Physiological oxygen tensions modulate expression of the *mdr1b* multidrug-resistance gene in primary rat hepatocyte cultures

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P-Glycoprotein transporters encoded by *mdr1* (multidrug resistance) genes mediate extrusion of an array of lipophilic xenobiotics from the cell. In rat liver, *mdr* transcripts have been shown to be expressed mainly in hepatocytes of the periportal region. Since gradients in oxygen tension (pO_2) may contribute towards zoned gene expression, the influence of arterial and venous pO_2 on mRNA expression of the *mdr1b* isoform was examined in primary rat hepatocytes cultured for up to 3 days. Maximal *mdr1b* mRNA levels (100%) were observed under arterial pO_2 after 72 h, whereas less than half-maximal mRNA levels (40%) were attained under venous pO_2 . Accordingly, expression of *mdr* protein and extrusion of the *mdr1* substrate rhodamine 123 were maximal under arterial pO_2 and reduced under venous pO_2 . Oxygen-dependent modulation of *mdr1b* mRNA expression was prevented by actinomycin D, indicating transcriptional regulation. Inhibition of haem synthesis by 25 μ M

$CoCl_2$ blocked *mdr1b* mRNA expression under both oxygen tensions, whereas 80 μ M desferrioxamine abolished modulation by O_2 . Haem (10 μ M) increased *mdr1b* mRNA levels under arterial and venous pO_2 . In hepatocytes treated with 50 μ M H_2O_2 , *mdr1b* mRNA expression was elevated by about 1.6-fold at venous pO_2 and 1.5-fold at arterial pO_2 . These results support the conclusion that haem proteins are crucial for modulation of *mdr1b* mRNA expression by O_2 in hepatocyte cultures and that reactive oxygen species may participate in O_2 -dependent signal transduction. Furthermore, the present study suggests that oxygen might be a critical modulator for zoned secretion of *mdr1* substrates into the bile.

Key words: cobalt chloride, desferrioxamine, haem, reactive oxygen species.

INTRODUCTION

Members of the ATP-binding cassette (ABC) protein superfamily are characterized by the presence of one or two highly conserved domains that bind and hydrolyse ATP [1]. The majority of ABC proteins are parts of transport systems involved in ATP-dependent translocation of substrates across biological membranes.

Hepatocytes, as major sites in the processing of nutrients, xenobiotics and endogenous metabolites, exhibit expression of several ABC transporters, which are involved in excreting a variety of endogenous and xenobiotic compounds into bile or blood. Several members of the multidrug-resistance transporter (MDR)/transporter associated with antigen processing (TAP) transporter subfamily are situated in the hepatocyte apical canalicular membrane, including the *mdr* proteins (also termed P-glycoproteins, P-gp [2]) and the bile salt-export pump, bsep, which is also termed sister of P-glycoprotein [3]. The human MDR1 and the rodent *mdr1a/mdr1b* isoforms transport numerous lipophilic, often cationic, xenobiotics and endobiotics into the bile [4]. *mdr1*-type P-glycoproteins are also expressed on the apical luminal surface of secretory cells in the small intestine, the colon and the proximal tubules of the kidney [5], as well as in the capillary endothelial cells of the brain and testis [6], supporting the concept of a protective role for *mdr1*-type proteins in elimination of potentially toxic xeno- and endobiotics.

Along the liver acinus, zonal heterogeneity has been demonstrated for specific hepatocyte functions, including carbohydrate and xenobiotic metabolism, or bile formation and hepatobiliary transport of bile constituents [7–9]. This heterogeneity is based on zoned gene expression. Secretion of bile acids and biliary transport of several xenobiotics, including the *mdr1* substrate rhodamine B, have been shown to occur predominantly in the periportal area [9]. Accordingly, *in situ* hybridization using an *mdr* riboprobe previously revealed highest *mdr* mRNA expression in the periportal region of the rat hepatic acinus, with transcript levels decreasing towards the perivenous area [10]. Sinusoidal gradients in nutrients, hormones, mediators or oxygen, which are formed due to metabolism of liver cells, may contribute to zoned gene expression in the liver [7,8]. The oxygen tension (pO_2) decreases by about 50% from the periportal to the perivenous regions. Thus the oxygen gradient has been considered to be a key regulator of zoned gene expression [7,8].

