Arsenic Induces Expression of the Multidrug Resistance-Associated Protein 2 (MRP2) Gene in Primary Rat and Human Hepatocytes

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ABSTRACT
Metals, such as arsenic or cadmium, have recently been demonstrated to interact with metabolic pathways, including phase I and phase II enzymes and the phase III efflux pump P-glycoprotein. In the present study, we investigated the effects of heavy metals and metalloids on the expression of the multidrug resistance-associated protein 2 (MRP2), a major hepatic transporter. Treatment of primary rat hepatocytes by sodium arsenite [As(III)], sodium arsenate and potassium antimony tartrate, but not cadmium chloride, was shown to markedly increase MRP2 mRNA and protein levels; As(III)-mediated induction was dose- and time-dependent and paralleled a strong increase in MRP2 amounts as assessed by Western blotting. As(III) was also demonstrated to markedly up-regulate MRP2 gene expression in primary human hepatocytes. MRP2 mRNA induction occurring in As(III)-treated rat hepatocytes was fully blocked by actinomycin D, indicating that it required active gene transcription. It was associated with an activation of the c-Jun N-terminal kinase pathway and with a reduction of cellular glutathione levels. Quercetin, a flavonoid compound known to block As(III)-related induction of P-glycoprotein, was also found to prevent up-regulation of MRP2 gene expression in rat hepatocytes exposed to As(III). Such an effect was unlikely to be due to alteration of JNK pathway since quercetin failed to abolish As(III)-induced JNK phosphorylation. It may rather be linked to the increase of cellular glutathione levels by quercetin, thus limiting the depleting effects of As(III) on glutathione amounts. Finally, these results confirm that some metals strongly regulate expression of detoxifying proteins, including biliary drug transporters.

Some heavy metals and metalloids are industrial and environmental contaminants to which human beings could be chronically exposed and that are generally regarded as potent toxic compounds (Goyer, 1986). Among them, arsenic and cadmium are recognized as responsible for various adverse health effects including hepatic disorders (Goyer, 1986). Indeed, skin, lung, and liver cancers have been attributed to chronic drinking of water contaminated with organic forms of arsenic (Abernaty et al., 1999) and acute exposure to high doses of cadmium has been shown to induce major liver damage characterized by hepatocyte necrosis (Dudley et al., 1982). On the other hand, some of these metals can display therapeutic properties: pentavalent antimonial salts have been demonstrated to be effective in the treatment of some tropical protozoan diseases (Berman, 1988), while arsenical salts are currently used in the treatment of acute promyelocytic leukemias (Soignet et al., 1998). Cellular effects of arsenic and cadmium are mainly related to their interaction with sulphydryl residues of endogenous compounds including glutathione (GSH) (Vallee and Ulmer, 1972; Snow, 1992) and to activation of mitogen-activated protein kinases such as the c-Jun N-terminal protein kinases (JNK) (Porter et al., 1999; Ding and Templeton, 2000). New targets of metals recently identified are liver metabolic pathways including phase I and phase II enzymes and phase III biliary transport proteins. Indeed, sodium arsenite treatment has been shown to decrease induction of enzymatic activities associated with different hepatic cytochrome P450s in xenobiotic-treated rat hepatocytes (Jacobs et al., 1999), thus outlining a down-regulation of oxidative drug metabolism in response to a metalloid. By contrast, exposures to arsenical and cadmium salts have been found to result in induction of phase II drug-metabolizing enzymes such as GSH S-transferases and quinone reductase (Bergelson et al., 1994). These metals also increase hepatic expression of the multidrug resistance P-glycoprotein (Kioka et al., 1992), an ATP-binding cassette efflux pump found at the canalicular pole of hepatocytes, encoded by mdr genes and involved in

