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In vivo carbon monoxide exposure and hypoxic hypoxia stimulate immediate early gene expression

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Abstract This study aimed to examine the influence of acute tissue hypoxygenation on the expression of immediate early genes in different rat tissues. To this end male Sprague-Dawley rats were exposed to 0.1% carbon monoxide for 0.5, 1 and 6 h or to 9% oxygen for 6 h and mRNA levels for c-*jun*, c-*fos*, c-*myc* and EGR-1 were assayed by RNase protection in hearts, kidneys, livers and lungs. We found that hypoxia increased c-*jun* mRNA levels between twofold (lung) and eightfold (liver) in all organs examined; c-*fos* mRNA increased between threefold (lung) and 20-fold (heart); c-*myc* mRNA increased between twofold (lung) and sixfold (heart); and EGR-1 mRNA increased between twofold (lung) and sixfold (heart). Our findings suggest that acute tissue hypoxygenation is a general stimulus of the expression of immediate early genes in vivo. With regard to the sensitivity to hypoxia, organ differences appear to exist in that the lung is rather insensitive, whilst the heart is rather sensitive.

Key words Acute tissue hypoxygenation · Immediate early genes · Carbon monoxide · Erythropoietin

Introduction

Accumulating evidence indicates that tissue hypoxygenation in vivo specifically induces a variety of gene products in order to match the cellular oxygen deficiency [34]. Evidence for a widespread oxygen-sensing mechanism mediating the transcriptional activation of genes during hypoxia has been elaborated [41]. According to this concept, hypoxia induces the appearance of a nuclear protein, named HIF-1, which binds to specific enhancer sites in oxygen-regulated genes and activates their transcription. There is evidence to indicate that not all hypoxia-induced genes are activated by this particular pathway, suggesting that alternative, as yet uncharacterized, signalling pathways exist [11, 44]. Since transcription factors encoded by the families of immediate early genes such as *jun*, *fos*, *myc* and EGR-1 among others are known to be rapidly activated by mechanisms that do not require de novo production of mediator proteins, it is in principle conceivable that these factors could also be involved in the triggering of gene expression during hypoxia. In fact, it has been observed that ischaemia and particularly reoxygenation of the brain [21], kidney [20, 26, 30], liver [33] or heart [43] induce the expression of certain immediate early genes such as c-*jun*, c-*fos* or EGR-1. Ischaemia and reoxygenation certainly produce a number of cellular effects and it is difficult therefore to distinguish whether the induction of immediate early genes in these cases was directly mediated by the fall of tissue oxygen tension, as is the case for the induction of classic oxygen-regulated genes such as erythropoietin [15], or whether it was mediated by other pathways. The information about the effects of a more cautious tissue hypoxygenation induced by systemic hypoxia on the tissue expression of immediate genes is scarce. There are a few reports that hypoxia per se exerts only minor, if any, effects on immediate early gene expression in the brain [1, 40] and no effect in the liver [6]. However, several in vitro studies exist which report that hypoxia substantially increases c-*fos* and c-*jun* expression in endothelial cells [2], adrenocortical [24], colon [45] and liver cancer [24] cell lines, in a fibroblast [24] cell line, in cultured dopaminergic cells [22] and in cultured cardiac myocytes [42]. In a neuroblastoma cell line [24], in primary cultures of hepatocytes [12] and in cultures of vascular smooth muscle cells [36], hypoxia was not found to change immediate early gene expression.

These in vitro findings suggest that hypoxia may stimulate immediate early gene expression in a type- and tissue-specific fashion, depending on the cell culture conditions.

Whether hypoxia also stimulates immediate early gene expression in vivo, however, cannot be deduced from these cell culture experiments, since previous evi-

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dence indicates that hypoxia-induced gene expression in cell culture may not parallel similar effects in vivo [32]. We were interested therefore to search for evidence indicating whether tissue hypoxygenation in vivo is a specific stimulus for the activation of immediate early genes by examining the influence of acute hypoxia on immediate early gene expression in different organs. Moreover, we aimed to investigate whether stimulation of early gene expression by hypoxia in vivo is similar to stimulation of classic oxygen-regulated genes such as that for erythropoietin (EPO). *epo* gene expression is stimulated not only by a fall of the arterial oxygen tension but also by a reduction of the oxygen-carrying capacity of the blood and by certain divalent metals such as cobalt [15].

