Reactive oxygen species participate in mdr1b mRNA and P-glycoprotein overexpression in primary rat hepatocyte cultures


Department of Toxicology, Institute of Pharmacology and Toxicology, University of Göttingen, Robert-Koch-Strasse 40, D-37075 Göttingen, Germany and 1 Division F0100, German Cancer Research Centre, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

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P-glycoproteins encoded by multidrug resistance type 1 (mdr1) genes mediate ATP-dependent efflux of numerous lipophilic xenobiotics, including several anticancer drugs, from cells. Overexpression of mdr1-type transporters in tumour cells contributes to a multidrug resistance phenotype. Several factors shown to induce mdr1 overexpression (UV irradiation, epidermal growth factor, tumour necrosis factor α, doxorubicin) have been associated with the generation of reactive oxygen species (ROS). In the present study, primary rat hepatocyte cultures that exhibit time-dependent overexpression of the mdr1b gene were used as a model system to investigate whether ROS might participate in the regulation of intrinsic mdr1b overexpression. Addition of H2O2 to the culture medium resulted in a significant increase in mdr1b mRNA and P-glycoprotein after 3 days of culture, with maximal (-2-fold) induction being observed with 0.5–1 mM H2O2. Furthermore, H2O2 led to activation of poly(ADP-ribose) polymerase, a nuclear enzyme activated by DNA strand breaks, indicating that ROS reached the nuclear compartment. Thus, extracellularly applied H2O2 elicited intracellular effects. Treatment of rat hepatocytes with the catalase inhibitor 3-amino-1,2,4-triazole (2–4 mM for 72 h or 10 mM for 1 h) following the hepatocyte attachment period also led to an up-regulation of mdr1b mRNA and P-glycoprotein expression. Conversely, antioxidants (1 mM ascorbate, 10 mM mannitol, 2% dimethyl sulphoxide, 10 mM N-acetylcysteine) markedly suppressed intrinsic mdr1b mRNA and P-glycoprotein overexpression. Intracellular steady-state levels of the mdr1 substrate rhodamine 123, determined as parameter of mdr1b-type transport activity, indicated that mdr1-dependent efflux was increased in hepatocytes pretreated with H2O2 or amino-triazole and decreased in antioxidant-treated cells. The induction of mdr1b mRNA and of functionally active mdr1b-type P-glycoproteins by elevation in intracellular ROS levels and the repression of intrinsic mdr1b mRNA and P-glycoprotein overexpression by antioxidant compounds support the conclusion that the expression of the mdr1b P-glycoprotein is regulated in a redox-sensitive manner.

Introduction

Multidrug resistance (mdr) is a general term for a variety of adaptation mechanisms observed in neoplastic cells, leading to evasion of cytotoxic effects of anticancer drugs. Overexpression of mdr1 genes is one of the factors recognized in the phenomenon of multidrug resistance. Mdr1-type P-glycoproteins (P-gps) function as membrane-bound transporters, mediating energy-dependent extrusion of various structurally and functionally unrelated substrates from the cell. While only one drug-transporting P-gp has been identified in humans (MDR1), two closely related isoforms (mdr1a and mdr1b) exist in rodents. Overexpression of mdr1 isoform(s) in tumour cells contributes to an mdr phenotype due to translocation of a broad spectrum of cytostatic drugs and antineoplastic compounds (reviewed in Ref. 1). P-gps encoded by mdr2 genes have been identified as phospholipid transporters not involved in drug resistance (2–4).

In normal liver, mdr1 protein is localized in the canalicular membrane region of hepatocytes and has been implicated in biliary elimination of numerous potentially toxic xenobiotics and perhaps endogenous metabolites (5). In vivo, mdr1 overexpression in the liver has been observed during cholestasis (6), liver regeneration (7–10) and experimental hepatocarcinogenesis (7, 9, 11–13). Untreated hepatocellular carcinomas of human and rodent origin have been shown to exhibit intrinsic mdr1 overexpression associated with primary drug resistance (12, 14–16). However, mechanisms by which mdr1 overexpression in the liver is induced are poorly understood. Hepatotrophic growth factors, thought to participate in tumour promotion and progression (17), may be critical modulators of mdr1 overexpression. Our previous work demonstrated the induction of mdr1b mRNA and of functional mdr1-type P-gp in primary rat hepatocyte cultures treated with epidermal growth factor (EGF) or insulin-like growth factor I (IGF-I) (18).

