
LVW/BW (mg/g)	1.147 \pm 0.040	1.405 \pm 0.068*
Hematocrit (%)	47.3 \pm 1.4	73.4 \pm 0.7*

Values are means \pm S.E.M.; *n*, number of rats; BW, body weight; RVW, right ventricular weight; LVW, left ventricular weight; SW, septum weight; RVW/BW, relative RVW; LVW/BW, relative LVW; * $p < 0.05$ vs. normoxic group.

Activities of MMPs

Enzymatic gelatinolytic activity in the range of approximately 63 kDa that corresponds to molecular weight of MMP-2 was detected in both ventricles. Significantly increased activation of this enzyme was observed in the RV of rats adapted to IHA hypoxia as compared to control normoxic group (Fig. 1A,C). In contrast, activity of MMP-2 in the LV was not influenced by IHA hypoxia (Fig. 1B,D).

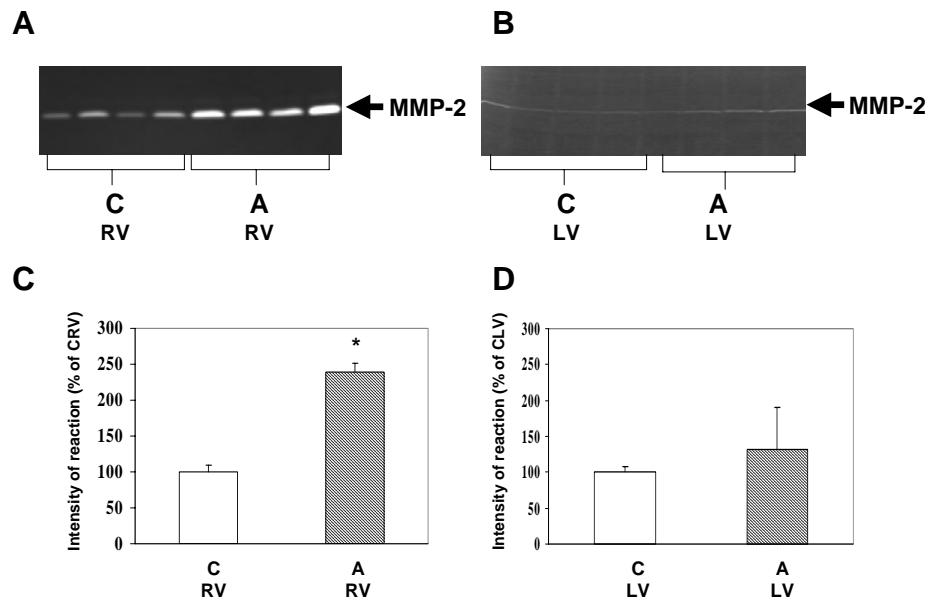


Figure 1. Effect of adaptation to IHA hypoxia on activities of matrix metalloproteinases (MMPs). The activities of MMPs in the RV (**A**) and LV (**B**) tissue samples were analyzed by zymography in 10% polyacrylamide gels containing 2% gelatine as a substrate. The arrow on the right shows the gelatinolytic activity in the range of 63 kDa (MMP-2). **C.** Quantitative analysis of MMP-2 activities in the RV. **D.** Quantitative analysis of MMP-2 activities in the LV. Data are expressed as a percentage of values for corresponding control tissue. Each bar represents mean \pm S.E.M. of 5–8 tissue samples *per* group. C, control rat hearts; A, rat hearts adapted to IHA hypoxia; RV, right ventricle; LV, left ventricle; * significant difference in the level $p < 0.05$.

Protein levels of CA IX

Western blot analysis revealed increased protein levels of CA IX in particulate fractions isolated from the RV of IHA rats (Fig. 2A,C) when compared with those in the control ones. No significant differences between the control and adapted hearts were observed in the CA IX levels in particulate fractions isolated from the LV (Fig. 2B,D). In soluble fractions, no significant differences were found between any groups. This is consistent with the plasma membrane localization of CA IX protein.

