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Induction of VEGF and VEGF receptor gene expression by hypoxia: Divergent regulation *in vivo* and *in vitro*

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Induction of VEGF and VEGF receptor gene expression by hypoxia: Divergent regulation *in vivo* and *in vitro*. This study examined the expression of EPO, VEGF and VEGF receptor gene under conditions of reduced oxygen supply in primary cultures of rat hepatocytes, and compared it with the expression of these genes in hypoxic rat livers *in vivo*. To this end we exposed male Sprague-Dawley rats to hypoxia (10% and 8% O_2), carbon monoxide (0.1% CO) or injected cobalt chloride (60 mg/kg $CoCl_2$) subcutaneously. For the *in vitro* experiments we used primary cultures of rat hepatocytes which were kept at high (20% O_2) and low (1% O_2) oxygen tensions for three hours. The EPO mRNA was up-regulated by hypoxia *in vitro* and *in vivo* about 10-fold. The VEGF mRNA was up-regulated fivefold in the hepatocytes only, whereas the *in vivo* mRNA levels remained unchanged. The mRNA levels of flt-1 were up-regulated threefold by 8% O_2 in livers, dependent on the strength of hypoxia (10% caused no changes in flt-1 gene expression) and on the kind of hypoxic stimulus (8% O_2 was as effective as 0.1% CO and more effective than cobalt). The mRNA levels of flk-1/KDR and flt-4 remained unchanged in the liver. *In vitro* there were no changes in the mRNA levels of flt-1, flt-4 and flk-1/KDR. Consequently, the *in vivo* regulation of VEGF, which might be modulated by induction of flt-1 receptor gene expression, differs from the *in vitro* cell culture situation and might be different from the EPO regulation *in vivo*.

Cells are able to sense low oxygen levels and to adapt to hypoxic environments so that whole tissues are able to survive under situations of insufficient oxygen delivery. For this adjustment to low oxygen tensions several genes are up-regulated, like erythropoietin (EPO) [1], which controls erythropoesis, followed by a higher oxygen carrying capacity of the blood or vascular endothelial growth factor (VEGF), which controls angiogenesis in order to improve oxygen delivery to hypoxic tissues [2, 3]. Also, glycolytic enzymes are induced which activate alternative metabolic pathways working without oxygen supply [4, 5]. The transcription of these genes is modulated by transcription factors like AP-1 or Hif-1 [6, 7] and post-transcriptionally by mRNA stability [8]. In cell culture experiments with cells, such as Hep3B and rat glioma cells, similarities have been found between the oxygen sensing mechanisms of EPO and VEGF [9]; both genes are up-regulated by hypoxia, carbon monoxide, cobalt chloride and other divalent cations [1, 2].

There is broad evidence that VEGF mRNA is up-regulated by hypoxia in isolated cells [10–12], but its not yet clear if this up-regulation is conserved in whole tissues [13, 14]. Recently, we found that there is only a minor effect of hypoxia on the VEGF gene expression *in vivo* [14], suggesting that there may be differences in the *in vivo* regulation between the EPO and VEGF gene. Recently evidence was provided that not only VEGF, but also its receptors may be up-regulated by hypoxia in rat lungs [15], raising the interesting question that the VEGF signal in tissues may be modulated by up-regulation of both ligand and receptor. VEGF receptors, like the receptors of other growth factors, such as platelet-derived growth factor receptors (PDGFR), belong to the family of tyrosine kinase receptors that show seven (or 5) immunglobulin-like loops on the cell surface, a single transmembrane segment, and an intracellular unit with a kinase domain insert (KDR) [16]. So far, two VEGF receptors have been identified: flt-1 and flk-1/KDR [17–19]. Two other receptors that are also tyrosine kinases, flt-3 and flt-4, are rather homologous to flt-1 and might also serve as VEGF receptors [20–22]. This variety of receptors should be able to fine tune the VEGF signal, which is composed of different VEGF proteins resulting from alternative exon splicing [23]. These VEGF isoforms may act as homo- or heterodimers or heterodimers with placental growth factor (PlGF) [24–26]. Recently, the ligand for flt-4 has been identified as "VEGF-related protein (VRP)" [27].