Reactive oxygen species (ROS) also appear to be involved in multistep carcinogenesis. In many cases, cancer cells of different origin are exposed to higher ROS concentrations than their normal counterparts, owing to a decrease in antioxidant enzyme activities (19) or to an induction of ROS-producing enzyme systems (20). Notably, hepatoma cells have been shown to be deficient or low in manganese superoxide dismutase and in the major H2O2-degrading enzymes glutathione peroxidase and catalase (19). A temporal decrease in catalase expression has also been observed in primary rat hepatocyte cultures (21). Such cultures also overexpress the mdr1b gene in a time-dependent manner (5, 18, 22–24). Mdr1b overexpression in hepatocytes or hepatoma-derived cell lines has been demonstrated to be further enhanced by various external stimuli, such as EGF (18), doxorubicin (22), tumour necrosis factor α (TNF-α) (24) or insulin (25). As the production of ROS has
been suggested to be one common signalling event in the course of signal transduction initiated by cytokines or growth factors (26), and as ROS are generated during biotransformation of doxorubicin (27), the hypothesis was raised that ROS might be critical mediators of mdr1 induction. Recently, H2O2 has been implicated in serving as a second messenger for external stimuli, thus affecting gene expression by modulating activity of transcription factors or regulatory enzymes (e.g. phosphotyrosine phosphatases or protein kinases) (28). As a small and highly diffusible molecule, which can rapidly be synthesized and destroyed, H2O2 fulfills important prerequisites for serving as an intracellular messenger. In the present study, we therefore directly addressed the question whether mdr1 expression might be induced by H2O2, and whether mdr1 overexpression might be reduced in the presence of antioxidants. Since primary rat hepatocyte cultures exhibit time-dependent mdr1b overexpression that is modulated in response to external stimuli (5,18,22–24) this culture system was used as a model to study regulation of intrinsic mdr1b overexpression.

Materials and methods

Animals and materials

Rat hepatocytes were isolated from adult male Wistar rats (180–240 g) that had been given free access to laboratory chow and water and had been maintained on a 12 h light/dark cycle. Collagenase CLS II was purchased from Biochrom (Berlin, Germany), fetal calf serum was from PAA (Coelbe, Germany). Polyvinylidene difluoride membranes were from Millipore (Bedford, MA). Secondary enzyme-conjugated anti-mouse and -rabbit IgG antibodies were from Sigma (Deisenhofen, Germany). H2O2 was purchased from Fluka (Neu-Ulm, Germany). T4 polynucleotide kinase and herring sperm DNA were purchased from Boehringer (Mannheim, Germany). Rhodamine 123, insulin, hydrocortisone, 3-amino-1,2,4-triazole (AT), verapamil, 3-aminobenzamide (3-AB) and other biochemicals were obtained from Sigma (Deisenhofen, Germany). H2O2 was purchased from Fluka (Neu-Ulm, Germany). Polyvinylidene difluoride membranes were from Millipore (Eschborn, Germany), Hybond N nylon membranes and the ECL system were purchased from Amersham (Braunschweig, Germany). [γ-32P]ATP was from DuPont/NEN (Bad Homburg, Germany). The P-gp antibody PC03 as well as the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin oligonucleotides used as probes were obtained from Oncogene Research Products (Cambridge, MA). Secondary enzyme-conjugated anti-mouse and anti-rabbit IgG antibodies were from Sigma.

Hepatocyte isolation and culture

Rat hepatocytes were isolated by in situ collagenase perfusion according to Seglen (29). Hepatocyte suspensions showed viabilities >90% as determined by trypan blue exclusion. Cells were suspended in MX-82 medium (30) containing 10% fetal calf serum and plated at a density of 8.6×106 cells/cm2 on culture dishes or coverslips coated with collagen type I (4.1 µg/cm2). Following an initial attachment period of 3 h at 37°C in a humidified atmosphere of 10% CO2 and 90% air, medium was replaced with serum-free MX-83 medium (30) that lacked arginine, but was supplemented with 1 µM insulin and 20 µM hydrocortisone hemisuccinate. Media changes were performed daily. In designated experiments, medium contained 3-aminobenzamide (3-AB), a potent and specific inhibitor of P-gp, or antioxidant compounds as indicated. At the end of the culture period hepatocytes were washed once with phosphate-buffered saline (PBS), pH 7.4, frozen in liquid nitrogen and stored at –80°C prior to isolation of total RNA or cytoplasmic membrane fractions.