Effect of IHA hypoxia on MAPK signaling pathways

Protein content and activation of p38-MAPK

Analysis with antibody specific for p38-MAPK showed no significant differences in the levels of p38-MAPK in soluble fractions from the RV of control and IHA rats (Fig. 3A, on the right). However, decreased phosphorylation of p38-MAPK

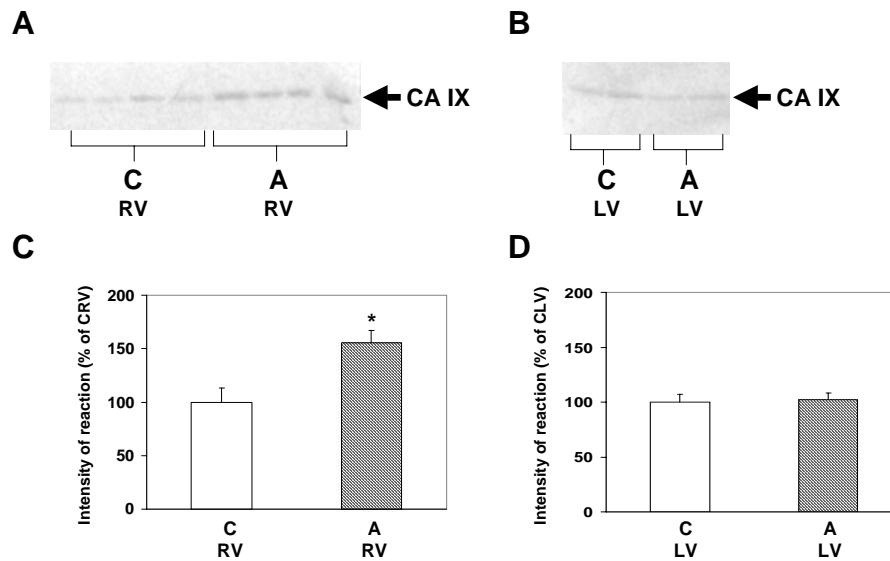


Figure 2. Effect of adaptation to IHA hypoxia on the protein levels of carbonic anhydrase IX (CA IX). The records show the changes in levels of CA IX in particulate fractions isolated from RV (**A**) and LV (**B**) tissue of the control and IHA hearts. The CA IX was determined using specific antibody and the arrow on the right shows the position of this enzyme. **C.** Quantitative analysis of CA IX content in the RV. **D.** Quantitative analysis of CA IX content in the LV. Data are expressed as a percentage of values for corresponding control tissue. Each bar represents mean \pm S.E.M. of 5–8 tissue samples *per* group. C, control rat hearts; A, rat hearts adapted to IHA hypoxia; RV, right ventricle; LV, left ventricle; * significant difference in the level $p < 0.05$.

on Thr180/Tyr182 was detected in the RV of IHA rats (Fig. 3B,C). Specific phosphorylation at these sites reflects the activation of p38-MAPK. Total content of p38-MAPK in the LV was not influenced by IHA hypoxia (Fig. 3A, on the left). In contrast, the activation of this kinase was moderately increased in the LV of animals adapted to IHA hypoxia (Fig. 3B). One of the direct substrates of p38-MAPK and a part of p38-MAPK pathway is a kinase known as MAP kinase-activated protein kinase 2 (MAPKAPK-2). For this kinase, we observed slightly increased levels in the RV of IHA group (Fig. 3D).

Protein content and activation of ERKs

IHA hypoxia induced partial up-regulation of protein levels of ERKs in soluble (Fig. 4A on the right, and D) and ERK-2 in particulate (Fig. 4C,D, on the right) fractions isolated from the RV of the adapted hearts as compared with normoxic control ones. Using antibody that reacts specifically with dual phosphorylated ERKs (Thr202/Tyr204) we found no differences in the content of activated ERK-1/2 in soluble fractions from RV of both investigated groups (Fig. 4B, on the right).