To this end we analysed mRNA levels for c-*jun*, c-*fos*, c-*myc* and for EGR-1 in hearts, kidneys, livers and lungs of rats exposed either to acute inspiratory hypoxia, of rats exposed to carbon monoxide and of rats treated with cobaltous chloride for 6 h. For comparison EPO mRNA levels were assayed in kidneys and livers.

Materials and methods

Animal experiments

Male Sprague-Dawley rats (220–250 g) with access to normal food and tap water were used for the studies.

Six groups of animals comprising five rats each were examined:

1. Controls receiving no treatment.
2. Animals exposed to 0.1% carbon monoxide (CO) for 0.5 h.
3. Animals exposed to 0.1% CO for 1 h.
4. Animals exposed to 0.1% CO for 6 h.
5. Animals exposed to 9% oxygen/balance nitrogen for 6 h.
6. Animals injected with cobaltous chloride (60 mg/kg, intraperitoneally) over 6 h.

At the end of the experiments the animals were killed by decapitation. Hearts, kidneys, livers and lungs were rapidly removed, frozen in liquid nitrogen and stored at –80°C until isolation of total RNA.

Extraction of RNA

Total RNA was extracted from the organs, which were stored at –80°C, according to the protocol of Chomczynski and Sacchi [8].

RNase protection for c-*fos*, c-*jun*, c-*myc* and EGR-1 mRNA

cDNA sequences for *fos* (170 bp), *jun* (314 bp), *myc* (194 bp) and EGR-1 (280 bp) were generated by RT-PCR according to standard protocols using the published sequences for c-*fos* [9], c-*jun* [31], *myc* [38] and EGR-1 [39]. The cDNA fragments were cloned into pSP73 vector. [^{32}P]-labelled cRNA probes were generated from the sense sequences using SP6 polymerase (Amersham International, Amersham, UK).

Transcripts were continuously labelled with [^{32}P]GTP (410 Ci/mmol or 15.2 GBq/mmol; Amersham International) and purified on a Sephadex G50 spin column. For hybridization organ total RNA was dissolved in a buffer containing 80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethane sulphonic acid), 400 mM NaCl, 1 mM ETDA. Total RNA was hybridized in a total volume of 50 µl at 60°C for 12 h with 5×10^5 cpm each of the radiolabelled cRNA probes. RNase dgestion with RNase A and T1 was carried out at 20°C for 30 min and terminated by incubation with proteinase K (0.1 mg/ml) and sodium dodecylsulphate (SDS, 0.4%) at 37°C for 30 min.

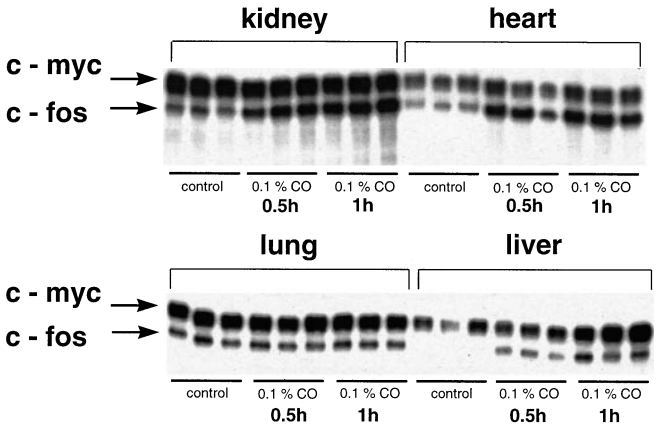

Fig. 1 Autoradiogram of an RNase protection assay with c-*myc* and c-*fos* mRNA probes using total RNA from hearts (40 µg), kidneys (50 µg), livers (40 µg) and lungs (20 µg) of three control rats and three rats each exposed to 0.1% CO for 0.5 and 1 h