The aim of the present study was to test the possible role of oxygen in modulating expression of the *mdr1b* gene. Primary rat hepatocytes were cultured under arterial (16% O_2) or venous (8% O_2) pO_2 . It was found that *mdr1b* mRNA expression was regulated by the ambient pO_2 . A haem protein leading to the generation of H_2O_2 has been proposed to function as an oxygen sensor in the oxygen-dependent regulation of gene expression [11]. The possible participation of haem proteins producing

Abbreviations used: ABC, ATP-binding cassette; AP-1, activator protein 1; DSF, desferrioxamine; HO-1, haem oxygenase 1; MDR/*mdr*, multidrug-resistance transporter; MRP/*mrp*, multidrug-resistance-associated protein; NF- κ B, nuclear factor κ B; PEPCK, phosphoenolpyruvate carboxykinase; pO_2 , oxygen tension; Rh123, rhodamine 123; ROS, reactive oxygen species; TAP, transporter associated with antigen processing; VEGF, vascular endothelial growth factor.

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H₂O₂ in the oxygen-sensing process regulating *mdr1b* mRNA expression was investigated, using the haem-synthesis inhibitor CoCl₂, the iron chelator desferrioxamine (DSF) or by exposing hepatocytes to exogenous H₂O₂.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade and were purchased from commercial suppliers. Collagenase, T4-polynucleotide kinase, digoxigenin-UTP, the digoxigenin nucleic acid detection kit and fetal calf serum were obtained from Roche Molecular Biochemicals (Mannheim, Germany). T3-RNA polymerase was purchased from United States Biochemical (Cleveland, OH, U.S.A.). The multidrug-resistance-associated protein (mrp) inhibitor MK571 (a leukotriene LTD₄ receptor antagonist) was from Biomol (Hamburg, Germany) and [γ -³²P]ATP was from NEN (Boston, MA, U.S.A.). PVDF membranes were purchased from Millipore (Eschborn, Germany); Hybond N nylon membranes and the enhanced chemiluminescence (ECL) detection kit were from Amersham (Braunschweig, Germany). The polyclonal anti-*mdr* antibody PC03 was obtained from Oncogene Research Products (Cambridge, MA, U.S.A.). Haemin chloride, actinomycin D and the secondary enzyme-conjugated antibody against rabbit IgG were purchased from Sigma (Munich, Germany).

Primary rat hepatocyte culture

Primary rat hepatocytes were isolated from adult male Wistar rats by collagenase perfusion and seeded on to culture dishes (8.5 cm diameter; Nunc, Roskilde, Denmark) at a density of 5×10^4 cells/cm² in M199 medium containing 1 nM insulin, 100 nM dexamethasone and 4% (v/v) fetal calf serum [12]. Following an initial attachment period of 4 h, hepatocytes were cultured in serum-free medium containing 1 nM insulin and 100 nM dexamethasone for up to 3 days with daily changes of the media. Cells were incubated under an atmosphere of 16% O₂/79% N₂/5% CO₂ to mimic arterial *p*O₂ or under an atmosphere of 8% O₂/87% N₂/5% CO₂ to mimic venous *p*O₂. The O₂ values take into account the O₂ diffusion gradient from the medium surface to the cells [13]. Cultures were incubated with 25 μ M CoCl₂ or 80 μ M DSF for 3 days as indicated. Haemin bound to BSA was prepared according to [14] and was added to the medium at a final concentration of 10 μ M, 24 h prior to harvesting of cells. In experiments involving actinomycin D treatment, hepatocytes were cultured for 1 or 2 days and then exposed to 4 μ M (5 μ g/ml) actinomycin D for 18 h or 2–10 h, respectively, as indicated in the Figure legends.

RNA preparation and Northern-blot analysis

Total RNA was isolated by guanidinium thiocyanate/phenol extraction [15] and separated by electrophoresis through formaldehyde/agarose gels (20 μ g/lane). Staining of gels with ethidium bromide was performed to ensure equal loading of lanes. Subsequently, RNA was transferred to Hybond N nylon membranes by capillary blotting. RNA blots were hybridized to an *mdr1b*-specific oligonucleotide probe which had been 5'-end-labelled by T4-polynucleotide kinase using [γ -³²P]ATP [16]. The *mdr1b* oligonucleotide probe [17] corresponded to rat *mdr1b* mRNA nucleotides 2038–2055 (GenBank® accession number M81855 [18]; 5'-CTC AGA GGC ACC AGT GTC-3'). Oligonucleotide hybridization was performed at 38 °C for 16 h as

described previously [19] and blots were washed up to a stringency of $0.1 \times$ SSC (15 mM NaCl/1.5 mM sodium citrate) and 0.1% (w/v) SDS at 38 °C. Transcript expression was quantified by a PhosphorImaging system (Raytest, Straubenhardt, Germany). Control hybridizations were performed employing β -actin or haem oxygenase-1 (HO-1) antisense RNA probes that were labelled by incorporation of digoxigenin-11-UTP during *in vitro* transcription. A human β -actin cDNA fragment and a HO-1 cDNA fragment, respectively, cloned into the pBS/Bluescript plasmid (Stratagene, Heidelberg, Germany), served as templates. Hybridization to the β -actin and HO-1 probes was performed at 68 °C with 40 ng of probe/ml for 12 h according to the manufacturer's application notes (Roche Molecular Biochemicals) of the digoxigenin nucleic acid detection kit. Hybrids were detected as described previously [20] and quantified with a video-densitometer (Biotech Fischer, Reiskirchen, Germany).