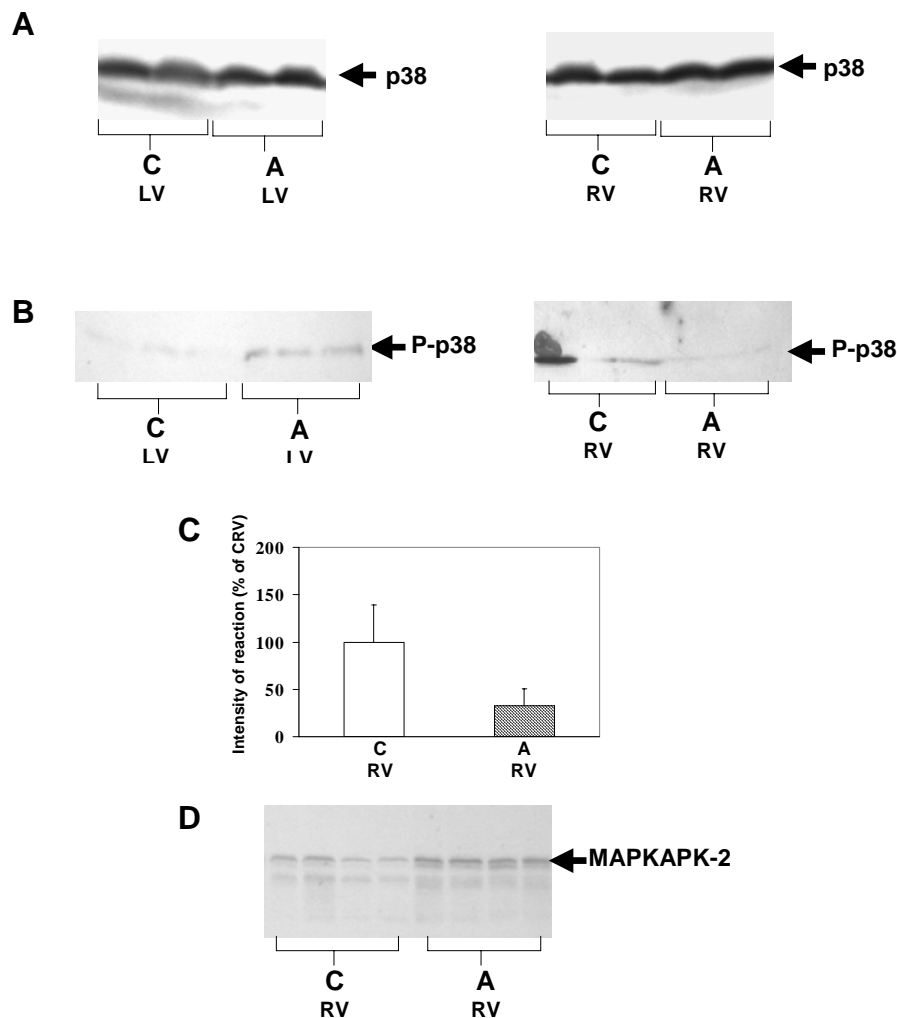


Figure 3. Effect of adaptation to IHA hypoxia on protein levels and activation of p38-MAPK (p38). **A.** The records show the changes in protein levels of p38-MAPK in soluble fractions isolated from the LV and RV tissue of hearts of control and IHA hypoxia adapted rats. The p38-MAPK levels were determined using specific antibody and the arrow on the right shows the position of this enzyme. **B.** The changes in specific phosphorylation of p38-MAPK (P-p38) in soluble fractions isolated from LV and RV tissue of hearts of control and IHA hypoxia adapted rats. The activation of p38-MAPK was determined using phospho-specific antibody (Thr180/Tyr182). **C.** Quantitative analysis of content of phosphorylated p38-MAPK in RV tissue. Data are expressed as a percentage of values for corresponding control tissue. Each bar represents mean \pm S.E.M. of 5–8 tissue samples *per* group. **D.** Influence of adaptation to IHA hypoxia on protein levels of MAPKAPK-2 in soluble fractions. The levels of MAPKAPK-2 were determined using specific antibody. C, control rat hearts; A, rat hearts adapted to IHA hypoxia; RV, right ventricle; LV, left ventricle.

