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Abstract

The formation of disulfide bonds in the endoplasmic reticulum requires protein disulfide isomerase (PDI) and endoplasmic reticulum oxidoreductin 1 (ERO1) that reoxidizes PDI. We report here that the expression of the rat, mouse and human homologues of ERO1-Like protein alpha but not of the isoform ERO1-Lbeta are stimulated by hypoxia in rats vivo and in rat, mouse and human cell cultures. The temporal pattern of hypoxic ERO1-Lalpha induction is very similar to that of genes triggered by the hypoxia inducible transcription factor (HIF-1) and is characteristically mimicked by cobalt and by deferoxamine, but is absent in cells with a defective aryl hydrocarbon receptor translocator (ARNT, HIF-1beta). We speculate from these findings that the expression of ERO1-Lalpha is probably regulated via the HIF-pathway and thus belongs to the family of classic oxygen regulated genes. Activation of the unfolded protein response (UPR) by tunicamycin, on the other hand, strongly induced ERO1-Lbeta and more moderately ERO1-Lalpha expression. The expression of the two ERO1-L isoforms therefore appears to be differently regulated, in the way that ERO1-Lalpha expression is mainly controlled by the cellular oxygen tension, whilst ERO1-Lbeta is triggered mainly by UPR. The physiological meaning of the oxygen regulation of ERO1-Lalpha expression likely is to maintain the transfer rate of oxidizing equivalents to PDI in situations of an altered cellular redox state induced by changes of the cellular oxygen tension.

THE CELLULAR OXYGEN TENSION REGULATES EXPRESSION OF THE ENDOPLASMIC OXIDOREDUCTASE ERO1-L

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Summary

The formation of disulfide bonds in the endoplasmic reticulum requires protein disulfide isomerase (PDI) and ERO1, which reoxidizes PDI. We here report that the expression of the rat homologue of ERO1-like protein is strongly stimulated by hypoxia in vivo and in vitro. The temporal pattern of hypoxic ERO1-l induction is very similar to that of genes triggered by the hypoxia inducible transcription factor (HIF-1) and is characteristically mimicked by cobalt and by desferoxamine, but is absent in cells with a defective ARNT (HIF-1β). We conclude from these findings that the expression of ERO1-l is directly regulated via the HIF-pathway and thus belongs to the family of classic oxygen regulated genes. The physiological meaning of the oxygen regulation of ERO1-l expression likely is to maintain the transfer rate of oxidizing equivalents to PDI in situations of an altered cellular redox state induced by changes of the cellular oxygen tension.

Introduction

Formation of dilsufide bonds is an essential event for the correct folding of proteins in the endoplasmic reticulum. It is well known that this process is catalyzed by protein disulfide-isomerase (PDI) (1). Until a few a years ago it remained, however, unclear how PDI is reoxidized in this reaction (2). It was the discovery of the ERO1 (endoplasmic reticulum oxidoreductin) -protein in yeast (3, 4) which provided evidence that this protein is essential to transfer oxidizing equivalents to PDI (5). It turned out that ERO1 is a highly conserved endoplasmic protein and for humans two ERO1-like proteins have meanwhile been identified, termed ERO1-l α (6) and -1β (7). The ERO1-l α protein which shares a higher homology with yeast ERO1 than ERO-1l ß, likely is a flavoprotein (8) that covalently binds to PDI (9), what explains it function to transfer oxidizing equivalents to PDI. ERO1-l α and ERO-1l ß display different tissue distributions (7), and moreover, appear to be differently regulated in their expression. Thus, only ERO-1l ß transcripts are induced in the course of unfolded protein response (7). In this pathway accumulation of misfolded proteins in the endoplasmic reticulum induces the expression of a number of proteins including those involved in the correct folding of proteins such as chaperones (10). How the expression of the ERO1-lα protein is regulated is yet unknown. Analyzing the protein expression pattern of a rat vascular smooth muscle cell line, we now found that a ERO1-like protein highly homologous to human ERO1-lα is strongly upregulated during cellular hypoxia. This study therefore aimed to characterize the effects of low oxygen tension on ERO1-l expression.

Materials and Methods

Cell Cultures

Rat aortic vascular smooth muscle cells (A7r5) from BDXI rats (ATCC CRL 1444) were cultured in 75cm^2 flasks (Sarstedt) with 15 ml Dulbecco´s MEM-medium containing 10% FCS and penicillin /streptomycin - P/S (10.000U/10.000µg/ml)(Biochrom), kept in room air with 10% CO_2 at 37°C. Medium was changed every second day and cells were confluent on day 7 to 10 after splitting which was achieved with trypsin-EDTA for 5 min at 37°C. For the experiments cell cultures (triplicates) were incubated at room air (21%O_2 i.e normoxia) or 1%O_2 or 0.5% O_2 (i.e. hypoxia) for up to 12 hours. Additional culture dishes were incubated at 21%O_2 with either cobaltous chloride (100µmol/l) or with desferoxamine (100µmol/l) for 12 hours.

Mouse hepatoma Hepa1 cells, and their subclone Hepa1C4, which produces defective ARNT (HIF-1β) (11) due to a point mutation (12) rendering the cells unable to form active HIF (13), were grown under the above mentioned conditions. For the experiments the cells were incubated either at 0.5%O_2 (i.e. hypoxia) or at 21%O_2 with desferoxamine (100µmol/l) for 4.5 hours.