Immunoblot analysis

Cytoplasmic membrane fractions were isolated from hepatocytes by differential centrifugation [21]. Buffers used for homogenization and centrifugation contained 1 mM PMSF. Membrane-protein samples (12 μ g/lane) were subjected to electrophoresis on SDS/polyacrylamide gels (7.5%) and were then transferred to PVDF membranes by the semi-dry blotting procedure [22] using a continuous transfer buffer system [48 mM Tris, 39 mM glycine, 0.038% (w/v) SDS and 15% (v/v) methanol, pH 9.0]. *mdr* proteins (P-glycoproteins) were detected on protein blots by employing the primary polyclonal antibody PC03, which is directed against a protein region conserved in all fully sequenced rat *mdr* isoforms [18,23]. A secondary peroxidase-conjugated antibody against rabbit IgG was used for visualization of immunoreactive protein in conjunction with the ECL detection kit.

Determination of intracellular rhodamine 123 (Rh123) accumulation

Hepatocytes were cultured on dishes with a diameter of 5.24 cm (Nunc) for 3 days under venous (8%) or arterial (16%) *p*O₂. Subsequently, cultures were incubated for 3 h under the corresponding *p*O₂ with medium containing 6.5 μ M Rh123, the fluorescent *mdr1* substrate, in the presence or absence of 10 μ M verapamil, an inhibitor of *mdr1*-dependent transport, or of 20 μ M MK571, an mrp inhibitor. The adherent cells were then washed five times at 4 °C with PBS and intracellular dye was extracted by incubation with *n*-butanol (2.5 ml/dish) for 10 min at 20 °C. Rh123 concentrations in butanol extracts were measured fluorimetrically (at the excitation wavelength of 518 nm and the emission wavelength of 531 nm) [24] and data were normalized to the protein content per dish.

RESULTS

Positive modulation of *mdr1b* mRNA expression by arterial *p*O₂

To investigate the possible effect of oxygen on expression of the *mdr1b* gene, primary rat hepatocytes were cultured under venous (8% O₂) or arterial (16% O₂) *p*O₂ for up to 3 days. Although *mdr1b* mRNA expression increased in a time-dependent manner under both *p*O₂s (Figure 1A), the temporal enhancement was greater under arterial *p*O₂ than under venous *p*O₂ (Figures 1A and 1B). The difference between *mdr1b* mRNA expression under arterial and venous *p*O₂ was significant even after 2 days of

Figure 1 Enhancement by arterial pO_2 of *mdr1b* mRNA and *mdr* protein expression in primary rat hepatocyte cultures

Hepatocytes were cultured under arterial (16%) or venous (8%) pO_2 for 1–3 days. Day 0 denotes freshly isolated hepatocytes. **(A)** In Northern-blot analysis, 20 μ g of total RNA was applied per lane, and hybridized to a 32 P-end-labelled *mdr1b* oligonucleotide probe as described in the Materials and methods section. Hybridization signals were quantified by PhosphorImaging analysis. *mdr1b* mRNA expression under 16% O_2 after 3 days of culture was set to 100%. Data represent means $\pm$ S.D. of three independent experiments. *, Significant difference between arterial and venous pO_2 ($P \leq 0.05$, Student's *t* test for unpaired values). **(B)** Representative Northern blot hybridized to the *mdr1b*-specific probe, using RNA isolated from hepatocytes cultured for 2 days. The ethidium bromide staining of the respective gel (28 and 18 S rRNA) demonstrates equal loading of lanes. **(C)** *mdr* protein (P-glycoprotein, P-gp) expression in hepatocytes cultured for 2 days. Cytoplasmic membrane fractions containing 12 μ g of protein were subjected to PAGE and transferred to a PVDF membrane. P-Glycoprotein was visualized in the immunoblot as outlined in the Materials and methods section. Two major immunoreactive protein bands with apparent molecular masses of 200 and 155 kDa were detected by the primary antibody, as described previously [17,19].

culture, and was maximal after 3 days: *mdr1b* mRNA levels under venous pO_2 amounted to only approx. 40% of maximal levels observed under arterial pO_2 (Figure 1A). Accordingly, immunodetectable *mdr* protein (P-glycoprotein) in isolated cytoplasmic membrane fractions was more abundant under arterial than under venous pO_2 (Figure 1C).