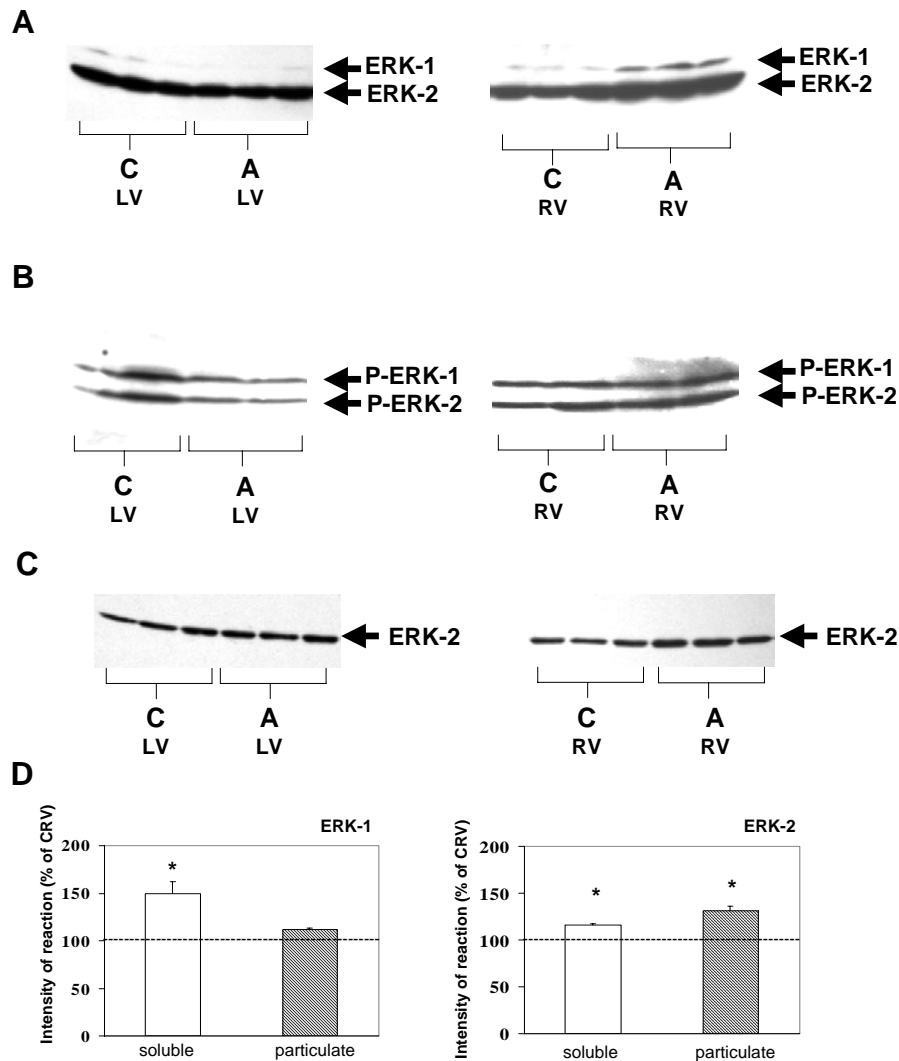


Figure 4. Effect of adaptation to IHA hypoxia on protein levels and activation of extracellular signal-regulated protein kinases (ERKs). **A.** The records show the changes in protein levels of ERK in soluble fractions isolated from LV and RV tissue of control and IHA hypoxia adapted rat hearts. The ERKs levels were determined using specific antibody and the arrows on the right show the position of these enzymes. **B.** The changes in specific phosphorylation of ERKs (P-ERK-1, P-ERK-2) in soluble fractions isolated from LV and RV tissue of hearts of control and IHA hypoxia adapted rats. The activation of ERK was determined using phospho-specific antibody (Thr202/Tyr204). **C.** The changes in protein levels of ERKs in particulate fractions isolated from LV and RV tissue of hearts of control and IHA hypoxia adapted rats. **D.** Quantification of ERKs content in RV tissue. Data for IHA hypoxia adapted hearts are expressed as a percentage of values for corresponding control RV tissue. Each bar represents mean \pm S.E.M. of 5–8 tissue samples *per* group. C, control rat hearts; A, rat hearts adapted to IHA hypoxia; RV, right ventricle; LV, left ventricle; * significant difference in the level $p < 0.05$.