Based on these previous findings, we were interested in the question of whether there are differences between the regulation of VEGF gene in the rat liver in comparison with rat hepatocytes during hypoxia, if this regulation is dependent on the strength of hypoxia or kind of hypoxic stimuli, and if the gene expressions of VEGF receptor, flt-1 flk-1/KDR and flt-4 are up-regulated, too.

Methods

Cell culture of hepatocytes

Rat hepatocytes were isolated as described in detail previously [28]. After isolation the cells were seeded in tissue culture dishes with gas permeable bottoms (Petriperm, Heraeus, Germany) and incubated 40% O_2 (5% CO_2, balance nitrogen) in humified chambers at 37°C for four hours. Then nonattached cells were removed by exchange of the culture medium and the cells were incubated for a further three hours at 20% O_2 (controls) or 1% O_2 Cells were harvested and total RNA was isolated as described previously [28].

Animal experiments

All animal experiments were conducted with the NIH *Guide for the Care and Use of Laboratory Animals* and the *German Law on the Protection of Animals*.

Male Sprague-Dawley rats (200 to 250 g) which had free access

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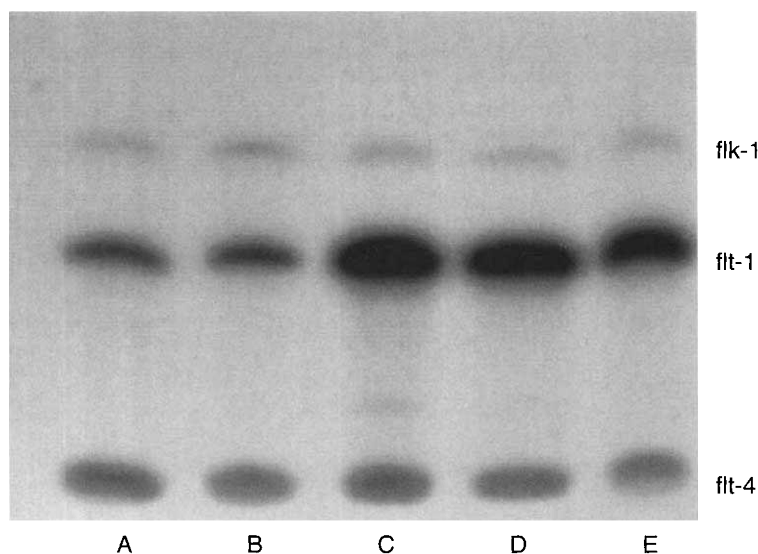

Fig. 1. *Autoradiographie of an RNase protection assay for flt-1, flt-4 and flk-1/KDR mRNA using 20 μg of total liver RNA of a control rat—21% O_2 (lane A), of hypoxic rats kept at 10% O_2 (lane B) or 8% O_2 (lane C), of rats treated with 0.1% CO (lane D) and 60 mg/kg cobalt (lane E).*

to food and water were used for the experiments. There were four groups of rats.

A. Controls. These rats received no treatment.

B. Hypoxia treatment. The animals were placed in a gas tight box and continuously gased with a mixture of 10% O_2/90% N_2 or 8% O_2/92% N_2 for six hours.

C. Carbon monoxide treatment. The animals were placed in a gas tight box and continuously gased with room air plus 0.1% carbon monoxide for six hours.

D. Cobalt treatment. These rats were subcutaneously injected with cobalt chloride (60 mg/kg) and then sacrificed six hours later.

At the end of the experiments the animals were killed by decapitation and blood was collected from the carotid arteries for the determination of hematocrit. The livers were rapidly removed, weighed, and rapidly frozen in liquid nitrogen. The organs were stored at −80°C until isolation of total RNA.

Isolation of RNA

Total RNA was extracted from the frozen tissues according to the protocol of as described previously [14]. The isolated RNA was finally dissolved in DEPC (diethylpyrocarbonate) treated water and stored at −80°C. The quality of obtained RNA was routinely tested by the control of the 28S/18S rRNA band on an 1% ethidium bromid stained agarose gel.