Several studies have previously demonstrated a substantial time-dependent increase in expression of the *mdr1b* gene during primary rat hepatocyte culture [17,19,25,26]. Considering the fact that, under standard culture conditions (as generally applied in former reports), primary hepatocytes are exposed to oxygen tensions resembling periportal pO_2 , the marked temporal enhancement of *mdr1b* mRNA levels observed here under 16% O_2 (Figure 1A) is in accordance with these previous studies. However, the present paper is the first to show that *mdr1b* mRNA expression in primary hepatocytes is modulated by ambient pO_2 .

Increased transcription and/or mRNA stabilization may contribute to enhanced mRNA accumulation. Therefore, the effect of the transcriptional inhibitor actinomycin D on modulation by pO_2 of *mdr1b* mRNA expression was examined. Hepatocytes in which a significant difference in *mdr1b* mRNA expression under arterial and venous pO_2 had not yet developed (cultured for 1 day; Figure 1A) were exposed to 4 μ M actinomycin D for 18 h. Actinomycin D inhibited further time-dependent *mdr1b* mRNA accumulation under both pO_2 conditions and prevented the enhanced expression of *mdr1b* mRNA under arterial pO_2 (Figure 2). This result suggests that the modulation by O_2 of *mdr1b* mRNA expression occurred on the transcriptional level.

In further experiments, *mdr1b* mRNA stability was examined under arterial and venous pO_2 . Hepatocytes were cultured for 2 days to obtain substantial *mdr1b* mRNA expression (Figure 1A) and then were exposed further to actinomycin D for 2–10 h. *mdr1b* mRNA levels demonstrated the tendency to increase

under both pO_2 conditions in a parallel manner, but did not decrease in the presence of actinomycin D (Figure 3). However, actinomycin D was effective in inhibiting transcription, since β -actin expression in the same cultures decreased under actinomycin D treatment in a time-dependent manner (Figure 3). Thus these data suggest that *mdr1b* mRNA after 2 days of culture exhibits a long half-life under both pO_2 conditions, but yield no indication of differential *mdr1b* mRNA stability or decay under the examined levels of pO_2 . These findings support the conclusion that enhanced transcription is the major factor contributing to higher *mdr1b* mRNA accumulation under arterial pO_2 in hepatocytes.

Positive modulation of *mdr1*-dependent transport activity by arterial pO_2

To investigate whether enhanced *mdr1b* mRNA expression under arterial pO_2 also resulted in elevated *mdr1*-dependent transport activity, the intracellular levels of the *mdr1* substrate Rh123 were measured after 3 h of incubation in hepatocytes cultured for 3 days under venous or arterial pO_2 . Higher Rh123 levels in hepatocytes were interpreted as being the result of lower Rh123 efflux and thus lower *mdr1*-dependent export activity. Relative Rh123 levels were approx. 20% higher in cells cultured under venous pO_2 than in cells maintained under arterial pO_2 , indicating that cells cultured under arterial pO_2 exhibited elevated *mdr1*-dependent transport activity (Figure 4). It appears improbable that lower *mdr1*-dependent transport activity in cells cultured under venous pO_2 reflected impaired energy metabolism, since comparable ATP/ADP ratios and energy-charge values were previously demonstrated in cells incubated under arterial and venous pO_2 [13]. The calcium-channel blocker verapamil is known to interfere with *mdr1*-dependent xenobiotic efflux and is there-

Figure 2 Prevention of modulation by O₂ of mdr1b mRNA expression by actinomycin D in primary rat hepatocyte cultures

Hepatocytes were cultured for 1 day under arterial or venous pO_2 and then cultured further in the absence (control) or presence of 4 μ M actinomycin D (actD) under 16 or 8% O₂ for 18 h. mdr1b mRNA expression was determined by Northern-blot analysis as outlined in Figure 1. (A) Hybridization signals were quantified by PhosphorImaging analysis. Expression of mdr1b mRNA under arterial pO_2 without actinomycin D was set to 100%. Data represent means $\pm$ S.D. of three independent experiments. *, Significant difference between hepatocytes cultured with actinomycin D and control cultures at 16% O₂; **, significant difference between hepatocytes cultured under 8% versus 16% O₂ ($P \leq 0.05$; Student's t test for unpaired values). (B) Representative Northern blot hybridized to the mdr1b-specific probe. The ethidium bromide staining of the respective gel (28 and 18 S rRNA) demonstrates equal loading of lanes.

fore regarded as a chemosensitizer [27]. Concomitant incubation of hepatocytes with Rh123 and verapamil resulted in increased intracellular accumulation of Rh123 (Figure 4); this effect was significant under 16% oxygen, confirming that the transport mechanism that was enhanced under arterial pO_2 was also sensitive to verapamil and was thus due to mdr1.