In vivo experiments

All experiments were conducted in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals and the German Law on the protection of Animals. Male Sprague-Dawley rats (200-250g) that had free access to food and water were used for the experiments and treated in the following way: 1) in the control group, animals received no treatment (n=6); 2) in the hypoxia group, the animals were placed in a gas-tight box that was continuously supplied with a gas mixture of 8% O_2-92%N_2 for 6 h(n=6); 3) in the carbon monoxide group, the animals were placed in a gas tight box that was continuously supplied with room air plus 0.1% carbon monoxide (CO) for 6h (n=6); and 4) for cobalt treatement, the rats were subcutaneously injected with cobalt chloride (60mg(kg), and the animals were killed 6 h later (n=6). At the end of the experiments, the animals were killed by decapitation. Brains, hearts, kidneys, livers and lungs were quickly removed, weighed, and rapidly frozen in liquid nitrogen. All organs were stored at –80°C until isolation of protein and total RNA.

Preparation of protein samples

After removal of cell culture medium, cell were washed three times with ice-cold PBS and then scraped off in lysis buffer (300µl/ 75 cm^2 flask) consisting of 7 mol/l urea, 2mol/l thiourea, 2%CHAPS, 1%DTT, Pharmalyte pH 3-10 l (Pharmacia, Uppsala, Sweden), supplemented with protease inhibitors (complete®, Boehringer Mannheim, Germany). The material was then homogenized with an Ultraturrax (3 x 10sec) and further sonicated with 3 x 10 sec. The homogenate was then allowed to stand at room temperature for 60 minutes prior to ultracentrifugation at 80.000 x g at 15°C for 1 hour. Aliquots of the clear supernatant were frozen in liquid nitrogen and stored at –80°C. For determination of the protein concentration, protein was precipitated with 10% TCA in acetone and resuspended in 0.1N NaOH. Protein concentration was then determined with the Bio-Rad protein assay (BIO-RAD, Int).

Two-dimensional PAGE

150µg protein (for silverstained gels) and 600 µg protein (for Coomassie-Blue staining) were loaded for each sample onto the first dimension strips. A linear immobilized pH gradient (pH 5.0-6.0 IPG 18cm; Pharmacia, Uppsala, Sweden) was used as the first dimension. Hydration of gel strips and sample application was performed at 50V for 15 h. For protein separation a step voltage protocol was applied (1h 150V, 3h 500V, 1h 1000V, gradient to 8000V within 0.5 h). A total volt-hour product of 60 kVh was used for 150µg protein and 110 kVh for 600µg protein. Afterwards the stripes were incubated in 50mmol/l Tris-HCl (pH 6.8), urea 6mol/l, glycerol 30%, DTT 65 mmol/l , 2% SDS for 20 min at room temperature followed by incubation in 50mmol/l Tris-HCl (pH 8.8)), urea 6mol/l, glycerol 30%, iodoacteamide 140 mmol/l, 2% SDS for another 20 min. For the second dimension, a vertical gradient slab gel of 8%-18% acrylamide was used and SDS/PAGE was performed at 8mA per gel at 13°C for 4 h followed by 30mA for 12 h. At the end of the second dimension, the gels were removed from the glass plates.

Staining of two-dimensional PAGE

The gels were fixed and stained with silver according to standard protocols (14). The gels were then scanned (Image Scanner Sharp JX-330, Amersham Biosciences) and analysed with the Image 3.1 analysis software package (Amersham Bioscience). Each spot was matched from one gel to another and the relative volume of matched spots was compared. For preparative protein analysis higher

amounts of protein were loaded for two-dimensional PAGE and the protein spots were then stained with colloidal Coomassie-Blue.

Protein sequence analysis

Coomassie-Blue stained spots were excised from the gels and were subjected to ESI-MS analysis (15). Sequences obtained with ESI-MS analysis were then compared with the mouserat.NCBInr.fasta protein database.

cDNA cloning

From the protein sequence of the obtained peptides the coding DNA sequence was obtained with database standard programs. A pair of sense primer 5´-CGG GAT CCT GCG AGC TAC AAG TAT TC-3´ and antisense downstream primer 5`- GGA ATT CTC CAC ATA CTC AGC ATC G-3´ was then used for standard RT-PCR cloning of a cDNA fragment of the sequenced protein. A 192 bp cDNA fragment with the sequence:. 5´- tcc aca tac tca gca tcg ggg gac tgt atg tca tca act tca cag aag ctg tct gaa gaa tca tcg tgt ttc gtc cac tga aga aca gcc ttc tgg gtc tcc tca ctc aga gat tcg tcc act gct ccg agc cgc tca gcc tgc tca cac tcc tca agg agg ttg gct tcc ttg gaa tac ttg tag ctc gca- 3´ was obtained. This sequence was then further used for sequence comparisons and to generate a cRNA probe for RNase protection.

RNA isolation

Total RNA was extracted from freshly harvested cells and from frozen tissues according to the protocol of Chomczynski and Sacchi (16).

RNase protection assay of ERO1-1, adrenomedullin (ADM) and β-actin mRNA

ERO1-1, ADM and β-actin mRNA levels were measured by RNase protection assay as described previously (17). In brief, radiolabelled antisense cRNA probes were synthesized by in vitro transcription of plasmid vectors carried subcloned cDNA fragments for ERO1-l, ADM and ß-actin with SP6 polymerase (Promega) in the presence of [α-^{32}P]GTP (Amersham). Labeled cRNA probes were hybridized with total RNA at 60°C for 16 h, then digested with RNase A/T1 at room temperature for 30 min and proteinase K at 37°C for 30 min. After phenol/chloroform extraction and ethanol precipitation, the protected RNA hybrids were separated by electrophoresis on 8% polyacrylamide gels. After drying of the gels, the amount of radioactivity was assessed by an Instant

Imager (Packard) in counts per minute (cpm) and autoradiography was performed at -80°C for 1 day. Results were expressed as in proportion to ß-actin mRNA as internal standard.
For analysis of Hepa1 and Hepa1C4 cells, which are of mouse origin, respective cDNA fragments were cloned from published sequences (gi|15718668).