RNase protection assay for EPO and VEGF

RNase protection assays for EPO [28] and VEGF [14] were performed as described previously.

RNase protection assay for flt-1, flt-4 and flk-1/KDR

Flt-1, flt-4 and flk-1/KDR mRNA levels were measured by RNase protection assay. To generate a cRNA probe able to detect all three receptor tyrosine kinases in one cup, a 286 bp fragment of flk-1/KDR, a 238 bp fragment of flt-1, and a 176 bp fragment of flt-4, and mRNA were amplified by RT/PCR. For flt-1 a combination of 5′Primer "flt-1A" (5′-CGGGATCCAAGGGACTCTACACTTGTC-3′) with 3′Primer "flt-1B" (5′-GGAATTCCCGAATAGCGAGCAGATTT-3′) was choosen in the published sequence [29], respectively, flt-4A (5′-CGGGATCCTGCTGTGGGATGACCGCC-3′) and flt-4B (5′-GGAATTCAGCTCCATTGACTTCTTGG-3′) [30], and for flk-1, 5′Primer "flk-1A" (5′-CGGGATCCCATGCTGTATCTCGAGGGC-3′) and 3′Primer "flk-B" (5′-GGAATTCGTTTGTCATCCCAGGGTAC-3′) for flk-1 [31]. For RT 1 μg of total lung RNA was reverse transcribed with 200 units of M-MLV Transcriptase (Gibco/BRL) using standard protocols and Oligo dt (Gibco/BRL) for priming reverse transcriptase reaction. From the total volume of 20 μl, 3 μl of the cDNA were used for the PCR. The reaction was performed in a final volume of 20 μl containing 2 μl 10 × concentrated buffer (supplied with the enzyme), 2 μl 2.5 mM/liter dNTPs and one unit of *Taq* Polymerase (Boehringer Mannheim). PCR conditions were 32 cycles with denaturation at 94°C (1 min), annealing at 60°C (1 min) and extension at 72°C (1 min) for flt-1 and flk-1 cDNAs, whereas flt-4 PCR annealing was done at 50°C and 32 cycles. Purified fragments were cloned in the Polylinker site of pGEM 4Z (Promega-Serva) for flk-1 after *Hin*cII (Pharmacia, Freiburg, Germany) digest and psp 73 (Promega-Serva) for flt-1 and flt-4 after *Bam*HI/*Eco*RI (Pharmacia, Freiburg, Germany) digest. Standard protocols for blunt-end cloning of flk-1 and sticky-end cloning of flt-1 and flt-4 were used. After transformation into *E. coli* DH 5a-positive clones were selected by size determination of the plasmid insert after *Sma*I/*Hin*dIII (Pharmacia) for flk-1 and *Bam*HI/*Eco*RI (Pharmacia) digest for flt-1 and flt-4. Sequencing of the inserts done by SEQUISERVE (Dr. Metzger; Vaterstetten, Germany) confirmed the identity of all cloned inserts with the published sequences [29–31] and the orientation in the polylinker site. All plasmids were linearized with *Hin*dIII and yielded after labeling with [α^{32}P] GTP and Sp6 polymerase probes of the expected size (345 bp for flk-1, 310 bp for flt-1 and 250 bp for flt-4). Figure 1 shows an autoradiography of the flt-1, flt-4 and flk-1 RNase protection with 20 μg of total liver RNA. For all assays 20 μg of total liver RNA were used.

Using the RNase protection assay for glycerine aldheyde phosphatedehydrogenase (GAPDH) GAPDH mRNA levels were measured as described previously [32].