Several members of the MRP transporter subfamily that are distantly related to the MDR/TAP protein subfamily of ABC transporters have been identified in hepatocytes. While the mrp2 isoform mediates translocation of glutathione and glucuronide conjugates into the bile, the mrp3 isoform may participate in secretion of anionic conjugates into the blood [28]. To test whether mrp proteins might participate in Rh123 transport, hepatocytes were incubated with Rh123 in the presence of the mrp inhibitor MK571 (a leukotriene LTD4 receptor antagonist). The mrp inhibitor was employed at a concentration of 20 μ M, which had formerly been demonstrated to effectively interfere with substrate transport mediated by human MRP1 or rabbit mrp2 [29,30]. However, MK571 did not increase Rh123 accumulation in hepatocytes, irrespective of the ambient pO_2 (Figure 4). It therefore appears unlikely that mrp proteins contributed to Rh123 transport in primary rat hepatocytes cultured for 3 days. Thus the presented Rh123-accumulation experiments

Figure 3 O₂-independent regulation of mdr1b mRNA levels in the presence of actinomycin D in cultured rat hepatocytes

Hepatocytes were cultured for 2 days under arterial or venous pO_2 . Cells were then incubated with 4 μ M actinomycin D under 16 or 8% O₂ for the periods indicated (2–10 h). Expression of mdr1b and β -actin (β -act) mRNA was determined by Northern-blot analysis as outlined in Figure 1. Following hybridization to the mdr1b-specific probe, blots were rehybridized to the β -actin antisense RNA probe. Hybridization signals were quantified by PhosphorImaging analysis. mdr1b and β -actin mRNA expression levels under arterial pO_2 were set to 100%. Data represent means $\pm$ S.D. of three independent experiments.

Figure 4 Decrease by arterial pO_2 in intracellular retention of the mdr1 substrate Rh123 in cultured rat hepatocytes

Hepatocytes were cultured for 3 days under arterial or venous pO_2 . Subsequently, the cells were incubated for 3 h with 6.5 μ M Rh123 and, when indicated, concomitantly with verapamil (Ver, 10 μ M), an inhibitor of mdr1-dependent transport, or MK571, an inhibitor of mrp-catalysed transport (MK, 20 μ M). Intracellular steady-state Rh123 levels were determined after butanol extraction as described in the Materials and methods section. Rh123 levels attained in the absence of verapamil in hepatocytes cultured under 16% O₂ were set to 100%. Data represent means $\pm$ S.D. of three independent experiments performed in triplicate. *, Significant difference between cells incubated with verapamil and control cultures under 16% O₂; **, significant difference between hepatocytes cultured under 8% versus 16% O₂ ($P \leq 0.05$, Student's t test for unpaired values).

support the conclusion that enhanced mdr1b mRNA expression under arterial pO_2 resulted in an increase in functional mdr1-type transporter protein.

Figure 5 Abrogation by $CoCl_2$ and DSF of the oxygen-dependent enhancement of *mdr1b* mRNA expression in primary rat hepatocyte cultures

Hepatocytes were cultured for 3 days under arterial or venous pO_2 in the presence or absence of 25 μ M $CoCl_2$ or 80 μ M DSF. *mdr1b* mRNA expression was determined by Northern-blot analysis with 20 μ g of total RNA/lane as outlined in Figure 1. (A) Hybridization signals were quantified by PhosphorImaging analysis. Expression of *mdr1b* mRNA under arterial pO_2 without $CoCl_2$ or DSF was set to 100%. Data represent means $\pm$ S.D. of seven (controls) or three independent experiments. *, Significant difference between hepatocytes cultured with $CoCl_2$ and control cultures at respective pO_2 values; **, significant difference between hepatocytes cultured under 8% versus 16% O_2 ($P \leq 0.05$; Student's *t* test for unpaired values). (B) Representative Northern blot. The blot was first hybridized to the *mdr1b*-specific oligonucleotide probe and then rehybridized to HO-1 and β -actin antisense RNA probes as described in the Materials and methods section.

Inhibition of *mdr1b* mRNA expression by $CoCl_2$ and DSF

The participation of a haem-dependent system in oxygen sensing was examined by treatment of primary rat hepatocytes with $CoCl_2$, a potent inhibitor of haem biosynthesis, and with DSF, an inhibitor of haem synthesis with antioxidant properties. In cells cultured for 3 days, exposure to 25 μ M $CoCl_2$ suppressed *mdr1b* mRNA expression to approx. 9% under venous and 16% under arterial pO_2 in comparison with control expression under arterial pO_2 without $CoCl_2$ (100%; Figure 5). β -Actin expression remained stable in the presence of $CoCl_2$, while HO-1 mRNA expression, which has been shown previously to be up-regulated by $CoCl_2$ and under hypoxic conditions [31,32], was induced (Figure 5). The differential regulation of *mdr1b*, β -actin and HO-1 mRNA expression by $CoCl_2$ indicates that the inhibition of *mdr1b* mRNA expression was a specific effect of Co^{2+} . In cells incubated in the presence of the iron chelator DSF (80 μ M) for 3 days, average *mdr1b* mRNA expression levels were clearly reduced under arterial pO_2 (Figure 5). Thus DSF alleviated the modulation of *mdr1b* mRNA expression by oxygen. These results are consistent with other studies, in which DSF mimicked low oxygen tensions in the regulation of expression of several other genes [33–35].