Statistics

Levels of significance between groups were calculated using ANOVA test and multi way comparisons analysis. $P<0.05$ was considered significant.

Results

Screening the rat vascular smooth muscle cell line A7r5 for hypoxia induced proteins by 2D-electrophoresis revealed a highly reproducible and marked (about 20-fold) upregulated abundance of a protein with an pI of around pH 5.7 and an apparent molecular mass of 58kD on SDS-PAGE (Fig.1). By ESI-MS tryptic peptides were identified that covered 45.9% of the aminoacid sequence of the mouse ERO1-like protein, which consists of a total of 464 amino acids (gi|7657067). Based on the sequenced peptides a cDNA fragment was cloned by RT-PCR standard techniques. The resulting 192 bp cDNA sequence shared a 100% homology with rat ERO-1(gi|18250365), 88% homology with mouse ERO1-1 (gi|15718668), 85% homology with human ERO1-1α (gi|7021225), but no significant homology with human ERO1-1β (gi|9845248).
It was concluded therefrom that the cloned cDNA was rat ERO1-l cDNA and the hypoxia induced protein was rat ERO1-l (rERO-l). The cloned cDNA was then used to generate cRNA probes for semiquantification of rERO1-l mRNA by RNAse protection.
It turned out that the abundance of rERO1-l mRNA in A7r5 cells at high oxygen tensions (21%O_2) was rather low, but increased strongly (20-fold) with a charateristic time pattern and reached a stable plateau level after exposure of the cells to low oxygen tensions (1%0_2) (Fig.2, upper). The temporal pattern of rERO1-l mRNA was very similar to that of classic oxygen regulated genes, such as adrenomedullin (ADM) (Fig.2, lower), the expression of which is triggered by the hypoxia inducible transcription factor HIF-1 (18). In addition, rERO1-l mRNA was like ADM mRNA upregulated by the divalent cation cobalt (100 µmol/l) and by the iron chelator desferoxamine (100 µmol/l) (Fig.3).
Hypoxia and desferoxamine also increased ERO-1 mRNA in the mouse hepatoma cell line Hepa1 (Fig.4), suggesting a species independent stimulatory effect of hypoxia on ERO-1 gene expression. Notably, in the mutant cell line Hepa1C4, which is unable to generate active HIF (13), hypoxia and desferoaxmine failed to increase ERO-1 mRNA (Fig.4). At high oxygen tensions (21%O_2) the abundance of ERO-1 mRNA was slightly lower in Hepa1C4 than in Hepa1 cells.

A next set of experiments was designed to test for the in vivo relevance of the findings obtained in vitro. For this goal rats were exposed either to room atmosphere (21%0_2) or to a low inspiratory oxygen tension (8%0_2) and the protein extracts from different organs were analyzed by 2D-electrophoresis, and rERO-1 mRNA was semiquantitated by RNAse protection. Again, we found a

pronounced protein spot in all hypoxic tissues, which displayed the same physical characteristics as in A7r5 cells. As shown in Fig.5 rERO1-l mRNA was also upregulated by hypoxia in all organs examined. To determine whether the upregulation of rERO1-l was not only related to a fall of the arterial oxygen tension but more generally to a fall of cellular oxygen tension, we also examined the effect of carbon monoxide (CO) inhalation (0.1%). 0.1% CO inhibits oxygen transport by hemoglobin by about 50% and thus diminishes oxygen delivery to the tissues without changing arterial oxygen tension. Depending on the rate of tissue oxygen consumption CO will therefore lower tissue oxygen tension. It turned out that also CO clearly stimulated rERO1-l mRNA levels in the different rat organs, with the exception of the lung, in which tissue oxygen tensions are directly related to inspiratory oxygen tensions rather than to the oxygen carrying capacity of the blood (Fig.5). Thus, the failure of CO to stimulate rERO1-l expression in the lung, can be taken as an arguement that CO did not itself increase rERO1-l expression. Like in vitro, rERO1-l in vivo was again stimulated by the divalent cation cobalt, that was subcutaneously administered (fig.5).

Discussion

Our data indicate that the expression of the rat and mouse isoform of ERO1-l , that are highly homologous to human ERO1-l α, are strongly upregulated if the cellular oxygen tension falls. Apparently, this phenomenon appears to be of major relevance also under in vivo conditions under which rERO1-l protein expression is also markedly increased during hypoxia. Our data also show that not only arterial hypoxia but also a reduction of the oxygen carrying capacity of the blood (by carbonmonoxide inhalation) stimulates rERO1-l gene expression in various tissues. The relative effects of arterial hypoxia and of CO-inhalation on ERO-l gene expression were organ dependent. This differential effect is likely explained by the fact that the effect of CO-inhalation on tissue oxygens tensions is dependent on the ratio of hemoglobin bound oxygen delivery over tissue oxygen consumption, which is different among the organs. This linkage becomes most obvious in the lung, in which tissue oxygen tensions are not dependent on hemoglobin bound oxygen delivery, since the lung is in direct contact with the gas athmosphere.