ABBREVIATIONS: GSH, glutathione; JNK, c-Jun N-terminal protein kinases; mdr, multidrug resistant/resistance; MRP2, multidrug resistance-associated protein 2; As(III), sodium arsenite; kb, kilobase(s).
the biliary secretion of cationic and amphiphilic compounds (Ambudkar et al., 1999). Whether other hepatic transporters, contributing together with P-glycoprotein to biliary secretion, may also be responsive to such metals has not been established yet. To gain insight about this point, we have analyzed in the present study the effects of arsenic, antimycin, and cadmium exposure on the expression of the multidrug resistance-associated protein 2 (MRP2). This 190-kDa ATP-binding cassette protein, also known as the canalicular multispecific organic anion transporter, belongs to the MRP transporter subfamily comprising other efflux pumps such as MRP1 and MRP3. MRP2 mediates biliary secretion of endogenous compounds including bilirubin and of various GSH, glucuronate, and sulfate conjugates of chemicals (König et al., 1999). Deficiency of MRP2 expression, as observed in Eisai hyperbilirubinemic rats (Ito et al., 1997) or transport deficient Wistar rats (Paulusma et al., 1996), therefore led to hyperbilirubinemia and reduced biliary secretion of drug conjugates; biliary elimination of heavy metals such as cadmium is also impaired (Dijkstra et al., 1996). The data reported in the present study demonstrated that treatment by arsenic, but not by cadmium, up-regulated MRP2 gene expression in primary rat and human hepatocytes. Such arsenic-mediated induction of MRP2 required active transcription and was found to be markedly inhibited by the flavonoid compound quercetin through a mechanism that seems to be unrelated to the JNK pathway but may rather be linked to alteration of cellular GSH levels.

**Materials and Methods**

**Chemicals.** Sodium arsenite [As(III)], sodium arsenate, potassium antimony tartrate, quercetin, and actinomycin D were supplied by Sigma (St. Louis, MO). Cadmium chloride was purchased from Cadmium Inc., Piscataway, NJ. The membrane was prehybridized, washed, dried and autoradiographed. The membranes were prehybridized with 32P-labeled probes, washed, dried and autoradiographed at ~80°C. MRP1, MRP2, and MRP3 mRNAs were analyzed with a 0.46-kb rat MRP1, a 0.86-kb rat or 0.6-kb human MRP2, or a 0.98-kb rat MRP3 cDNA probe, respectively, generated by reverse transcription-polymerase chain reaction (Payen et al., 1999, 2000). MRP3 probe was prepared using total RNA from rat liver, which is known to display high expression of the MRP2 gene. The specific primers used were sense GACAGGCATGGAAGCTGA and antisense CCTCTTGACTCCTCCACGG. The identity of the cDNA fragment was verified by sequencing and was found to be similar to the previously published MRP3 sequence (GenBank accession number AF072816). The mr RNAs were detected using the pCHP1 probe (Riordan et al., 1985). Equal gel loading and transfer efficiency were checked using an 18S rRNA probe.

**Preparation of Whole Cell Extracts.** Whole cell extracts were prepared for immunoblotting of MRP2 protein as previously described (Payen et al., 2000). For analysis of JNK phosphorylation, cells were first lysed in a solution containing 62.5 mM Tris-HCl (pH 8.6), 2% sodium dodecyl sulfate, 1% β-mercaptoethanol, and 0.1% glycerol. Cell extracts were further sonicated for 10 s at 0°C and then stored at ~20°C.

**Western Blot Analysis.** Proteins were separated on a 7.5% SDS polyacrylamide gel and then transferred onto nitrocellulose membranes. Membranes were blocked for 2 h with Tris-buffered saline containing 3% bovine serum albumin and 2% milk and were next incubated with either the rabbit anti-rat MRP2 polyclonal antibody (provided kindly by Dr. D. Keppler, Deutsches Kreforschungszenzter, Heidelberg, Germany), the rabbit anti-human MRP2 monoclonal antibody M2 III-6 (kindly provided by Dr. R. Scheper, Free University Hospital, Amsterdam, The Netherlands) or a monoclonal anti-phospho-JNK (New England Biolabs, Inc., Beverly, MA). A peroxidase-conjugated anti-rabbit antibody was used as secondary antibody. After washing, blots were developed by chemiluminescence using a 100 mM Tris-HCl, pH 8.5, solution containing 0.9% (w/v) H2O2, 225 μM coumaric acid, and 1.25 mM luminol. For phospho-JNK immunoblotting, equal gel loading and transfer efficiency were checked by rehybridizing the blot with a polyclonal antibody raised against total JNK, i.e., unphosphorylated and phosphorylated forms (Calbiochem-Novabiochem Corporation, La Jolla, CA).