Figure 6 Induction of *mdr1b* mRNA expression by haem in primary rat hepatocyte cultures

Hepatocytes were cultured for 3 days under arterial or venous pO_2 . In designated cultures, haem (at a final concentration of 10 μ M, bound to BSA) was added to the medium 24 h prior to harvesting of the cells. Expression of *mdr1b* mRNA was determined by Northern-blot analysis with 20 μ g of total RNA/lane as described in Figure 1. (A) Hybridization signals were quantified by PhosphorImaging analysis. *mdr1b* mRNA expression under arterial pO_2 was set to 100%. Data represent means $\pm$ S.D. of seven (control) or three (haem) independent experiments. *, Significantly different from control values under respective oxygen tensions; **, significant difference between cells cultured under 8% versus 16% O_2 ($P \leq 0.05$; Student's *t* test for unpaired values). (B) Representative Northern blot. The blot was first hybridized to an *mdr1b*-specific oligonucleotide probe and then rehybridized to HO-1 and β -actin antisense RNA probes as outlined in the Materials and methods section.

Induction of *mdr1b* mRNA expression by haem

Since the inhibitor of haem biosynthesis $CoCl_2$ suppressed *mdr1b* mRNA expression, the effect of haem itself on *mdr1b* transcript levels under venous and arterial pO_2 was examined. Treatment of hepatocytes with 10 μ M haem for the last 24 h of a 3-day culture period increased *mdr1b* mRNA levels 2–3-fold under both oxygen tensions (Figure 6). As shown in control hybridizations, β -actin mRNA expression was not affected by haem, whereas HO-1 mRNA expression was induced. Haem-dependent up-regulation of HO-1 mRNA has been described for hepatocytes previously [32] and is in accordance with the haem-degrading role of the HO-1 enzyme.

Enhancement by H_2O_2 of *mdr1b* mRNA expression under venous and arterial pO_2

Haem-containing oxidases, leading to enhanced intracellular H_2O_2 levels, have been implicated as sensors in oxygen-regulated gene expression [11,33–37]. Therefore, the effect of the potential second messenger for oxygen, H_2O_2 , was investigated on *mdr1b*

Figure 7 Enhancement by H₂O₂ of *mdrlb* mRNA expression under venous and arterial *p*O₂ in primary rat hepatocyte cultures

Hepatocytes were cultured for 3 days under arterial or venous *p*O₂ in the absence or presence of exogenous H₂O₂. As indicated, H₂O₂ was added to the culture medium at an initial concentration of 50 μM once daily. *mdrlb* mRNA expression was determined by Northern-blot analysis with 20 μg of RNA/lane as outlined in Figure 1. (A) Hybridization signals were quantified by PhosphorImaging analysis. *mdrlb* mRNA expression without H₂O₂ under arterial *p*O₂ was set to 100%. Data represent means ± S.D. of three independent experiments. *, Significant difference between H₂O₂-treated and control cultures under respective *p*O₂ values; **, significant difference between cells cultured under 8% versus 16% O₂ ($P \leq 0.05$; Student's *t* test for paired values). (B) Representative Northern blot. The blot was first hybridized to the *mdrlb*-specific oligonucleotide probe and then rehybridized to a β-actin antisense RNA probe as described in the Materials and methods section.

mRNA expression under arterial and venous *p*O₂. H₂O₂ was added to the culture medium at an initial concentration of 50 μM once daily for 3 days. In all of the three independent experiments, H₂O₂ slightly but significantly increased *mdrlb* mRNA levels under both oxygen tensions (average elevation, 1.6-fold under venous *p*O₂ and 1.5-fold under arterial *p*O₂; see Figure 7). The specificity of the H₂O₂ action was demonstrated by the lack of an H₂O₂ effect on the expression of β-actin and HO-1 mRNAs, as shown in previous studies: using the same culture system, expression of the oxidative-stress-inducible HO-1 mRNA was only elevated by H₂O₂ concentrations above 100 μM [12].