Protected mRNA fragments were purified by phenol/chloroform extraction, ethanol precipitation and subsequent electrophoresis on a denaturing 10% polyacrylamide gel. After autoradiography of the dried gel at –80°C for 1 day, bands representing protected mRNA fragments were excised from the gel and radioactivity was counted with a liquid scintillation counter (1500 Tri-CarbTm, Packard Instrument Company, Downers Grove, Ill., USA). Figure 1 shows a representative autoradiogram of an RNase protection for a combination of c-*myc* and c-*fos* cRNA probes using total RNA from heart, kidney, liver and lung of control animals and of animals exposed to 0.1% CO for 0.5 and 1 h.

Determination of β-actin mRNA

The abundance of rat cytoplasmic β-actin mRNA was determined by RNase protection. An actin cRNA probe containing the 76-nucelotide and around 200 bp of the surrounding sequence was generated by transcription with SP6 polymerase from a pAM19 vector carrying an *Ava*I/*Hind*III restriction fragment of β-actin cDNA [27].

Determination of EPO mRNA by RNase protection

EPO mRNA was determined by RNase protection as described in detail previously [27]. For the assays 100 µg of kidney total RNA and 200 µg of liver total RNA were used. The assay was performed as described above.

Statistics

Levels of significance were calculated by ANOVA followed by Student's unpaired *t*-test. $P < 0.05$ was considered significant.

Results

Basal expression of immediate early gene mRNAs in different rat organs

To allow an estimate about the relative abundance of the mRNAs for the immediate early gene in the organs of

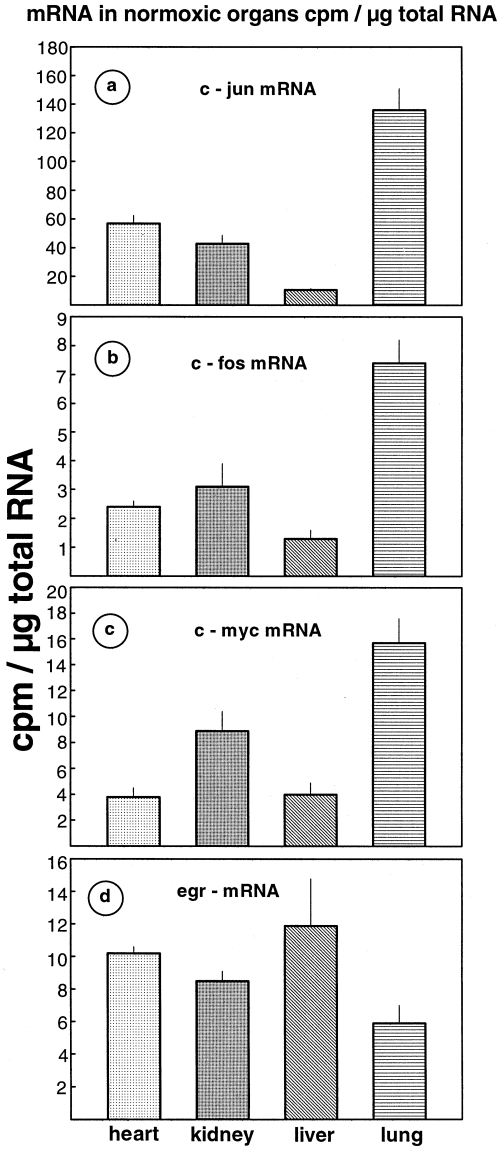

Fig. 2 Expression of c-*jun* (**a**), c-*fos* (**b**), c-*myc* (**c**) and EGR-1 (**d**) mRNA expression in different organs of normoxic rats. The abundance of mRNA is given by relating the respective hybridization signal obtained by RNase protection assay to the amount of total RNA used for the assay. Data are means ± SEM from five animals

normoxic rats, the hybridization signals obtained with the respective cRNAs were normalized to the amount of total RNA used for the RNase protection assays.