Northern blot analysis

Total cellular RNA was isolated by guanidinium thiocyanate–phenol-chloroform extraction according to (31). For northern blots, 25 µg of total RNA per lane were separated electrophoretically through 1% formaldehyde agarose gels. RNA was subsequently blotted onto Hybond N nylon membranes by capillary transfer (32) using 20× SSC (3 M NaCl, 0.3 M trisodium citrate) as transfer buffer. RNA blots were hybridized to oligonucleotide probes specific for mdr1a (24), mdr2 (18,24), mdr1b (24,33), GAPDH or β-actin mRNA, which had been 5′-end-labelled with T4 polynucleotide kinase using [γ-32P]ATP (34). Membranes were prehybridized for 2 h and hybridized for 16 h at 38°C in hybridization buffer containing 1 M NaCl, 0.1 M trisodium citrate, 50% (v/v) formamide, 0.5% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) Denhard’s solution and 400 µg/ml herring sperm DNA. The blots were then washed to a stringency of 0.1× SSC (15 mM NaCl, 1.5 mM trisodium citrate) and 0.1% SDS (w/v) at 38°C (mdr1a, mdr1b, mdr2) or 72°C (GAPDH, β-actin, respectively). The expression of specific mRNA was quantitated by a phosphorimaging system (BAS 1500 Bio-Imaging Analyzer; Raytest, Straubenhart, Germany).

Immunoblot analysis

Cytoplasmic membrane fractions of ~107 cells per sample were isolated by sucrose gradient centrifugation according to Simpson et al. (35). The buffers used for homogenization and centrifugation contained 1 mM phenylmethylsulphonyl fluoride. Aliquots of 10–15 µg of protein per lane, determined according to Lowry et al. (36), were subjected to electrophoresis through 7.5% SDS–polyacrylamide gels (37). Membrane proteins were subsequently transferred to polyvinylidene difluoride membranes by semidyblotting (38) using a continuous buffer system [48 mM Tris, 39 mM glycine, 0.038% (w/v) SDS and 15% (v/v) methanol, pH 9.0], P-gp was detected by utilizing the primary polyclonal antibody PC03 directed against a protein region conserved in all fully sequenced rat P-gps (39,40). Peroxidase-conjugated anti-rabbit IgG antibody was used as the secondary antibody. Protein bands were visualized by enhanced chemiluminescence utilizing the ECL system.

Rhodamine 123 accumulation assay

Rat hepatocytes preincubated for 72 h at a density of 8.6×106 cells/cm2 on 21.5 cm2 dishes were incubated with MX-83 medium containing 6.5 µM rhodamine 123 in the presence or absence of 10 µM (±) verapamil for 3 h. Subsequently, cells were washed five times with ice-cold PBS, and the dye was extracted by incubation with 2.5 ml n-butanol per dish for 10 min at room temperature. Rhodamine 123 concentrations were measured fluorimetrically in butanol extracts as described previously (18,24,41). Data were normalized for protein content per dish.