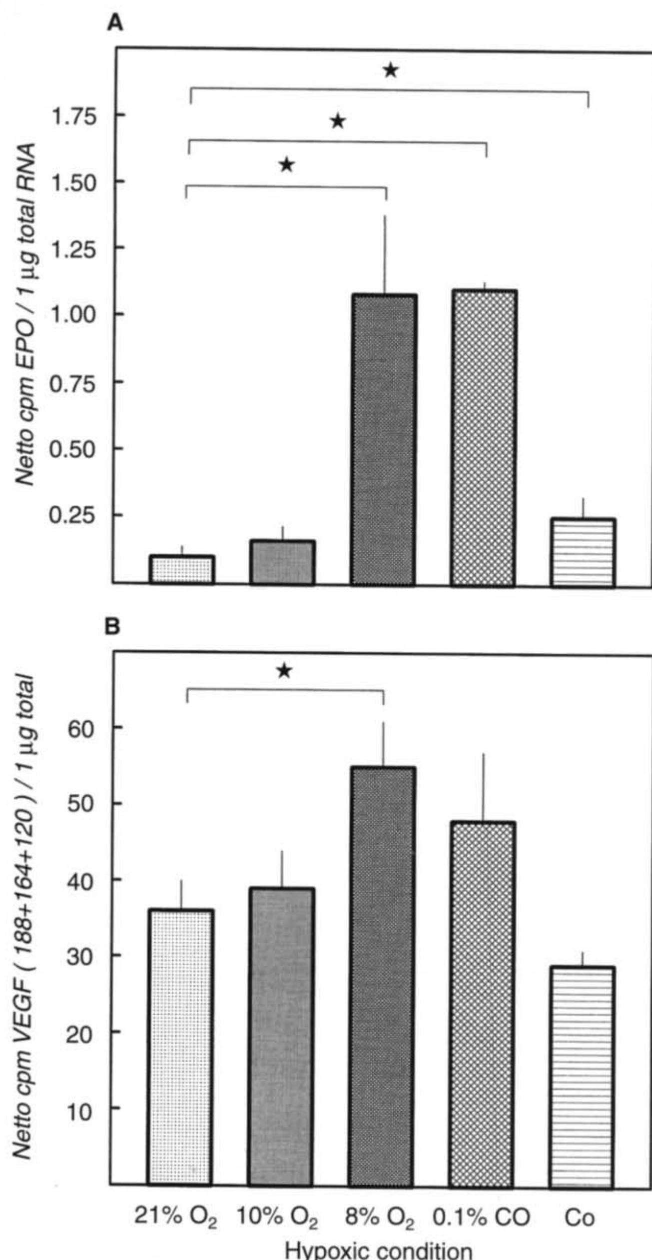

Fig. 2. *Abundance of EPO mRNA (A) and VEGF mRNA (B) in livers of animals exposed to normoxia (21% O_2), hypoxia (10% and 8% O_2), carbon monoxide or cobalt chloride.* Values are expressed as radioactivity found in the protected fragments (cpm) related to1 μg total liver RNA. Data are means ± SEM of 6 animals in each group.

Statistics

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

Results

EPO mRNA expression in the rat liver

Figure 2 shows the EPO mRNA abundance in the rat livers under normoxic conditions (21% O_2) and the up-regulation of EPO gene expression during hypoxia. EPO mRNA abundance in the livers was not affected by 10% O_2 inspiratory hypoxia in comparison with the control group, but was significantly increased (10-fold) by 8% O_2 and to the same extent by CO inhalation. EPO mRNA was also induced by cobalt administration about 2.5-fold. For quality control we measured the housekeeping gene, GAPDH, by the RNase protection assay for all RNAs used in this study

VEGF mRNA expression in the rat liver

VEGF mRNA levels which were presented as the sum of VEGF-188, VEGF-164 and VEGF-120, three amino acid isoforms of VEGF formed by alternative exon splicing from three mRNAs [23], were also quantitated in normoxic and hypoxic rat livers. The rank order of relative abundance in livers was VEGF-188 > VEGF-164 > VEGF-120, which was not changed under hypoxic conditions (not shown). No changes in VEGF mRNA expression were found between the control group and the 10% O_2, CO inhalation and cobalt injection groups (Fig. 2). The latter two maneuvers were able to stimulate EPO mRNA in the same livers about 10-fold and 2.5-fold, respectively. Only the 8% O_2 group tended to increase VEGF levels from about 40 up to 50%, on the borderline of significance. mRNA levels in all Figures are expressed in counts per minute (cpm)/1 μg total liver or hepatocyte RNA, allowing a comparison of the absolute abundance of the quantitated mRNAs directly. The abundance of VEGF mRNA in livers is markedly higher than that of EPO mRNA, which is under normoxic conditions at the threshold of detectability.