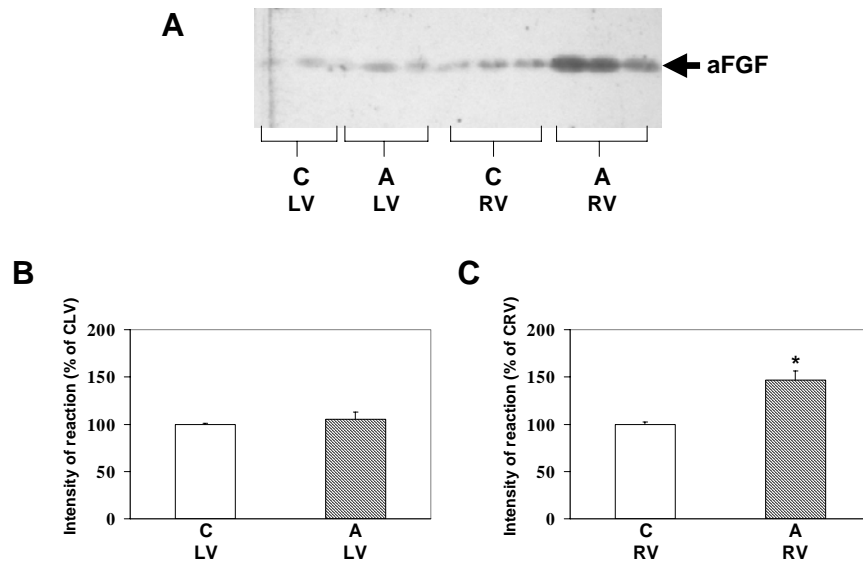


Figure 5. Effect of adaptation to IHA hypoxia on protein levels of acidic fibroblast growth factor (aFGF). **A.** The records show the changes in levels of aFGF in soluble protein fractions isolated from RV and LV tissue of hearts of control and IHA hypoxia adapted rats. The aFGF was determined using specific antibody and the arrow on the right shows the position of this protein. **B.** Quantification of aFGF content in LV tissue. Data for IHA hypoxia adapted hearts are expressed as a percentage of values for corresponding control LV tissue. Each bar represents mean \pm S.E.M. of 4 tissue samples *per* group. **C.** Quantification of aFGF content in RV tissue. Data for IHA hypoxia adapted hearts are expressed as a percentage of values for corresponding control RV tissue. Each bar represents mean \pm S.E.M. of 4 tissue samples *per* group. C, control rat hearts; A, rat hearts adapted to IHA hypoxia; RV, right ventricle; LV, left ventricle; * significant difference in the level $p < 0.05$.

In the LV, we did not detect significant differences in the levels of ERKs between the control and adapted hearts (Fig. 4A,C, on the left), however, the activation of both ERK-1 and ERK-2 was moderately decreased in hearts adapted to IHA hypoxia (Fig. 4B, on the left).

Protein levels of aFGF

Chronic IHA hypoxia induced significant up-regulation of aFGF protein levels in the RV as compared with the normoxic group (Fig. 5A,C). These changes corresponded to up-regulation of ERKs in the RV. There were no differences in content of aFGF in the LV (Fig. 5A,B).

Protein content and activation of JNKs

Results of immunochemical analysis with an antibody specific for c-Jun N-terminal protein kinases (JNKs) are shown in Fig. 6. In the soluble fractions isolated from