Immunofluorescence staining of poly(ADP-ribose) in cell nuclei

To analyse H2O2-induced poly(ADP-ribose) formation in rat hepatocytes, cells were preincubated on coverslips for 48 h. Hepatocytes were then incubated with MX-83 medium containing 1 or 10 µM H2O2 at 37°C for 5 min as indicated. To inhibit poly(ADP-ribose) formation, some hepatocyte samples were preincubated with 1.5 h with 1 mM 3-aminobenzamide (3-AB) alone prior to treatment with H2O2 in combination with 3-AB. Fixation and immunofluorescence analysis were performed as described previously (42), with some modifications. Briefly, coverslips were rinsed in ice-cold PBS and immediately fixed with 10% (w/v) ice-cold trichloroacetic acid for 30 min. Coverslips were then successively washed with 70%, 90% and undiluted ethanol, respectively, at –20°C for 10 min each, followed by air drying. Unspecific binding sites were blocked by incubation for 30 min in PBS containing 5% (w/v) non-fat dry milk and 0.05% (w/v) Tween 20 at room temperature. Protein A-column-purified mouse monoclonal antibody 10H, recognizing poly(ADP-ribose) (43), was used as primary antibody at a concentration of 5 µg/ml in blocking solution. Fluorescein isothiocyanate-conjugated anti-mouse IgG was diluted 1:50 in blocking solution and used as the secondary antibody. Nuclei were counterstained with Hoechst 33258 dye.

Results

Induction of mdr1b mRNA and functional P-gp expression in primary rat hepatocyte cultures by treatment with H2O2

Primary rat hepatocyte cultures exhibit an increase in P-gp expression with culture duration that predominantly involves the mdr1b isoform, as shown by a dramatic increase in mdr1b mRNA (5,18,22–24). Using a specific oligonucleotide probe for the detection of mdr1b mRNA, a time-dependent increase in mdr1b mRNA (migrating at 4.5 kb) was observed in the presently applied culture system, with maximal levels being obtained after 2–3 days in culture (Figure 1A). The expression of the mdr1a gene (transcript migrating at 5.0 kb) showed only a modest transient increase during hepatocyte culture (Figure 1A), which is in agreement with other studies (44). In contrast, the expression of the multidrug resistance-unrelated mdr2 gene as determined by mdr2 mRNA levels (migrating at 4.2 kb), remained uninfluenced with time in culture. To ensure optimal hepatocyte viability, cells were not cultivated for >3 days.

To investigate whether ROS might modulate mdr1b mRNA expression, rat hepatocytes were cultured with medium supplemented with H2O2 for 18–72 h. H2O2 was added to the medium
daily, at an initial concentration of 1 mM, which had previously been shown to modulate mRNA expression of antioxidant (21) and cytochrome P450A1 (45) enzymes in rat hepatocyte cultures. As demonstrated in Figure 1A, treatment with 1 mM H₂O₂ markedly enhanced basal mdr1b mRNA overexpression. Within the time frame of 3 days, maximal mdr1b mRNA induction under 1 mM H₂O₂ was observed after 72 h in culture and amounted to an ∼2-fold increase in comparison with control cells (Figure 1B). Control hybridizations were performed with oligonucleotide probes detecting mdr1a, mdr2 and GAPDH mRNA, respectively. Mdr1a mRNA expression was slightly enhanced by H₂O₂ treatment on day 3 of culture compared with basal expression, whereas expression of the mdr2 gene was not altered by H₂O₂ (Figure 1A). Furthermore, levels of GAPDH mRNA (migrating at 1.4 kb) exhibited a slight time-dependent increase during culture, but remained uninfluenced under H₂O₂ treatment, thus indicating specific up-regulation of mdr1b mRNA expression by the oxidant compound. Maximal induction of mdr1b mRNA after 3 days of culture was observed at a concentration of 0.5–1 mM (Figure 2A).

To determine whether H₂O₂ led to overexpression of P-gp, rat hepatocytes were treated with 0.1–1 mM H₂O₂ for 72 h, and P-gp was detected immunologically in isolated plasma membrane fractions. The primary antibody reacted with two major protein bands with apparent molecular weights of 200 and 155 kDa, respectively, as described previously (18,24). Concomitant with the concentration-dependent induction of mdr1b mRNA (Figure 2A), H₂O₂-treatment of cells also resulted in a concentration-dependent up-regulation of basal P-gp overexpression. The concentration of 1 mM H₂O₂ exhibited the strongest effect (Figure 2B), amounting to an increase in P-gp content of ∼2-fold with respect to control cells. A single treatment with 1 mM H₂O₂ for only 1 h following the hepatocyte attachment period also markedly increased P-gp expression as determined 72 h later (Figure 2B). Within the time frame of 3 days, P-gp induction under 1 mM H₂O₂ was maximal after 72 h in culture, with respect to control hepatocytes (data not shown).