Flt-1, flt-4 and flk-1/KDR mRNA expression in the rat liver

Figures 1 and 3 show the hepatic mRNA levels of the tyrosine kinase receptors flt-1, flt-4 and flk-1/KDR. The absolute abundance for flt-1, flt-4 and flk-1/KDR was about 1:1:0.2. Inspiratory hypoxia with 10% O_2 had no effect on the mRNA levels of flt-1, flt-4 and flk-1/KDR; 8% O_2 increased flt-1 mRNA threefold, but had no influence on the flt-4 and flk-1/KDR gene expression. CO was as effective as 8% O_2 and stimulated flt-1 gene expression about threefold, whereas cobalt has a minor stimulating effect on flt-1 gene that was up-regulated twofold. The mRNA levels of flt-4 and flk-1/KDR were not changed by CO and cobalt (Figs. 1 and 3).

EPO mRNA expression in rat hepatocytes

After incubating cells three hours with 20% O_2 (control group) or 1% O_2, EPO was not changed in the control preparation but was substantially increased by hypoxia (Fig. 4).

VEGF mRNA expression in rat hepatocytes

The abundance of VEGF mRNAs and the rank order (VEGF188 > VEGF-164 > VEGF-120) did not change between the rat liver and the primary culture of the hepatocytes, but was markedly higher than the EPO mRNA in intact livers and hepatocytes, as described above. VEGF mRNA levels significantly dropped the incubation of the cells with 20% O_2 for three hours. After incubation at 1% O_2 VEGF mRNA levels were increased five- to sixfold in the hypoxic hepatocytes (Fig. 4).

Flt-1, flt-4 and flk-1/KDR mRNA expression in rat hepatocytes

As shown in Figure 4, flt-1, flt-4 and flk-1/KDR mRNA levels were markedly lower than in intact livers, and we found only about

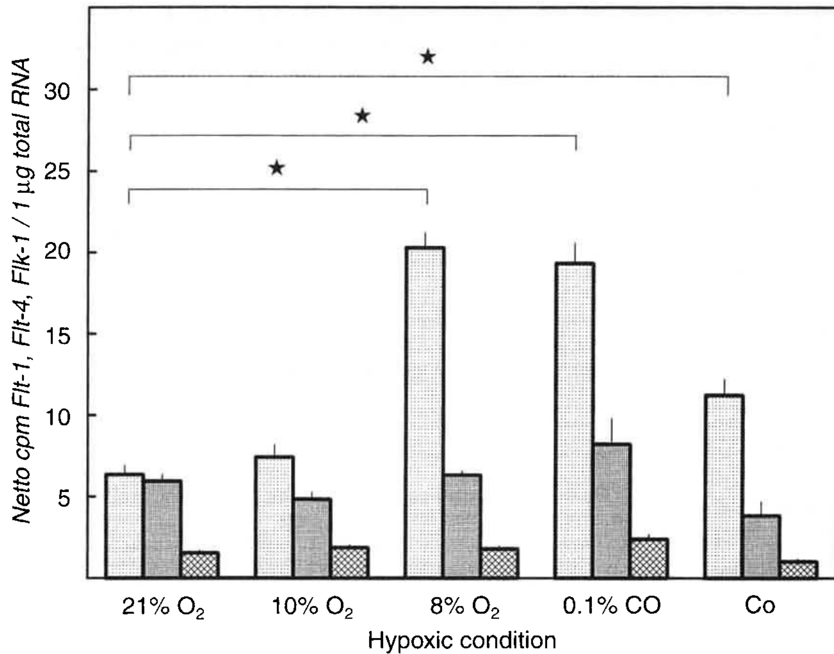

Fig. 3. *Abundance of flt-1 (▨), flt-4 (▨) and flk-1/KDR (▩) mRNA in livers of animals exposed to normoxia (21% O_2), hypoxia (10% and 8% O_2), carbon monoixide or cobalt chloride.* Values are expressed as radioactivity found in the protected fragments (cpm) related to 1 µg total liver RNA. Data are means ± SEM of 6 animals in each group.