It is well known that a variety of endoplasmic proteins with chaperone function are induced by severe cellular hypoxia (anoxia) as well as by glucose deprivation (19). It is thought that the expression of these proteins in response to anoxia is triggered by the unfolded protein response (URP) which regulates the activity of chaperone genes (20). Unfolding or misfolding of proteins in the endoplasmic reticulum during anoxia probably results from ATP depletion and also from changes of redox potentials. In fact, yeast ERO1 (3) and ERO-1l ß in human tissues (7) are also stimulated by URP. Interestingly, human ERO1-l α is not affected by URP (7) suggesting that ERO1-l α is differently regulated in its expression, likely via the HIF-pathway. HIF-1 is a heterodimer consisting of an α- and a β-subunit (21). HIF-1α stability is regulated by the cellular oxygen tension, in the way that an oxygen/iron dependent prolyl-hydroxylation leads to increased ubiquitinilation and finally proteasomal degradation of HIF-1α (22, 23). In consequence, a decrease of prolyl-hydroxylase activity by low oxygen tensions, by iron chelation or by cobalt increase HIF-1α protein levels and therefore the activity of the HIF-1 transcription factor (24). Notably, the HIF prolyl-hydroxylases themselves are also induced by hypoxia (25).

Our data provide several lines of evidence to indicate that the expression of mouse and rat ERO1-l, that are homologous to human ERO1-l α, is importantly triggered by the hypoxia-inducible transcription factor (HIF-1). Thus, the temporal pattern of the induction of rERO1-l expression by hypoxia in vitro is very similar to HIF-1 regulated genes, such like adrenomedullin (18). Moreover, the effect of hypoxia on rERO1-l gene expression can be mimicked in a very characteristic fashion

by cobalt and by the iron chelator desferioxamine, which do not change cellular oxygen tension but increase HIF-1α and therefore stimulate HIF-1 activity (26, 27). Finally, the stimulation of ERO-1 gene expression was absent in a cell line with a functional mutation in the HIF-1β gene, which causes an inability to form active HIF (13). The localization of HIF-binding sites on the rERO1-l gene, however, must await the cloning of the genomic rat ERO1-l sequence (28). The observation that HIF-deficient cells also express ERO-l mRNA at all, suggests that additional transcription factors to HIF tigger the ERO-l gene.

HIF-1 regulated genes as identified so far encode proteins that mainly serve to match the cellular energy deficit resulting from insuffient oxygen supply (29). Thus, glucose transporters and key enzymes of the glycolytic pathway are regulated by HIF-1 and are upregulated during hypoxia. Also secreted proteins such as erythropoietin which stimulates red cell formation (and thus increases the oxygen carrying capacity of the blood) or vascular endothelial growth factor (VEGF), which induces capillary formation, or adrenomedullin (ADM), which causes vasodilation, are stimulated by HIF-1 in response to hypoxia (for rev. see 29).

With the regulation of proteins that are involved in correct folding of proteins in the endoplasmic reticulum, HIF-1 would aquire a new responsibility for cellular function. A regulation of ERO1-l production by HIF-1 means that chaperone formation during hypoxia is uncoupled from energy depletion (which intiates the UPR), and thus allows a counterregulation in situations in which the cellular redox state is already altered whilst the energy state is still normal. A number of endo- or paracrine signals involved in the hypoxia defense such as for example erythropoietin (30), vascular endothelial growth factor (31) or adrenomedullin (32) in fact contain disulfide bonds that are indispensible for their biological function. Problems with disulfide bond formation during a fall of the oxygen tension may arise from the change of the redox potential of the cell, which impairs the flow rate of oxidizing equivalents from ERO1-l to PDI. Under reducing conditions PDI would actually catalyze the reduction of protein disulfides (1). The relevance of PDI in this context was underlined previously by the finding that overexpression of PDI attenuated the loss of cell vilability induced by hypoxia in a neuroblastoma cell line (33). Since ERO1-l exists as a collection of oxidized and reduced forms (34) increasing the total number of ERO1-l molecules during hypoxia would therefore compensate for the diminuation of the redox gradient and maintain a constant flow of oxidizing equivalents to PDI over a broad range of cellular oxygen tension.

The oxygen regulation of ERO1-l expression appears to be part of a more general network in which the expression of chaperones is regulated by the oxygen tension through HIF-1. Thus, it was shown previously that hypoxia increases the expression of PDI itself in brain cells in vitro and in vivo (33), although it was not further examined in that study as to whether the upregulation of PDI was mediated by URP or by the HIF-1 pathway. PDI also serves as the ß-subunit of the prolyl-4-hydroxylase, which is a heterotetramer consisting of 2α and -2β subunits (35). It was reported previously for cultured fibroblasts that hypoxia induces the expression of α-subunit of the prolyl-4-hydroxylase (I) through the HIF-1 pathway (36).

All together, our findings suggest that a fall of the cellular oxygen tension compensatorily increases the expression of a protein that is required to transfer oxidizing equivalents to PDI, and is therefore required for correct protein folding in the endoplasmic reticulum.

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Figure legends

Figure 1

2D-electrophoresis of proteins isolated from the rat vascular smooth muscle cell line A7r5 kept at either 21%O_2 (a) or 1%O_2 (b) for 12 hours. Note the upregulation of the indicated protein spot.

Figure 2

Time course of rERO1-l mRNA (upper panel) and of adrenomedullin mRNA (lower panel) in A7r5 cells after exposure of the cells to 1%O_2. Data are means +/- SEM of five experiments. * indicates $p<0.05$ hypoxia (1%O_2) vs normoxia (21%O_2).