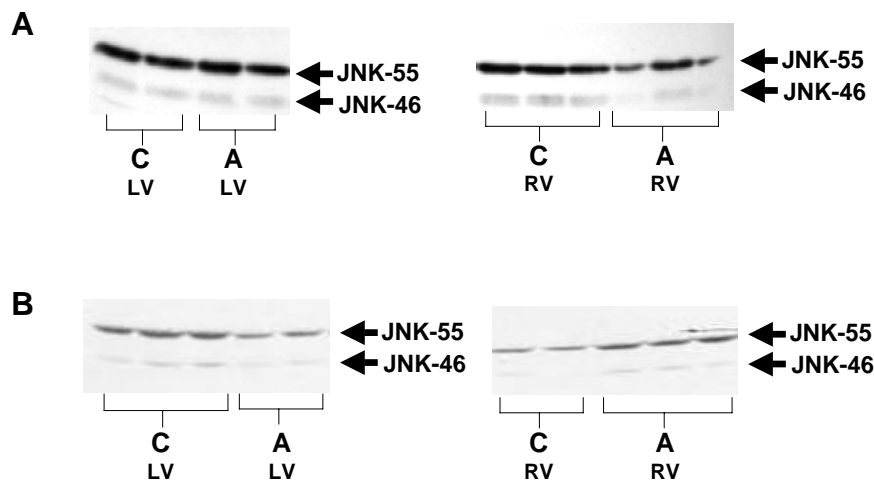


Figure 6. Effect of adaptation to IHA hypoxia on protein levels c-Jun-N-terminal kinases (JNKs). **A.** The records show the changes in protein levels of JNKs in soluble fractions isolated from LV and RV tissue of control and IHA hypoxia adapted rat hearts. The JNKs levels were determined using specific antibody and the arrows on the right show the position of the JNK-46 and JNK-55. **B.** The content of JNKs in particulate fractions isolated from LV and RV tissue of hearts of control and IHA hypoxia adapted rats. C, control rat hearts; A, rat hearts adapted to IHA hypoxia; RV, right ventricle; LV, left ventricle.

the RV and LV myocardium (Fig. 6A) we did not observe differences in JNKs levels between the control and adapted hearts. Different effects of IHA hypoxia on the total content of JNKs we found in the particulate fractions. While the levels of JNKs were increased in the RV, they were decreased in the LV (Fig. 6B) in comparison with those in the control hearts. Changes in the activation of JNKs were not observed (data not shown).

Effect of IHA hypoxia on PI3K/Akt signaling pathway

To explore the influence of IHA hypoxia on the protein levels and activation of Akt kinase, we performed Western blot analysis with tissue samples from the RV and LV of the control normoxic and adapted animals. Analysis with the antibody specific for Akt kinase revealed no differences in the levels of Akt kinase between the control and IHA hearts (Fig. 7A). On the other hand, using a phosphospecific antibody (Ser473) we found increased activation of Akt kinase in soluble fractions from the LV of IHA rats (Fig. 7B,C) as compared with that in the normoxic controls. There were no differences in Akt kinase activation in the RV between the samples from the IHA-adapted and normoxic animals. Observed changes in the levels and/or activities of the examined regulatory proteins induced by IHA hypoxia in both ventricles are summarized in Table 2.

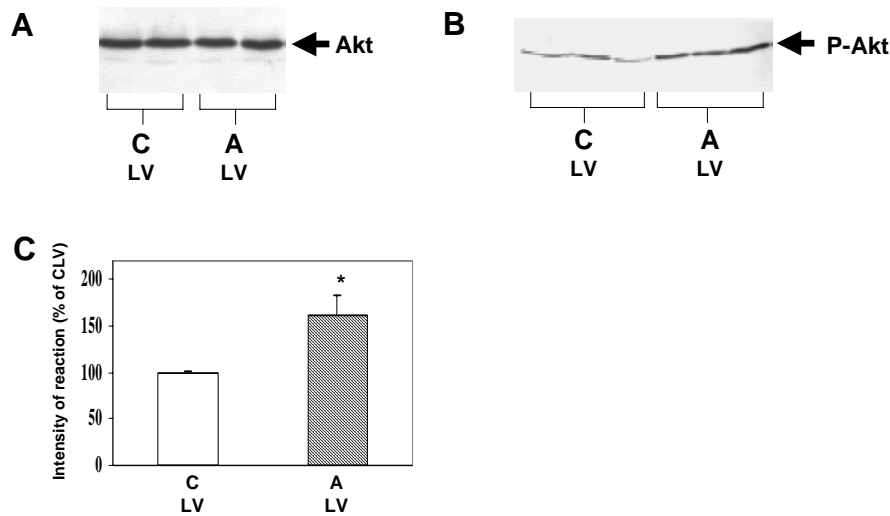


Figure 7. Influence of adaptation to IHA hypoxia on protein levels and activation of Akt kinase (Akt). **A.** The record shows the protein levels of Akt kinase in soluble fractions isolated from LV tissue of control and IHA hypoxia adapted rat hearts. The Akt kinase levels were determined using specific antibody. **B.** The changes in specific phosphorylation of Akt kinase in soluble fractions isolated from LV tissue of hearts of control and IHA hypoxia adapted rats. The activation of Akt kinase was determined using phospho-specific antibody (Ser473). **C.** Quantification of phospho-Akt kinase (P-Akt) content in the LV. Data are expressed as a percentage of values for corresponding control tissue. Each bar represents mean \pm S.E.M. of 5–8 tissue samples per group. C, control rat hearts; LV, left ventricle; A, rat hearts adapted to IHA hypoxia; * significant difference in the level $p < 0.05$.