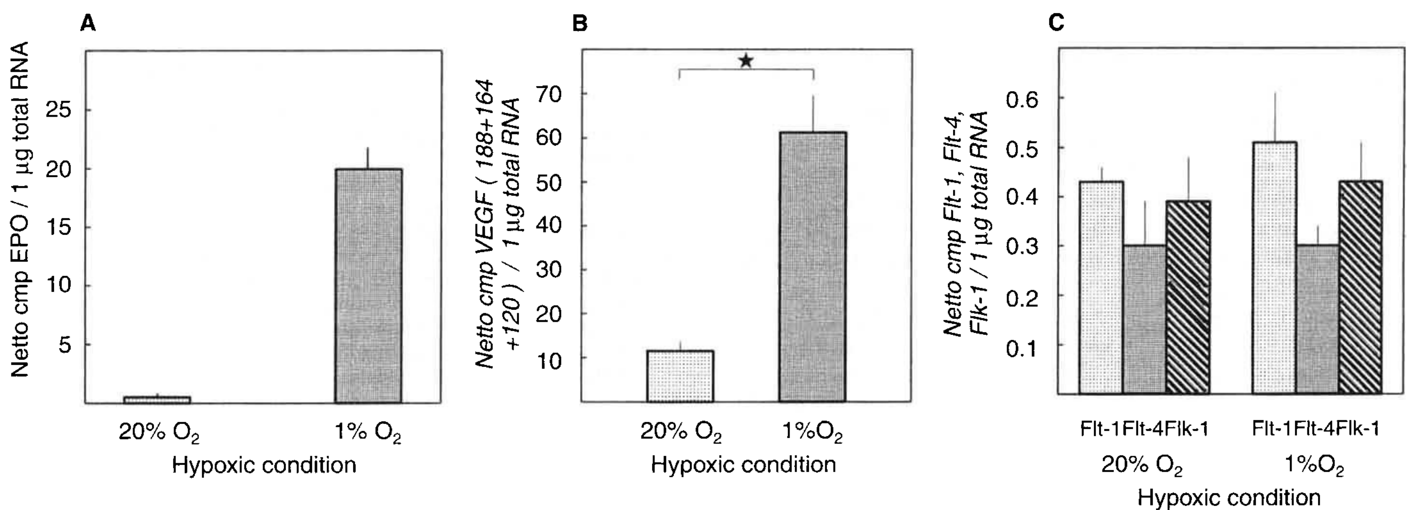

Fig. 4. *Abundance of EPO* (**A**), *VEGF* (**B**) *and flt-1, flt-4, flk-1/KDR mRNA* (**C**) *in rat hepatocytes after three hours incubation at 20% O_2 or 1% O_2.* Values are expressed as radioactivity found in the protected fragments (cpm) related to 1 µg total liver RNA. Data are means ± SEM of three preparations in each group.

1/10 of the abundance in hepatocytes after isolation and incubation at 20% O_2. EPO and VEGF mRNA levels were unchanged in intact livers and the primary cultures of the hepatocytes, as mentioned before. After incubation at 1% instead of 20% O_2 no up-regulation of flt-1, flt-4 or flk-1/KDR mRNA could be measured, which was in sharp contrast to EPO and VEGF mRNA which were substantially up-regulated in the cell culture by 1% O_2. This differed from the *in vivo* experiments where flt-1 gene expression could be up-regulated threefold during hypoxia (Fig. 3).

Discussion

The present study investigated and compared the expression of EPO, VEGF and flt-1, flt-4 and flk-1/KDR gene expression in rat livers and hepatocyte cell cultures under classical conditions of hypoxia.

We observed a substantial up-regulation of EPO mRNA *in vivo* and *in vitro* by all kind of hypoxic stimuli, which confirmed previous findings [14, 28]. Cobalt was less effective in stimulating EPO mRNA in total livers, which is also in keeping with our previous findings [14].