The expression of functional mdr1-type protein was further investigated by an accumulation assay utilizing the fluorescent mdr1 substrate rhodamine 123 (46). High intracellular dye levels are indicative of a low mdr1-dependent transport activity and vice versa. Rhodamine 123 accumulation assays performed in parallel to immunological P-gp detection demonstrated that the enhancement of basal P-gp overexpression under H₂O₂ comprised functionally active mdr1-type P-gp. H₂O₂-pretreated hepatocytes exhibited a concentration-dependent reduction in intracellular rhodamine 123 accumulation (to 58 ± 3% of control, for 1 mM H₂O₂) (Figure 2C). The decrease in rhodamine 123 retention under H₂O₂ was completely reversed by co-incubation with dye and 10 μM (±) verapamil (Figure 3), a well-known chemosensitizer interfering with mdr1-type P-gp transport activity (47), thus indicating a specific up-regulation of mdr1-dependent transport activity in H₂O₂-treated rat hepatocytes. The strong increase in rhodamine 123 accumulation observed in control cells co-incubated with dye and verapamil (Figure 2C) revealed high basal mdr1-dependent transport activity in rat hepatocytes after 72 h in culture, which is in agreement with the high basal mdr1b mRNA expression after the same culture duration (Figure 1A).

To determine whether H₂O₂ might directly activate pre-existing mdr1 protein, rhodamine 123 accumulation assays were performed in hepatocytes precultured under control conditions for 24 h (low mdr1b gene expression) or 72 h (high mdr1b gene expression). Rhodamine 123 accumulation was not significantly reduced when directly evaluated after short H₂O₂-treatments from 20 min up to 2 h (Table I), thus supporting the conclusion that pre-existing P-gp was not activated directly by H₂O₂. At the most, in cells preincubated for 1 day, a brief exposure to H₂O₂ rather increased dye accumulation slightly, suggesting inhibition of mdr1-dependent transport activity (Table I). These results indicate that increased mdr1-dependent transport activity in H₂O₂-pretreated cells was due to induction of P-gp expression, but not to activation of pre-existing mdr1 protein.

**Poly(ADP-ribose) formation in nuclei of H₂O₂-treated rat hepatocytes**

Treatment of cell lines with H₂O₂ is known to cause DNA strand break formation, which has previously been shown to
result in activation of poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) in living cells (42). To determine whether in our system exogenously added H$_2$O$_2$ actually reached the nuclear compartment and caused DNA damage, we utilized an antibody directed against poly(ADP-ribose), the product of PARP catalysis.

In the present study, the concentration of 1 mM H$_2$O$_2$, which was shown to be effective in induction of functional mdr1b overexpression (Figures 1 and 2), indeed led to poly(ADP-ribose) formation (Figure 4A, right panel). Poly(ADP-ribose) was visualized as fine-granular nuclear fluorescence spots not appearing in untreated hepatocytes (Figure 4A, left panel).

The specificity of observed immunofluorescence was confirmed in experiments using the inhibitor of ADP-ribosylation, 3-aminobenzamide (3-AB). Hepatocytes exposed to 10 mM H$_2$O$_2$ showed strong nuclear staining whereas appearance of nuclear fluorescence spots was completely inhibited in cells pretreated with 1 mM 3-AB for 1.5 h prior to H$_2$O$_2$ treatment (Figure 4B). Notably, PARP was demonstrated to be transiently activated under H$_2$O$_2$, exhibiting maximal activity at ~3–5 min following H$_2$O$_2$ addition (data not shown).

Increase in mdr1b mRNA, P-gp expression and mdr1-dependent transport activity after treatment of hepatocytes with the catalase inhibitor AT

To support the notion that ROS, in particular H$_2$O$_2$, may contribute to mdr1b mRNA overexpression, further experiments were performed with the catalase inhibitor AT, which decreases H$_2$O$_2$ decomposition and thereby increases intracellular ROS levels. In rat hepatocytes exposed to 0.5–4 mM AT (Figure 5A), added to the culture medium once daily for 72 h or exposed to 10 mM AT for only 1 h following the attachment period, an increase in basal mdr1b mRNA overexpression of ~1.4-fold for 0.5 mM AT, 1.7-fold for 2 mM AT, 1.5-fold for 4 mM AT (Figure 5A) and ~2-fold for 10 mM AT (data not shown) was demonstrated on day 3 of culture,
Regulation of mdr1b expression by reactive oxygen species