Figure 3

rERO1-l mRNA (upper panel) and adrenomedullin mRNA (lower panel) in A7r5 cells after exposure to 0.5%O_2 or to cobaltous chloride (100µmol/l) or desferioxamine (100µmol/l) for 12 hours at 21%O_2. Data are means +/- SEM of five experiments each. * indicates $p<0.05$ vs control (21%O_2).

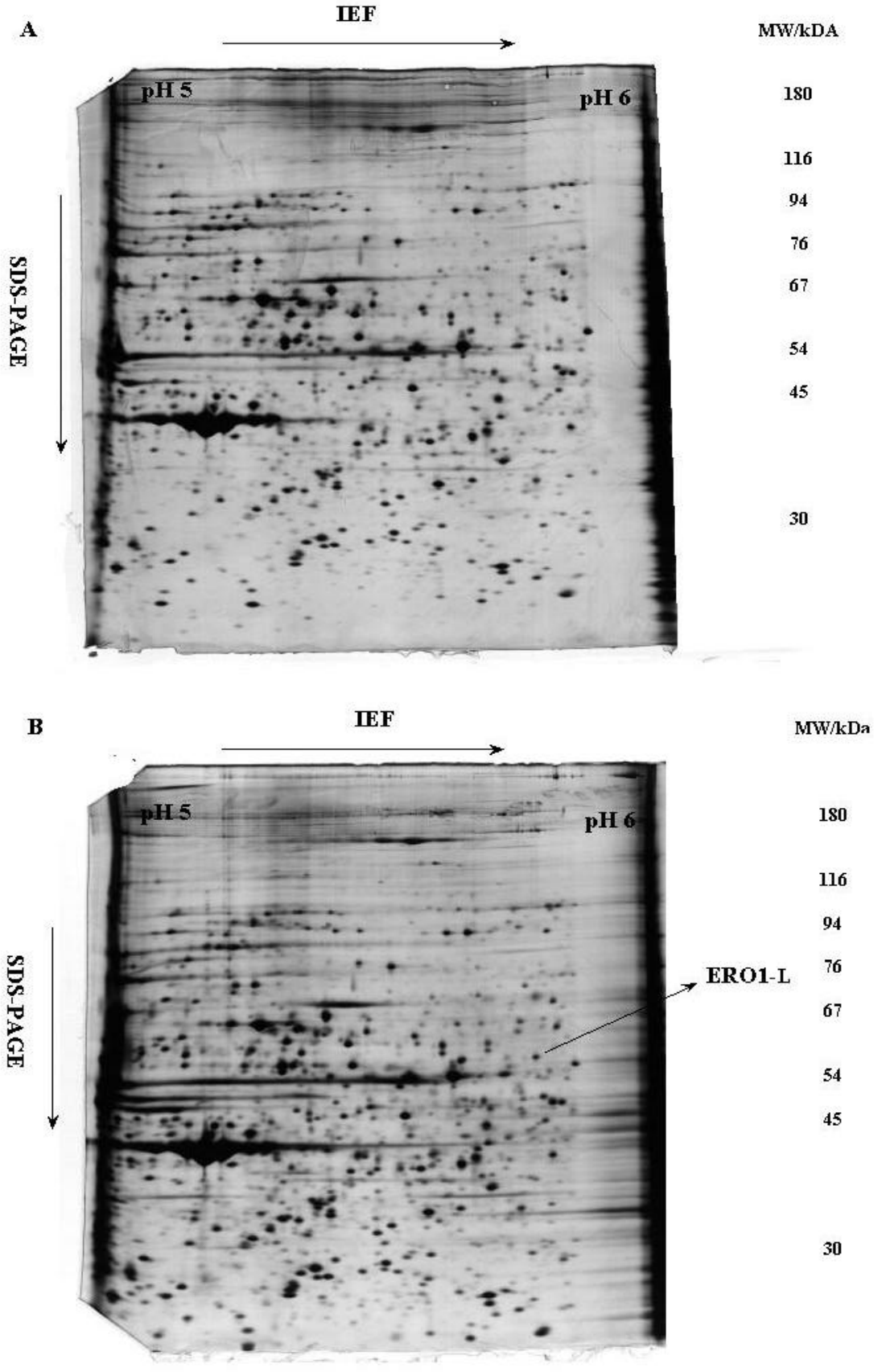
Figure 4

Mouse ERO1-l mRNA in Hepa1 (upper panel) and in Hepa1C4 cells (lower panel) after exposure to hypoxia (0.5% O_2) (100µmol/l) or to desferioxamine (100µmol/l) at 21%O_2 for 4.5 hours Data are means +/- SEM of five experiments each. * indicates $p<0.05$ vs control (21%O_2).

Figure 5

Effect of hypoxia (8%O_2), carbon monoxixde (0.1%) inhalation and of administration of 60mg/kg cobaltous chloride on rERO1-l mRNA in rat liver, kidney and lung. The experiments were performed for 6 hours each. Data are means +/- SEM of 6 rats in each group. Asterisks indicate $p<0.05$ vs normoxia control (21%O_2).