Inspiratory hypoxia with 8% O_2 was a rather modest stimulus for VEGF gene transcription in the rat liver in comparison with the 10-fold increase of EPO mRNA in the same livers (Fig. 2). Thus our study provides further evidence that VEGF mRNA expression *in vivo* does not follow the same pathway as EPO gene expression [14].

VEGF mRNA expression observed *in vivo* contrasted to the up-regulation of VEGF mRNA in the hepatocyte cell cultures where hypoxia caused a fivefold increase of VEGF mRNA (Fig. 4). This up-regulation of VEGF mRNA in cell culture experiments by hypoxia fits with previous reports covering a variety of cell types, such as hepatoma cell lines [9] or glioma cells [11], where this up-regulation during hypoxia has been described. One could imagine that the relatively high basal expression of VEGF mRNA in rat livers, which is about 350-fold higher than basal EPO mRNA expression, reflects a constitutive expression that appears to be oxygen independent and that can be stimulated to a minor extent only by hypoxia. This raises the question if there are other determinants which regulate VEGF gene expression in a more substantially way.

In our study we also aimed to characterize the effect of acute hypoxia on the gene expression of the receptor tyrosine kinases flt-1, flt-4 and flk-1/KDR in the rat liver and in hepatocytes. Flt-1 bind VEGF and PlGf with high affinity, whereas flk-1 only binds VEGF homodimers [24–26]. The ligand of flt-4 was recently identified as a VEGF related protein [27]. Our results show that flt-1 and flt-4 were substantially expressed in the rat liver while the expression of flk-1/KDR is relativly low (Figs. 1 and 3). This distribution of flt-1 and flk-1/KDR has already been reported [22, 29]. In the livers we observed a threefold induction of Flt-1 mRNA by 8% O_2 and CO and a twofold induction by cobalt, but no effect on the flk-1/KDR and flt-4 mRNA levels. Inspiratory hypoxia by 10% O_2 did not influence the mRNA levels of the three tyrosine kinase receptors after six hours of exposure (Figs. 1 and 3). An up-regulation of flt-1 and flk-1 has recently described in lungs of rats exposed to an altitude of 16,000 feet [15]. Therefore, the regulation of flt-1 gene paralleled that of EPO gene expression (Fig. 2) which was up-regulated by 8% O_2 and CO, and to a minor extent by cobalt, suggesting that the regulation of both genes would follow similar pathways under hypoxic environments.

In the hepatocytes the basal expression of flt-1, flt-4 and flk-1/KDR were markedly lower in comparison with the *in vivo* mRNA levels (Figs. 3 and 4) and were not changed by incubation at 1% O_2. Two main explanations arise for this discrepancy. The expression of flt-1, flt-4 and flk-1/KDR may depend on the age of the rats used for the experiments. For the *in vivo* experiments we used adult male rats (200 to 250 g) whereas for isolation of hepatocytes young male rats (80 to 100 g) were used. For flt-1 and flk-1/KDR such a dependence of gene expression on the age of rats has already been described [19, 33]. Finally, it cannot be excluded that the main production site of the tyrosine kinase receptors in livers is not localized in the hepatocytes. One can speculate if Ito or Kupffer cells produce this mRNA to a much more higher extent. For Ito cells such an expression for another growth factor receptor, fibroblast growth factor receptor, bek, has already been reported [34], and evidence has also been provided that EPO is produced by Ito cells [35].

Taken together, our findings suggest that the expression of flt-1 gene, but not the expression of VEGF gene in livers, is regulated in a way very similar to that of EPO gene. There were substantial differences in the regulation of VEGF gene and flt-1, flt-4 and flk-1/KDR genes in intact livers and in cell culture experiments, assuming that the answer to hypoxia of VEGF and the VEGF receptors follows different pathways *in vivo* and *in vitro*. Hypoxia up-regulated flt-1 mRNA selectively but not the other VEGF receptor, flk-1/KDR mRNA, nor the related flt-4 mRNA. Thereby it appears conceivable that hypoxia enhances the action of VEGF not only via increased formation of the protein, but also via induction of a particular VEGF receptor.

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