As shown in Fig. 2 c-*jun* was abundantly expressed in all the organs examined. Lowest mRNA levels were generally found for c-*jun* – the intraorgan c-*jun* mRNA/c-*fos* mRNA ratio ranging between 10 and 20. The relative abundance of c-*myc* mRNA and of EGR-1 mRNA was organ specific (Fig. 2).

For evaluation of the effect of hypoxia on mRNA levels of the immediate early genes in the different organs, the respective hybridization signals obtained in the RNase protection assay are expressed as a proportion of the hybridization signal obtained with a cRNA for β-actin and are always given as a percentage of the respective

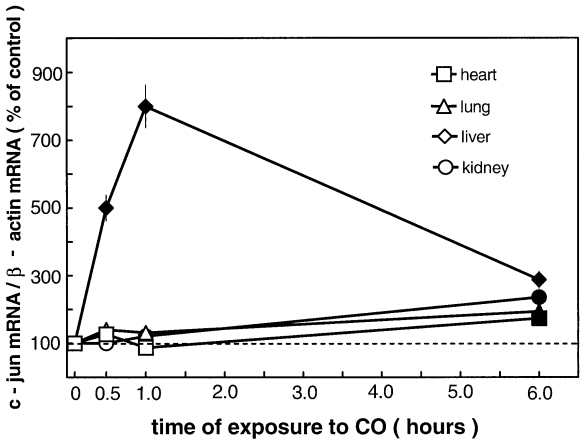

Fig. 3 c-*jun* mRNA/β-actin mRNA ratio for hearts, kidneys, livers and lungs of rats exposed to 0.1% CO for 0.5, 1 and 6 h. Data are means ± SEM from five animals in each case. *Filled symbols* indicate significant changes vs control ($P < 0.05$)

mean mRNA/β-actin mRNA ratio of control (normoxic) animals. The levels of β-actin mRNA were organ dependent and radioactivity values for actin mRNA obtained by RNase protection assay were 3440 ± 180, 1450 ± 160, 820 ± 75 and 340 ± 20 cpm/µg total RNA for lungs, kidneys, hearts and livers, respectively, but were not influenced by any of the experimental manoeuvres of this study.

Effect of hypoxia on c-*jun* mRNA levels (Fig. 3)

Exposure of the animals to 0.1% CO did not change c-*jun* mRNA expression in heart, lung and kidney within 1 h. After 6 h of exposure to CO c-*jun* mRNA levels had increased by about 100% in these organs. It was found that 6 h of inspiratory hypoxia also produced a moderate increase of c-*jun* mRNA levels in hearts and lungs but not in the kidney.

In livers, CO induced a prominent and temporally transient increase of c-*jun* mRNA levels. After 1 h of exposure to CO c-*jun* mRNA levels had increased about sevenfold over control. c-*jun* mRNA level in livers were still elevated over control after 6 h of exposure to CO or low oxygen tensions (Figs. 3 and 7).

Effect of hypoxia on c-*fos* mRNA levels (Fig. 4)

In livers CO also produced a more transient increase of c-*fos* mRNA levels, which were still elevated after 6 h of exposure to low oxygen tensions or to CO (Figs. 4 and 7). In hearts, kidneys and lungs CO produced a more continuous increase of c-*fos* mRNA levels over 6 h. The most prominent changes of c-*fos* mRNA levels were found in the heart, where c-*fos* mRNA levels had increased 20-fold and 12-fold after 6 h of exposure to CO and low oxygen tensions, respectively (Figs. 4 and 7).

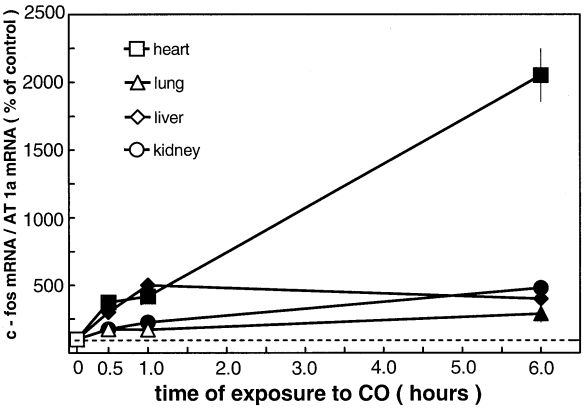

Fig. 4 c-*fos* mRNA/β-actin mRNA ratio for hearts, kidneys, livers and lungs of rats exposed to 0.1% CO for 0.5, 1 and 6 h. Data are means ± SEM from five animals in each case. *Filled symbols* indicate significant changes vs control ($P < 0.05$)