Fig. 4. Immunofluorescence detection of poly(ADP-ribose) synthesized in nuclei of intact rat hepatocytes after treatment with H\textsubscript{2}O\textsubscript{2}. Cells precultured on coverslips for 48 h were incubated with prewarmed control medium or culture medium supplemented with H\textsubscript{2}O\textsubscript{2} for 5 min at 37°C. Coverslips were subsequently processed for immunofluorescence as described in Materials and methods. To localize the nuclear compartment, cells were counterstained with Hoechst dye (right panels). (A) Cultures were treated with or without 1 mM H\textsubscript{2}O\textsubscript{2}, as indicated. (B) Hepatocytes were treated with or without 10 mM H\textsubscript{2}O\textsubscript{2}, as indicated. To confirm the specificity of poly(ADP-ribose) immunodetection, cells designated with 3-AB were preincubated for 1.5 h in medium supplemented with 1 mM 3-AB prior to H\textsubscript{2}O\textsubscript{2} exposure in the same medium. Note the typical, granular pattern of poly(ADP-ribose) immunofluorescence in the nuclear region.

whereas GAPDH or β-actin mRNA expression remained unaffected by AT (Figure 5A). In different cell preparations, maximal mdr1b mRNA induction was observed between 0.5 and 2 mM AT.

Under AT-treatment P-gp expression, as determined immunologically in isolated plasma membrane fractions, was greatest at 2–4 mM AT (Figure 5B), with an ~3-fold increase on day 3 of culture. In the same set of cells, an example of which is shown in Figure 5, more AT was required to elicit maximal protein induction (at 2–4 mM) than was necessary for maximal mdr1b mRNA induction (2 mM AT), indicating that additional post-translational mechanisms may contribute to P-gp expression under AT.

Parallel rhodamine 123 accumulation studies, performed in triplicate, indicated increased mdr1-type transport activities in AT-pretreated hepatocytes as demonstrated by diminished intracellular rhodamine 123 accumulation. AT-pretreated rat hepatocytes exhibited dye levels amounting to 84 ± 4.4% (2 mM AT), 81 ± 3.1% (4 mM AT) (Figure 5C) and 82 ± 0.7% (10 mM AT) (data not shown) of control levels, respectively.

Reduction in basal mdr1b mRNA and functional P-gp overexpression in primary rat hepatocytes cultured in the presence of antioxidant compounds

To test the hypothesis that ROS might participate in basal (intrinsic) mdr1b mRNA and P-gp overexpression during hepatocyte culture, we investigated whether antioxidants might suppress basal mdr1b mRNA expression. As expected, basal mdr1b mRNA overexpression after 72 h in culture was markedly reduced in rat hepatocytes cultured with medium supplemented with 1 mM ascorbate, 10 mM mannitol, 2% DMSO (added to the medium once daily) or 10 mM N-acetylcysteine (added to the medium only once for 1.5 h following the attachment period), respectively (Figure 6A). Down-regulation of mdr1b mRNA expression did not result from non-specific toxicity as GAPDH mRNA levels remained uninfluenced by antioxidant treatment.

Western blot analyses demonstrated a parallel reduction in basal P-gp overexpression in cells cultured for 72 h in the presence of 1 mM ascorbate, 10 mM mannitol or 2% DMSO (Figure 6B). Furthermore, rhodamine 123 accumulation assays revealed that down-regulation of the P-gp content comprised
mdr1-type P-gp, as antioxidant-pretreated hepatocytes exhibited markedly higher dye steady-state levels (reduced mdr1-dependent transport activities) compared with the respective controls. Any direct effect of antioxidants on rhodamine 123 accumulation could be ruled out, as was apparent from concomitant incubation with rhodamine 123 during accumulation assays (data not shown). Dye levels of hepatocytes preincubated with antioxidant compounds reached mean levels of 131 ± 9% for ascorbate and of 150 ± 10% for DMSO (Figure 6C). Single experiments performed in triplicate with mannitol- and N-acetylcysteine-pretreated hepatocytes demonstrated rhodamine 123 accumulation amounting to 148 ± 4% and 162 ± 0.1%, respectively, compared with untreated hepatocytes (data not shown). Notably, N-acetylcysteine abolished AT-dependent induction of mdr1b mRNA and mdr1-dependent transport activity on day 3 of culture when cells were pretreated with 10 mM N-acetylcysteine for 1.5 h and subsequently incubated with 10 mM N-acetylcysteine and 10 mM AT for another hour (data not shown).