Figure 1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MW/kDA
pH 5
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SDS-PAGE
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116
94
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54
45
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ERO1-L
180
116
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30

Figure 2

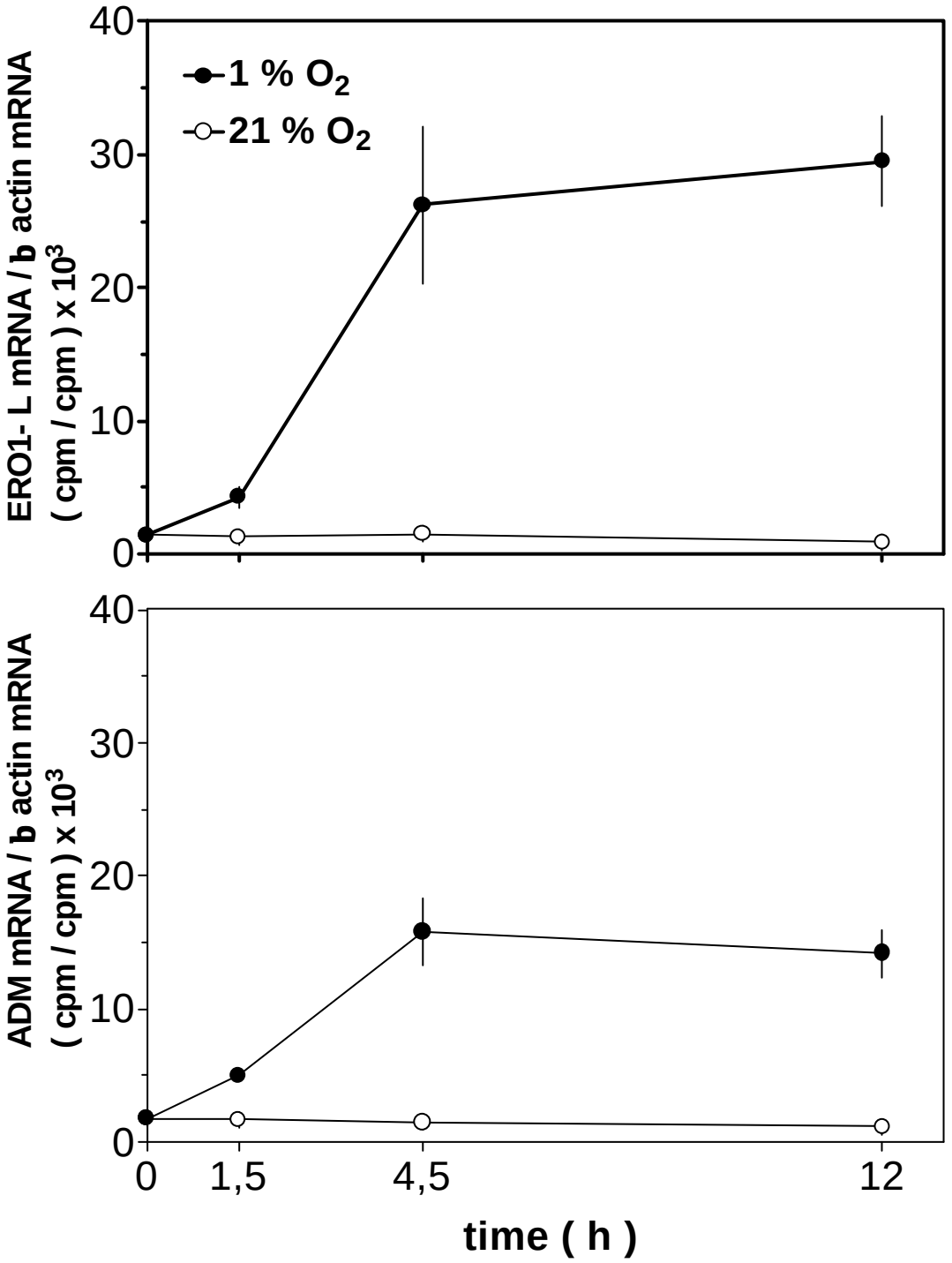

1 % O2
21 % O2
ERO1- L mRNA / b actin mRNA
(cpm / cpm) x 10³
ADM mRNA / b actin mRNA
(cpm / cpm) x 10³
0
10
20
30
40
0
1,5
4,5
12
time (h)