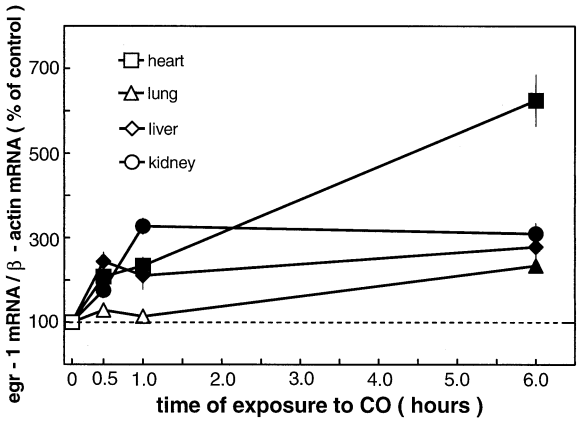

Fig. 6 EGR-1 mRNA/β-actin mRNA ratio for hearts, kidneys, livers and lungs of rats exposed to 0.1% CO for 0.5, 1 and 6 h. Data are means ± SEM of five animals in each case. *Filled symbols* indicate significant changes vs control ($P < 0.05$)

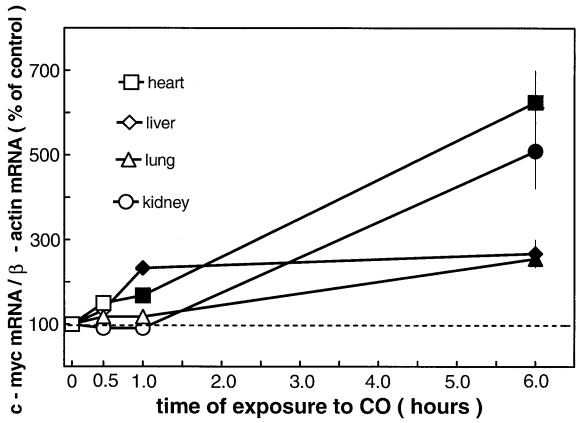

Fig. 5 c-*myc* mRNA/β-actin mRNA ratio for hearts, kidneys, livers and lungs of rats exposed to 0.1% CO for 0.5, 1 and 6 h. Data are means ± SEM from five animals in each case. *Filled symbols* indicate significant changes vs control ($P < 0.05$)

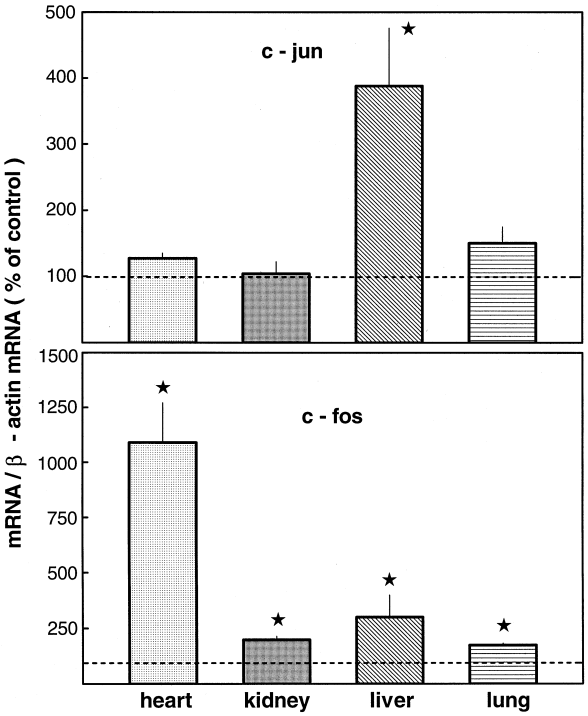

Fig. 7 c-*jun* mRNA/β-mRNA ratio (*upper*) and c-*fos* mRNA/β-actin mRNA ratio (*lower*) for hearts, kidneys, livers and lungs of rats exposed to 9% oxygen 6 h. Data are means ± SEM of five animals in each case. *Asterisks* indicate $P < 0.05$ vs controls