DISCUSSION

In the present study, arterial *p*O₂ as compared with venous *p*O₂ enhanced *mdrlb* mRNA and *mdr* protein expression as well as transport of the *mdr1* substrate Rh123 in primary rat hepatocyte cultures. The positive modulation by arterial *p*O₂ was abolished by the haem-synthesis inhibitor CoCl₂ and the iron chelator DSF, whereas haem itself induced *mdrlb* mRNA expression

under both oxygen tensions. H₂O₂, applied at a concentration of 50 μM, elevated *mdrlb* mRNA levels, which supports the notion that H₂O₂ might act as a second messenger in the O₂-signalling pathway.

Oxygen as a modulator of gene expression

In the rat liver, biliary transport of the *mdr1* substrate rhodamine B has been shown to occur primarily in the periportal region [9]. Furthermore, predominant expression of *mdr* transcripts has been described for hepatocytes of the periportal area [10]. The present results suggest that a higher *p*O₂ in the periportal region may constitute a crucial determinant for zoned expression of the *mdr1*-type multidrug transporter *mdrlb* in the rat liver, which would contribute to predominant periportal secretion of lipophilic xeno- and endobiotics into the bile.

An increasing number of genes in eukaryotic organisms have been demonstrated to be regulated in an oxygen-dependent manner [33]. Examples of induction under hypoxic conditions include erythropoietin and glycolytic-enzyme gene expression in Hep3B hepatoma cells [38,39] and expression of vascular endothelial growth factor (VEGF) in PC-12 cells [40]. Furthermore, in primary rat hepatocyte cultures, insulin-dependent induction of the perivenously located glucokinase occurred to a greater extent under venous than under arterial *p*O₂ [35]. A positive modulation of gene expression by arterial *p*O₂, as found here with the *mdrlb* gene, has been demonstrated before with the glucagon-dependent induction of the periportal phosphoenolpyruvate carboxykinase (PEPCK) in primary rat hepatocytes [20,37]. However, the present study demonstrates for the first time that apart from metabolic processes, expression of a transporter gene (the *mdrlb* multidrug transporter gene) is subject to modulation by oxygen in hepatocytes. Thus the *p*O₂ in the liver may play a crucial role in co-ordination of metabolic and secretory functions of the liver.

Haem-dependent oxygen sensing

Mechanisms by which oxygen might modulate gene expression are only beginning to be resolved. Evidence has been provided previously that haem proteins might function as oxygen sensors in eukaryotic cells. The transition-metal ions Co²⁺ and Ni²⁺ substitute for Fe²⁺ during haem biosynthesis [38,41], and are incorporated by ferrochelatase into protoporphyrin IX, for which oxygen binding is thus impaired or impossible [38]. Both Co²⁺ and Ni²⁺ have been shown to mimic hypoxia in stimulating expression of erythropoietin [38]. Co²⁺ also mimicked hypoxia by enhancing expression of other genes [33,42], including genes encoding glycolytic-enzyme and VEGF [39,40], or by decreasing the glucagon-dependent induction of PEPCK [20]. The notion of haem proteins as oxygen sensors has been substantiated further by the fact that CO, which binds to haem proteins like O₂ and thus locks them into the oxy-conformation, has been found to affect oxygen-dependent modulation of the expression of several genes in cell culture, including the genes encoding erythropoietin, VEGF [40] and PEPCK [43].

In the present culture system, *mdrlb* mRNA expression was markedly reduced in the presence of the haem-synthesis inhibitor CoCl₂, regardless of the *p*O₂ (Figure 5). This supports the proposal that a haem protein, possibly with a rapid turnover, might be involved in oxygen sensing, leading to regulation of *mdrlb* expression. Haem itself further up-regulated *mdrlb* mRNA expression under both oxygen tensions (Figure 6),

possibly by increasing the level of the haem-containing oxygen sensor. Interestingly, the repression of *mdr1b* mRNA expression by CoCl_2 was not abolished by addition of haem for the last day of culture (results not shown). The fact that haem did not abrogate repression of *mdr1b* mRNA expression by CoCl_2 might be due to a rapid degradation of exogenous haem by HO-1, which was strongly induced in hepatocytes treated with CoCl_2 (Figure 5). Induction of the HO-1 enzyme by CoCl_2 has been observed previously in primary rat hepatocytes [32].

Haem-containing oxidases as O_2 sensors

An NAD(P)H oxidase-type flavohaem protein has been proposed to function as an oxygen sensor, leading to the generation of H_2O_2 which then serves as a mediator in oxygen-regulated gene expression [11,33,34]. H_2O_2 as a highly diffusible molecule is ideally suited to be a second messenger, which in the presence of iron yields highly reactive hydroxyl radicals via the Fenton reaction [42]. These radicals, in turn, may lead to oxidation of proteins, thereby altering protein function or stability, e.g. of transcription factors [44,45].