Figure 3

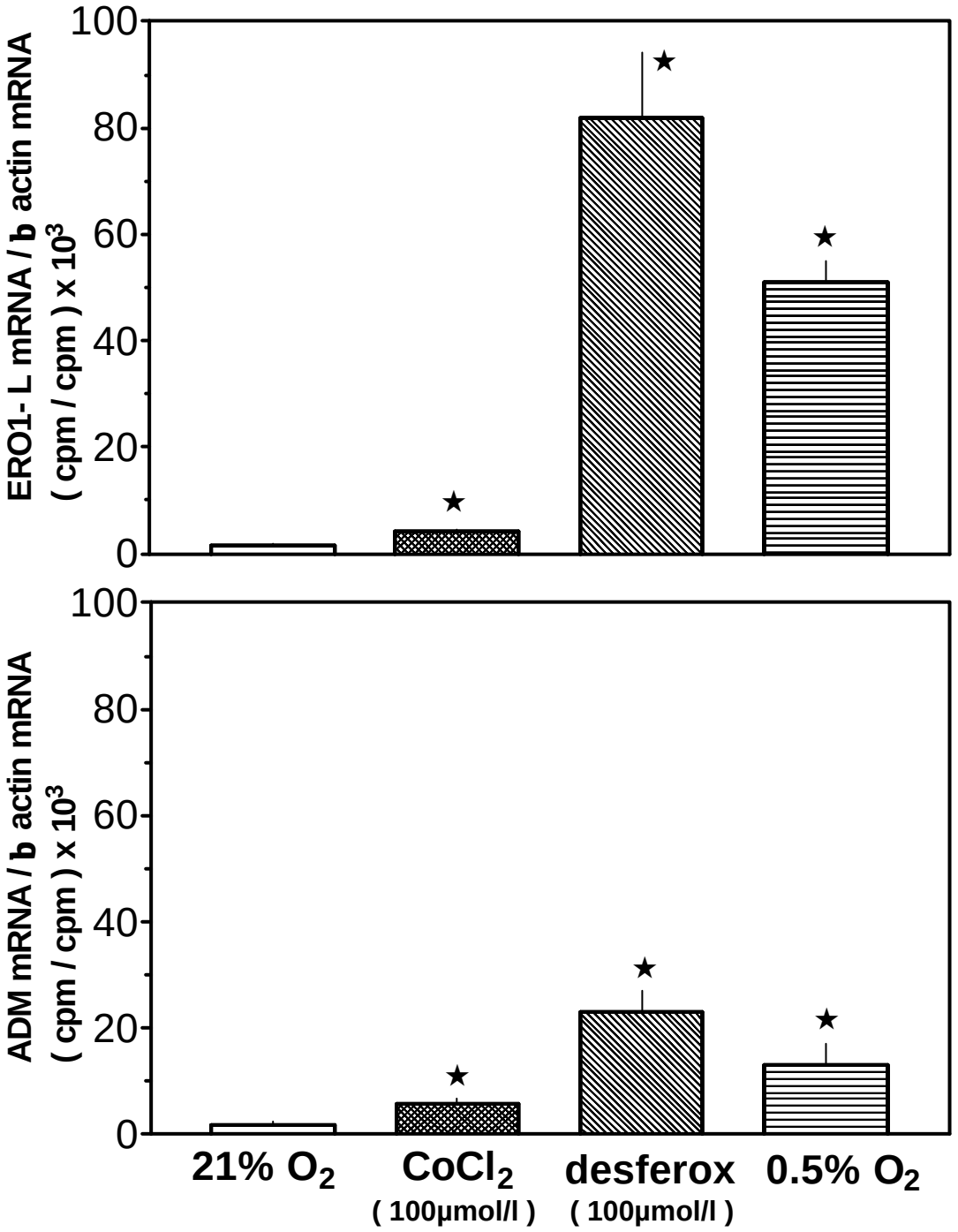
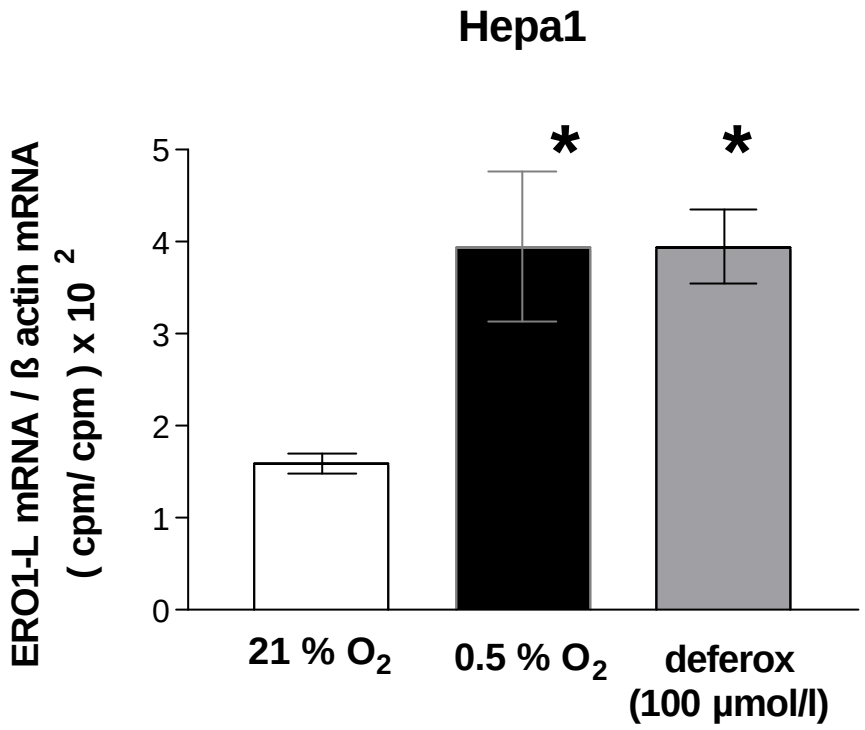

ERO1- L mRNA / b actin mRNA
(cpm / cpm) x 10³
ADM mRNA / b actin mRNA
(cpm / cpm) x 10³
100
80
60
40
20
0
21% O2
CoCl2
(100µmol/l)
desferox
(100µmol/l)
0.5% O2

Figure 4


Hepa1
ERO1-L mRNA / ß actin mRNA
(cpm/ cpm) x 10 2
0
1
2
3
4
5
*
*
21 % O2
0.5 % O2
deferox
(100 µmol/l)