Effect of hypoxia on c-*myc* mRNA levels (Fig. 5)

c-*myc* mRNA levels increased continuously over 6 h in heart, kidney and lung in response to CO. A more transient increase of c-*myc* mRNA was again found for the liver. Nonetheless c-*myc* mRNA was still elevated in the liver after 6 h of CO inhalation or after 6 h of inspiratory hypoxia (Figs. 5 and 8).

Effect of hypoxia on EGR-1 mRNA levels (Fig. 6)

EGR-1 mRNA increased more continuously over 6 h in response to CO inhalation in hearts and lungs. In livers and kidneys EGR-1 mRNA levels appeared to reach a plateau after 1 h. However, our data do not rule out the possibility that EGR-1 mRNA might have reached a peak value between 1 and 6 h after exposure to CO or hypoxia. EGR-1 mRNA levels were also elevated after 6 h of inspiratory hypoxia (Fig. 8).

Effect of hypoxia on EPO mRNA levels (Fig. 9)

For comparison we also measured the time course of EPO mRNA expression in kidneys and livers of rats exposed to CO, since EPO is considered as a classic oxygen-regulated gene product. As shown in Fig. 9, EPO mRNA levels were elevated after 30 min in the liver, whilst the first rise of EPO mRNA in kidneys was detected after 1 h of exposure to CO. After 6 h of hypoxic hypoxia EPO mRNA levels were increased 80-

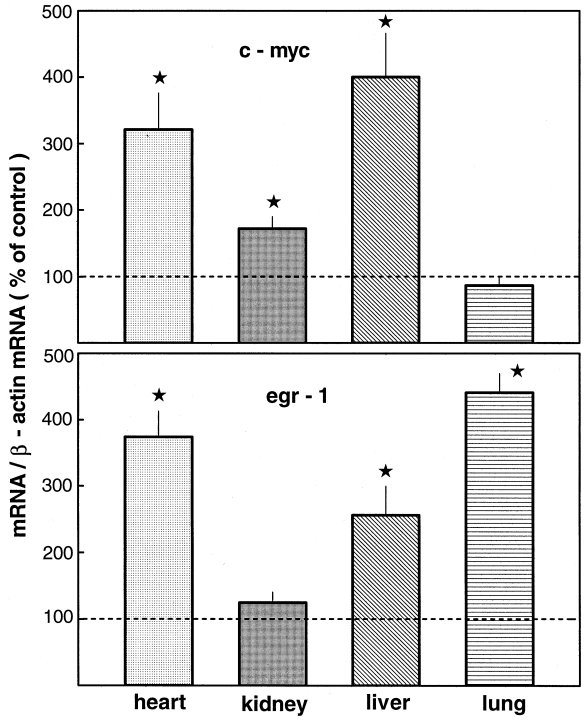

Fig. 8 c-*myc* mRNA/β-actin mRNA ratio (*upper*) and EGR-1 mRNA/β-actin mRNA ratio (*lower*) for hearts, kidneys, livers and lungs of rats exposed to 9% oxygen 6 h. Data are means ± SEM of five animals in each case. *Asterisks* indicate $P < 0.05$ vs controls