In HepG2 hepatoma cells and in primary rat hepatocytes, H_2O_2 was produced proportionally to ambient $p\text{O}_2$ [36,37]. Addition of exogenous H_2O_2 to the culture medium inhibited hypoxia-induced erythropoietin production in HepG2 cells [36]. Furthermore, H_2O_2 mimicked periportal oxygen pressures in enhancing glucagon-dependent PEPCK expression, and reciprocally in reducing the insulin-dependent glucokinase gene activation in primary rat hepatocyte cultures, thus abolishing modulation of gene expression by ambient $p\text{O}_2$ [35,37].

Recently, evidence has been provided that reactive oxygen species (ROS) might play a pivotal role in enhancing *mdr1b* mRNA expression: in primary rat hepatocytes cultured under conditions corresponding to periportal $p\text{O}_2$, initial concentrations of H_2O_2 above $100 \mu\text{M}$ and exposure to the catalase inhibitor aminotriazole led to elevation of *mdr1b* mRNA levels [19]. Accordingly, several antioxidants (DMSO, ascorbic acid and *N*-acetylcysteine) suppressed the time-dependent increase in *mdr1b* expression, which was observed during hepatocyte culture [19]. However, the effect of H_2O_2 on hepatocytes cultured under venous $p\text{O}_2$ had not been examined.

In the present study, the lower initial H_2O_2 concentration of $50 \mu\text{M}$ significantly elevated *mdr1b* mRNA levels under venous and arterial $p\text{O}_2$ (Figure 7). Thus enhancement of *mdr1b* mRNA expression by low H_2O_2 concentrations further substantiates the proposal that H_2O_2 might function as a second messenger in regulation of *mdr1b* expression by oxygen.

As demonstrated in Figure 6, exogenous haem also increased *mdr1b* mRNA expression. On the one hand, exogenously added haem might be incorporated into the putative haem-protein oxygen sensor (as suggested above), thus possibly indirectly resulting in haem-protein-catalysed enhanced ROS production. On the other hand, ROS generation may also occur by non-enzymic reactions catalysed by iron or iron complexes such as haem, e.g. via the Fenton reaction [46]. Therefore, haem may participate in oxygen sensing via a direct (ROS-generating) mechanism or as a prosthetic group of a ROS-generating haem protein with a rapid turnover.

The tendency of the iron-chelating agent DSF to lower *mdr1b* mRNA expression under arterial $p\text{O}_2$ in the present study could be explained either by the ability of DSF to impair haem biosynthesis [47] (not as efficiently as CoCl_2) or by its interference with hydroxyl radical (ROS) formation via the Fenton reaction [42,48].

The participation of ROS in the regulation of *mdr1b* expression does not rule out the possibility that several signal-transduction pathways (leading to generation of ROS on the one hand and involving a different haem-protein-dependent mechanism on the other) may contribute to modulation of *mdr1b* expression under ambient $p\text{O}_2$. Recently, it has been suggested that $p\text{O}_2$ and ROS might be sensed independently in the regulation of erythropoietin expression in Hep3B cells [49].

Both transcriptional activation and mRNA stabilization have been shown to contribute to *mdr1b* mRNA accumulation during hepatocyte culture [26,50,51]. In the present study, prevention by actinomycin D of the enhancement of *mdr1b* mRNA expression levels under arterial $p\text{O}_2$ (Figure 2) indicated that transcriptional activation played a major role in the modulation of *mdr1b* mRNA expression by O_2 . Several *cis*-regulatory elements are situated in the rat *mdr1b* promoter, including C/EBP- (CCAAT-enhancer-binding protein), CRE- (cAMP-responsive element), AP-1- (activator protein 1), NF- κ B- (nuclear factor κ B), SP-1- and p53-binding sites for transcription factors [51–53]. Many of these factors exhibit redox sensitivity [54–56]; apart from the potential of NF- κ B and AP-1 to confer gene activation under oxidative stress [55,56], all of these transcription factors have been associated with gene activation under hypoxic conditions [57–63]. Thus it cannot be predicted whether one of these factors might confer *mdr1b* mRNA induction under arterial $p\text{O}_2$. Interesting candidates might be p53 and NF- κ B, which have been shown to mediate *mdr1b* gene activation by daunorubicin (p53) or insulin (NF- κ B) in H-4-II-E hepatoma cells [52,53]. However, it remains to be clarified whether these or other factors might participate in mediating the modulation of *mdr1b* mRNA expression by ambient $p\text{O}_2$.

In conclusion, the present results demonstrate that oxygen positively modulated *mdr1b* expression in rat hepatocyte cultures and provide further evidence for the involvement of a haem-dependent and oxygen-radical-generating process in oxygen sensing. It is suggested that the $p\text{O}_2$ might function as a critical determinant for zonated secretion of *mdr1* substrates into the bile.

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