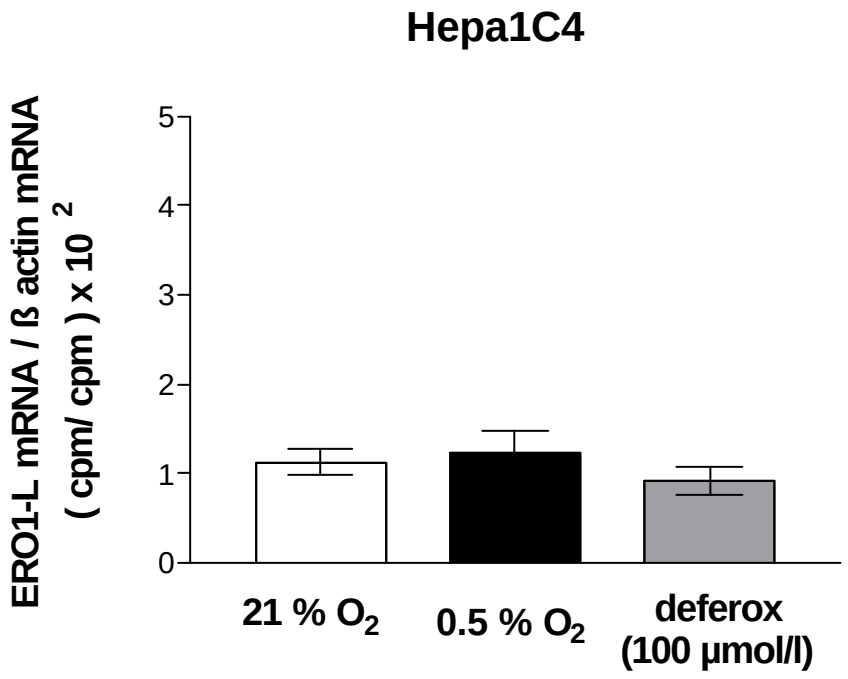
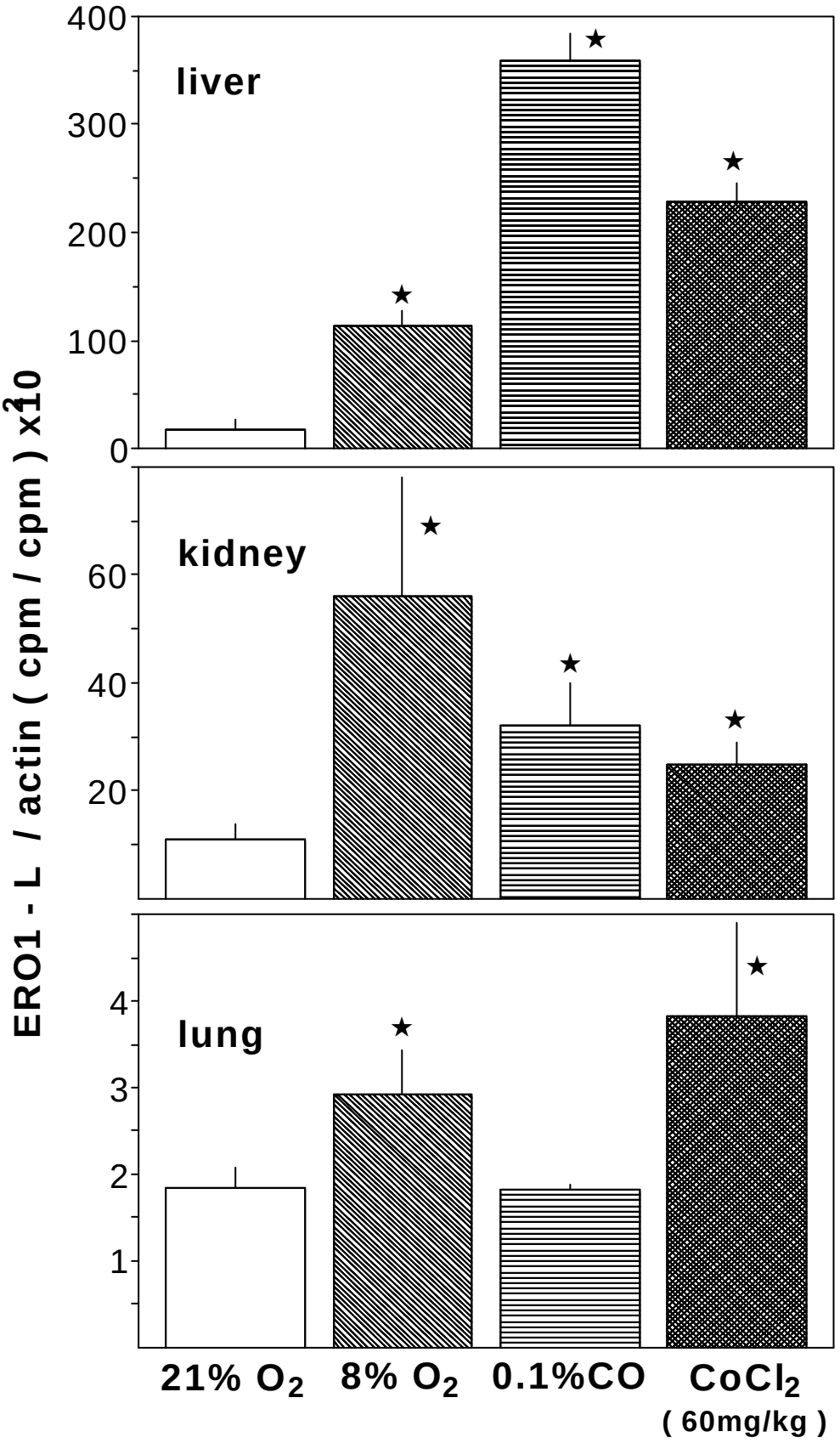

Hepa1C4
ERO1-L mRNA / ß actin mRNA
(cpm/ cpm) x 10 2
0
1
2
3
4
5
21 % O2
0.5 % O2
deferox
(100 µmol/l)

Figure 5


liver
kidney
lung
ERO1 - L / actin (cpm / cpm) x10²
400
300
200
100
0
60
40
20
4
3
2
1
21% O2
8% O2
0.1%CO
CoCl2
(60mg/kg)