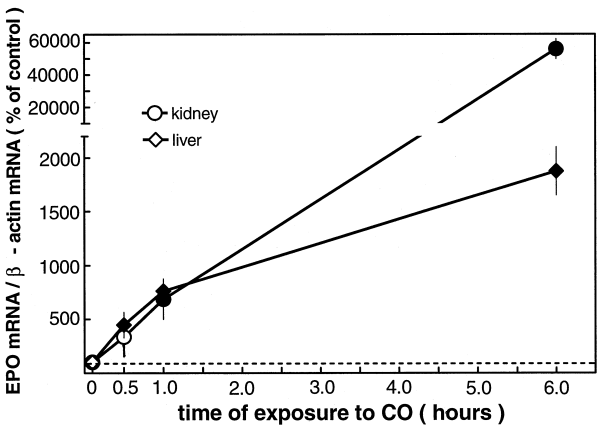

Fig. 9 EPO mRNA/β-mRNA ratio for kidneys and livers of rats exposed to 0.1% CO for 0.5, 1 and 6 h. Data are means ± SEM of five animals in each case. *Filled symbols* indicate significant changes vs control ($P < 0.05$)

fold ± 14-fold and 18-fold ± 4-fold, in kidneys and livers, respectively. Apart from the tissue oxygen tension *epo* gene expression is also stimulated by certain divalent metals, in particular by cobalt. It was found that 6 h after an intraperitoneal injection of 60 mg/kg cobaltous chloride EPO mRNA levels had increased 64-fold ± 11-fold and 7.1-fold ± 0.4-fold in kidneys and livers, respectively.

We also examined the mRNA levels of immediate early genes in these animals treated with cobalt.

Table 1 Effect of a 6-h treatment with cobaltous chloride (60 mg/kg) on mRNA levels of immediate early genes

Organ	mRNA levels (% of untreated controls)			
	c-*jun*	c-*fos*	c-*myc*	Egr-1
Heart	149±11	154±8*	71±3*	144±22
Kidney	73±7	98±6*	71±6*	130±6
Liver	106±11	115±15	29±4*	150±30
Lung	237±4*	224±9*	154±3*	195±19*

Data are means±SEM of five rats
Asterisks indicate $P<0.05$ vs controls

Cobalt treatment increased c-*jun* mRNA and c-*fos* mRNA in hearts and lungs up to twofold but not in kidney and liver (Table 1). c-*myc* mRNA was downregulated by cobalt in heart, kidney and livers but was moderately increased in lungs (Table 1). EGR-1 mRNA was consistently upregulated by cobalt up to twofold in all organs (Table 1).

Discussion

This study aimed to examine the influence of acute tissue hypoxygenation on the gene expression of immediate early genes in rat tissues. Tissue hypoxia was induced by CO inhalation which reduces the oxygen-carrying capacity of the blood or by inspiratory hypoxia. The effectiveness of these experimental manoeuvres is indicated by the significant stimulation of *epo* gene expression in livers and lungs of these animals, because *epo* gene transcription is considered to be directly triggered by a fall of the tissue oxygen tension [13].

We found that the mRNA levels of all immediate early genes examined in this study, namely c-*jun*, c-*fos*, c-*myc* and EGR-1, were increased by hypoxia in all organs within 6 h, albeit in a type-, organ- and time-specific fashion.

With the exception of the liver, c-*jun* mRNA expression was only moderately stimulated by tissue hypoxygenation. c-*fos* mRNA expression was in general more sensitive to hypoxia than c-*jun* mRNA expression and increased up to 22-fold in the heart. c-*myc* mRNA and EGR-1 mRNA expression displayed a similar sensitivity towards hypoxia and increased between two- and sixfold in an organ-dependent fashion.

With the exception of c-*jun* mRNA expression, the heart showed the most prominent inductions of immediate early gene expression by hypoxia among the organs examined in this study. On the other hand the smallest increments of immediate early gene expression were found in the lung. This could indicate that the sensitivity towards systemic oxygen deficiency is less in the lung. The relatively small increments of mRNA levels for immediate early genes in the lung could also be due to the fact that in the lungs of normoxic animals mRNA levels for immediate early genes are already high in comparison with the other organs. This high basal level of expression of immediate early gene expression in the lung

could indicate that these genes are already strongly stimulated by factors unrelated to hypoxia. Supportive of this explanation is the observation that in the heart, which appears to be rather sensitive to hypoxia with regard to immediate early gene expression, c-*jun* mRNA expression was already strong in normoxic animals and hypoxia exerted only a moderate further stimulatory effect.