Table 2. Summary of changes in the levels and/or activities of the examined regulatory proteins observed in the animals adapted to IHA hypoxia compared to the corresponding normoxic controls

Protein	RV	LV
MMP-2 (gel)	↑	≈
CA IX (part)	↑	≈
p38-MAPK (sol)	≈	≈
phospho-p38-MAPK (sol)	↓	↑
ERK-2 (sol, part)	↑	≈
phospho-ERK-1/2 (sol)	≈	↓
aFGF (sol)	↑	nd
JNKs (part)	↑	↓
phospho-JNK (part)	≈	≈
Akt (sol)	≈	≈
phospho-Akt (sol)	≈	↑

Symbols indicate: increased (↑), decreased (↓) and similar (≈) protein levels; gel, gelatinolytic activity; part, particulate fractions; sol, soluble (postmitochondrial) fractions; nd, not determined.

Discussion

The main consequences of adaptation to IHA hypoxia are an increased cardiac resistance to acute ischemic injury and myocardial hypertrophy, and both might be regulated by the same products of gene expression induced by hypoxia (Semenza 2001). Cardiac hypertrophy is an adaptive compensatory response that in advanced stages impairs myocardial tolerance to ischemia as demonstrated in the models of pressure overload (Friebs and del Nido 2003). However, despite severe hypertrophy of the RV of the hypoxic heart, increased ischemic tolerance appears to be a characteristic feature of both ventricles in this model (Kolar and Ostadal 1991; Ostadal et al. 1994; Deindl et al. 2003). Thus, it is conceivable to speculate that factors involved in the cardioprotective response would be influenced by IHA hypoxia in both markedly hypertrophic RV and only marginally hypertrophic LV, whereas changes in the levels of proteins and/or their activity observed in the RV only could be related to hypertrophic response.

To the best of our knowledge, this is the first study that attempted to search for the changes induced by IHA hypoxia in several potentially protective protein kinases cascades and other specific regulatory proteins in the rat heart, with particular regards to the differences in protein expression and/or activation between the LV and RV. Our data clearly show that IHA hypoxia modulated the levels and/or activities of several regulatory proteins. Moreover, these changes appeared to be unequal in the LV and RV myocardium of the hypoxic animals.

With respect to the mechanisms of cardioprotection, the major important observation of this study is a finding of significant stimulation of Akt kinase in the LV of the adapted hearts, accompanied by a moderately increased activity of p38-MAPK. MAPK and PI3K/Akt kinase pathways have been implicated in the mechanisms of various forms of cardioprotection (short-term and long-term), such as classical ischemic or hypoxic preconditioning and its delayed (second window) phase, as well as pharmacologically induced preconditioning (Strohm et al. 2000; Fryer et al. 2001; Hausenloy et al. 2004; Uchiyama et al. 2004; Wang et al. 2004). Recent *in vivo* studies indicate that Akt kinase activation plays an important role not only in the attenuation of cell death, but also in preservation of cardiac function (Matsui et al. 2001; Gross et al. 2004). However, there is only limited evidence of the potential role of MAPKs in adaptive responses of myocardium to chronic hypoxia, and the role of Akt kinase activation has not been explored so far in a setting of chronic IHA hypoxia. In a study of Rafiee et al. (2002) it was shown that infant human and rabbit hearts adapt to chronic hypoxia through p38-MAPK and JNK activation but not through ERK (p42/44-MAPK). In concept, our study also demonstrated a moderate activation of p38-MAPK, but not of ERKs, in the LV of IHA rats, in conjunction with an increased activity of Akt kinase. These changes may be considered as cardioprotective but the fact that they occurred only in the LV seems to reflect on their involvement in the mechanism of increased cardiac ischemic tolerance induced by IHA hypoxia.