Discussion

Counteracting intrinsic mdr1-mediated drug resistance of hepatocellular carcinomas could, in principle, be an attractive therapeutic option. From this point of view, it is of special interest to clarify whether different mdr1-inducing compounds might share common signal transduction pathways. On closer examination, a broad spectrum of structurally and functionally unrelated mdr1-inducing agents such as EGF (18), TNF-α (24), doxorubicin (22), benzo[a]pyrene (48) or UV irradiation (49) are known to trigger the production of ROS (including the superoxide anion radical, the hydroxyl radical or H_2O_2). We therefore raised the question whether ROS might induce mdr1 overexpression.

In the present study, the influence of H_2O_2 on basal (intrinsic) expression of the mdr1b gene was examined in primary rat hepatocyte cultures. Treatment of cultures with 0.5–1 mM H_2O_2 for 3 days led to a marked increase in mdr1b mRNA levels, to an elevation in immunodetectable P-gp and to a decrease in intracellular accumulation of the mdr1 substrate rhodamine 123, supporting the conclusion that elevated mdr1b mRNA levels resulted in elevated levels of functional transporter protein.

It has been postulated that mdr1b gene induction by carcino gens ( aflatoxin B_1 or 2-acetylaminofluorene) or by the cytostatic drug mitoxantrone might be mediated through DNA damage, e.g. by adduct formation with active metabolites (50–52). Treatment of cells with H_2O_2 has previously been shown to result in DNA damage, including DNA strand break formation, which triggers the catalytic function of PARP and thus leads to a rapid increase in cellular poly(ADP-ribose) levels (42).
In our system exogenously added H$_2$O$_2$, at a concentration effective in inducing mdr1b mRNA (1 mM), caused DNA strand break formation, presumably by H$_2$O$_2$-derived hydroxyl radicals, as determined by immunocytochemical analysis of poly(ADP-ribose) formation. Due to their high reactivity, hydroxyl radicals react at their site of generation, indicating that exogenously added H$_2$O$_2$ actually reached the nuclear compartment. In our system, PARP was demonstrated to be transiently activated under H$_2$O$_2$, exhibiting maximal activity at ~3–5 min following H$_2$O$_2$ addition. Given the dynamic equilibrium of poly(ADP-ribose) synthesis and degradation under conditions of DNA breakage (53), the transient presence of polymer immunofluorescence signals is likely to reflect the transient presence of DNA strand breaks, which are known to be an essential trigger for ongoing polymer synthesis. This implies that H$_2$O$_2$ added to the cultures lost its ability to cause the ongoing formation of strand breaks, suggesting a rapid H$_2$O$_2$ detoxification in rat hepatocytes. It is noteworthy that a single treatment with 1 mM H$_2$O$_2$ for 1 h following the attachment period also affected P-gp expression and mdr1-dependent transport activity, which were both determined 3 days later, emphasizing a fast and persistent effect of H$_2$O$_2$ on functional P-gp expression. It remains to be clarified whether DNA damage elicited by H$_2$O$_2$ treatment constitutes a critical or independent event in the signal transduction pathway leading to mdr1b induction.

The notion that ROS contribute to mdr1b mRNA overexpression was supported by experiments performed with the catalase inhibitor AT. Inhibition of catalase leads to a decrease in H$_2$O$_2$ decomposition, resulting in elevated intracellular H$_2$O$_2$ levels (54). In our experiments AT was able to mimic H$_2$O$_2$ action on mdr1b mRNA and P-gp expression, and mdr1-dependent transport activity, thus indicating that endogenously liberated H$_2$O$_2$ was also effective in enhancing functional mdr1b overexpression.