There were also obvious differences in the time courses of immediate early gene expression in response to hypoxia between the different organs. In the liver stimulation of immediate early gene expression turned out to be of rapid onset, reaching peak values within the first 6 h after the start of tissue hypoxygenation. In the other organs, in particular in the heart, mRNA levels for immediate early genes increased more continuously during acute hypoxia.

Considering our findings in the light of background data relating to the influence of hypoxia on immediate early gene expression in cell cultures as listed above, our findings support the prevous findings that hypoxia stimulates c-*fos* and c-*jun* gene expression in various cell types in vitro in a tissue-specific fashion and, for the first time, suggest the in vivo relevance of these findings. Stimulation of c-*myc* and EGR-1 gene expression by acute hypoxia has not been reported previously, either in vitro or in vivo.

Previously, a concept was developed in which the transcriptional activation of oxygen-regulated genes in response to hypoxia was hypothesized to follow very similar intracellular signalling pathways [19]. It has been suggested that the primary oxygen sensor is a haem protein [13] which in the desoxy-state or in the cobalt-substituted state induces a sequence of events which lead to the activation of a transcription factor termed HIF-1 [41], which then bind to a specific enhancer site [25] on the gene and consequently enhances transcriptional activity. This concept has been elaborated for EPO in particular, which is considered to be a classic oxygen-regulated gene [15]. It appeared of interest, therefore, to us to compare the expression of immediate early genes with the *epo* gene expression in response to hypoxia, to obtain some evidence as to whether immediate early gene expression is similarily triggered by hypoxia as is *epo* gene expression. When comparing the time course of immediate early gene expression in the liver and kidney, in particular that of c-*jun* and c-*fos*, with that of EPO it becomes clear that they have significantly different kinet. Moreover, *epo* gene expression in livers and kidneys can clearly be stimulated by cobalt, whilst c-*jun* and c-*fos* gene expression are not. One may infer from this that the induction pathways of c-*jun* and c-*fos* transcription in response to acute hypoxia are different from that of EPO. This conclusion is supported by the concept that early activation of c-*jun* and c-*fos* gene transcription does not require de novo protein synthesis whilst the activation of *epo* gene transcription clearly does. It is likely, therefore, that the increase of mRNAs of immediate early genes during hypoxia was not mediated via the “haem protein HIF-1”-pathway. This inference is indirectly supported by the observation that hypoxia stimulates *epo* but not c-*fos* or c-*jun* gene expression in short-term primary cultures of hepatocytes [12]. Moreover, it has been reported that hypoxia stimulates VEGF but not c-*myc* gene expression in cultured vascular smooth muscle cells [36] and cultured endothelial cells [37].

Since transcription factors encoded by immediate early genes are involved in a number of gene activations it appears quite likely that activation of immediate early genes could mediate, or at least contribute to, either up- or downregulation of genes during hypoxia. c-*jun* and c-*fos* may be of particular interest for genes containing AP-1 bindings sites, such as vascular endothelial growth factor and lactate dehydrogenase A [29, 35]. It is also generally agreed that immediate early gene products could act as growth regulators for cells relevant to tumorigenesis and also for physiological growth processes such as organ hypertrophy, hyperplasia or organ repair.

In this view hypoxia-induced c-*myc* gene expression in the lung could be involved in hypoxia-induced media-hyperplasia of pulmonary vessels [14], because c-*myc* stimulates proliferation of vascular smooth muscle cells [3]. A wealth of information has meanwhile been accumulated which suggests that immediate early genes could be involved in the development of cardiac hypertrophy [4, 7, 10, 16, 18, 28]. In this context immediate early genes are thought to be also relevant to the induction of the embryonic gene repertoire [5, 17], such as the atrial natriuretic peptide or the β-myosin gene, which both are characteristic and functionally relevant molecular markers of the development of ventricular hypertrophy. Chronic hypoxia indeed induces atrial natriuretic peptide and β-myosin gene expression and also ventricular hypertrophy [23].

In summary, our findings show that systemic hypoxia leads to a widespread upregulation of immediate early gene expression in the whole organism.

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