In the RV, IHA hypoxia led to upregulation of proteins involved in cell signaling (ERK, JNK), growth (aFGF), cardiac remodeling (MMPs) and those related to the changes in the oxidative state of the myocardium (CA IX). MMPs are essential for extracellular matrix turnover and their activation facilitates angiogenesis and development of cardiac fibrosis through breakdown of the extracellular matrix (Burbridge et al. 2002; Siwik and Colucci 2004). Regulation of MMPs expression and activities involves intracellular signaling mechanisms mediated by reactive oxygen species, MAPK (ERK, p38-MAPK) and PI3K/Akt kinase pathway, as well as several growth and transcription factors (Choi et al. 2004; Siwik and Colucci 2004; Zhang et al. 2004). These changes induced by IHA hypoxia were manifested mainly on the level of protein expression and were not observed in the LV. The latter may indicate their relationship with the processes of hypertrophy and remodeling that were predominant in the RV, whereas the LV underwent only marginal hypertrophy that reached statistical significance only when normalized to body weight.

In our study, zymographic analysis revealed a significant increase in gelatinolytic activity of MMP-2 in the RV of IHA rats. This finding is in accord with an earlier study demonstrating that IHA hypoxia-induced remodeling of ventricular myocardium was associated with the changes in contents of extracellular matrix proteins, particularly fibrillar collagenous proteins (Pelouch et al. 1997). Enhanced production of reactive oxygen species is a common feature of a number of chronic processes associated with myocardial hypertrophy and remodeling including IHA hypoxia, and might lead to observed activation of MMP-2. Higher level of fibroblast growth factor-1 (aFGF) was also detected in the right ventricle of IHA rats. Recent studies demonstrated that another member of FGF family (basic FGF) significantly stimulated activities of MMP-9 (Allen et al. 2003), and that angiogenic basic FGF upregulated the expression of different MMPs including gelatinases (MMP-2 and MMP-9), whereas vascular endothelial growth factor led to a marked increase in the expression of MMP-2 only (Choi et al. 2004). Thus, it seems plausible that up-regulation of aFGF shown in our study might also contribute to observed increase in MMP-2 activity.

The FGFs are effective activators of ERK pathway. Moreover, a link between this kinase pathway and regulation of MMP-2 activities was demonstrated in the coronary endothelial cells (Donnini et al. 2004) as the induction of ERK-1 and ERK-2 phosphorylation resulted in MMP-2 up-regulation and activation. In our study, only protein levels of ERKs but not ERK activity were increased in the RV of IHA rats, suggesting that this pathway is not involved in the observed modulation of MMP-2 activity.

Although the role of CA IX in the hypoxic heart has not been yet established, its increased protein levels in the RV may also reflect the response of myocardium to severe hypoxia associated with myocardial hypertrophy. The expression of this protein is regulated through well-characterized hypoxia-inducible factor-1 (HIF-1) (Svastova et al. 2004), a heterodimeric transcriptional factor directly induced by severe tissue or cellular hypoxia (Semenza 2001). Expression and activity of α subunit of HIF-1 may be increased by growth factors or cytokines stimulating the ERKs or

PI3K/Akt kinase pathway (Jiang et al. 2001; Minet et al. 2001; Lim et al. 2004). However, using Western blot analysis we found no changes in the protein level of HIF-1- α in the LV or RV of IHA rats when compared to the control rats (data not shown). This is similar to the results obtained in the study of Deindl et al. (2003), where the exposure of rats to IHA hypoxia simulated in hypobaric chamber (5000 m) induced significant adaptive responses, but the changes in the protein levels of HIF-1- α were not observed. This was suggested to be a consequence of HIF-1- α ubiquitination and proteosomal degradation during the period of normoxia. In contrast to HIF-1- α , whose half-life in normoxia is less than 5 min, CA IX is a highly stable protein with a half-life of about 38 h persisting in reoxygenated cells for several days (Rafajova et al. 2004).

Certain limitation of this study was the fact that all investigations were performed in the phase of the established cardiac adaptation and, therefore, factors activated during the initial phase and acting as potential triggers of protective cascades in that early period had not been necessarily active at the time of our observations.

In conclusion, adaptation of rats to IHA hypoxia led to modulation of the levels and/or activities of several regulatory proteins. Moreover, these changes differed in the RV and LV. The latter may be attributed to chronic hypoxia-induced processes of cardiac remodeling and hypertrophy on one hand, and to activation of protective signaling mechanisms leading to cell survival on the other hand. A complexity of divergent pathways involved in cardioprotection requires a more detailed study where different temporal patterns of their activation are also taken into consideration.

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