The observed H$_2$O$_2$- and AT-induced up-regulation of mdr1b mRNA overexpression during rat hepatocyte culture may be based on increased transcription, increased RNA stabilization, or both. While a previous study demonstrated that mdr1b transcription remained unchanged during hepatocyte culture (23), suggesting that mRNA stabilization was crucial in mdr1b mRNA accumulation observed with time, nuclear run-on analysis performed in another study indicated that transcriptional activation might also contribute to basal mdr1b mRNA overexpression (51). The induction of mdr1b mRNA expression by 2-AAF (51,55), mitoxantrone (52), aflatoxin B$_1$, methyl methanesulphonate (50) or insulin (25), as well as the induction of the human MDR1 gene by UV irradiation (49), are also thought to occur at the transcriptional level. As the generation of ROS in response to various external stimuli has been related to the activation of redox-sensitive transcription factors, such as activator protein-1 (AP-1) or nuclear factor κB (NF-κB) (45), modifications of their activity may be involved in mdr1b gene regulation. The rat mdr1b gene promoter (50) contains putative AP-1- and NF-κB-responsive elements as well as a potential antioxidant responsive element, which has previously been shown to respond to H$_2$O$_2$ in the induction of the glutathione S-transferase Ya subunit gene (56). It has recently been demonstrated that transcriptional induction of the mdr1b gene in H-4-II-E rat hepatoma cells under insulin treatment involved NF-κB activation (25). As inhibition of cytochrome P4501A gene expression by insulin has previously been shown to be mimicked by H$_2$O$_2$ (45), NF-κB may also participate in mdr1b induction under H$_2$O$_2$ and AT. Furthermore, a functional p53 binding site has recently been identified in the rat mdr1b promoter, which is responsive to activation by daunorubicin (57), an anticancer drug that is subject to redox cycling.

Keeping in mind that rat hepatocyte cultures show a time-dependent decrease in catalase activity in culture (21) and that H$_2$O$_2$ or treatment with the catalase inhibitor AT resulted in an increase in mdr1b mRNA overexpression, basal (intrinsically) mdr1b mRNA overexpression observed with time in primary rat hepatocyte cultures might be a consequence of reduced antioxidant capacity. It was therefore reasonable to hypothesize that antioxidant compounds might counteract spontaneously occurring mdr1b mRNA overexpression. Our results show that overexpression of mdr1b mRNA and functional P-gp in rat hepatocyte cultures was indeed markedly suppressed by ascorbate (universal radical scavenger), mannitol and DMSO (hydroxyl radical scavengers), or N-acetylcysteine (replenisher of reduced glutathione-pools). Due to the fact that hydroxyl radical scavengers (mannitol, DMSO) were able to reduce functional mdr1b overexpression, it seems likely that hydroxyl radicals, derived from H$_2$O$_2$ decomposition via the Fenton reaction, might constitute a critical event in temporal mdr1b overexpression. Interestingly, acid-condensation products of indole-3-carbinol, a constituent of cruciferous vegetables, have been demonstrated to reverse multidrug resistance exhibited by human P-gp-expressing B16F10/MDR-1 tumour cells in nude mice (58). As we found intrinsic mdr1b mRNA overexpression in primary rat hepatocytes to be also suppressed by indole-3-carbinol condensation products (unpublished data) and these products are thought to comprise antioxidant capacity (59,60), this dietary constituent might participate in mdr1b down-regulation by enhancing antioxidant capacity.

In summary, the present study demonstrates that overexpression of functional mdr1b transporters in primary rat hepatocyte cultures was increased by the addition of H$_2$O$_2$ or AT and lowered by antioxidant compounds, thus indicating a redox-sensitive regulation of the resistance-related mdr1b isofrom. It appears likely that ROS may also participate in the regulation of mdr1 genes during pathophysiological processes associated with an impaired antioxidant capacity or increased ROS liberation such as hepatocarcinogenesis (19), known to lead to intrinsic mdr1 overexpression (12,14–16). Further studies are required to clarify whether enhancement of antioxidant capacity might constitute an effective strategy to counteract intrinsic mdr1 overexpression in vivo and might thus contribute to reversal of multidrug resistance.

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