**Measurement of GSH Levels.** Cellular GSH levels were measured using the recycling method of Tietze (1969). Briefly, hepatocytes were scraped and first resuspended in phosphate-buffered saline to determine cellular protein contents using the Bradford assay (Bradford, 1976). Cells were then centrifuged and resuspended in 3% (w/v) 5-sulfosalicylic acid in water. After centrifugation, 18 μl of triethanolamine (1:3 v/v in water) were added to 100 μl of supernatant to bring the pH to 7.0 to 7.5, and GSH was assayed as previously reported (Tietze, 1969). Results were normalized to total cellular protein contents.

**Statistical Analysis.** Data on GSH levels were processed by Student’s t test. The criterion of significance of the difference between means (± S.E.M.) was p < 0.05.

**Results**

Primary rat hepatocytes were first treated for 48 h with different concentrations of metals found to be ineffective on protein content; MRP2 gene expression was then analyzed by Northern blot and Western blot. As shown in Fig. 1, 8 mM arsenic-induced MRP2 gene expression 235

![Fig. 1](https://i.imgur.com/3.png)
As(III), 100 μM sodium arsenate, and 4 μM potassium antimony tartrate increased both MRP2 mRNA and protein levels. In contrast, cadmium had no effect on MRP2 gene expression in hepatocytes when tested at 1 μM, the highest nontoxic concentration (Fig. 2).

Since As(III) appeared to be a stronger inducer of MRP2 gene than arsenate or antimony, this compound was retained for our further studies. The dose dependence of the As(III) effects on MRP2 gene expression was further analyzed by Northern blot. As shown in Fig. 3A, As(III) used at 6 or 8 μM similarly enhanced MRP2 mRNA levels in primary rat hepatocytes whereas lower concentrations were inactive. As previously described (Büchler et al., 1996; Paulusma et al., 1996), different sizes of MRP2 mRNAs, mainly 5.5 and 7.5 kb, which likely represent alternative mRNA splicing variants with different 3'-untranslated region lengths, were often detected on the blots. The time course of the induction of MRP2 mRNAs was thereafter determined in rat hepatocytes treated with 8 μM As(III) (Fig. 3B). MRP2 mRNA amounts were clearly increased as soon as 4 h after starting incubation of rat hepatocytes with the metalloid and MRP2 mRNA induction was maximal after 24 to 48 h of exposure. Besides its effect toward MRP2 gene expression, we found that As(III) treatment also increased mdr mRNA levels in primary rat hepatocytes, being ineffective on MRP1 and MRP3 gene expression (Fig. 4).

To determine whether As(III) also mediated induction of MRP2 in human cells, Northern and Western blot analyses were performed using untreated and As(III)-treated primary cultures of human hepatocytes. The As(III) concentration retained for these experiments was 4 μM since higher doses (6–8 μM) were found to be cytotoxic toward human hepatocytes. Figure 5A shows that human hepatocytes exposed to As(III) for 48 h displayed enhanced MRP2 mRNA levels when compared with their untreated counterparts; such data were observed with liver cells obtained from three different donors. In parallel, Western blot analysis of whole cell extracts clearly indicated that MRP2 protein levels were also increased in As(III)-treated human hepatocytes (Fig. 5B).

We next investigated whether active transcription was required for As(III)-mediated induction of MRP2 gene expression. As indicated in Fig. 6, the transcription inhibitor actinomycin D used at the concentration of 3 μg/ml, previously found to decrease RNA synthesis to less than 1% of control value in liver cells (Fardel et al., 1997), fully blocked the As(III)-related MRP2 mRNA increase in rat hepatocytes.

Actinomycin D, however, had no major effects on basal levels of MRP2 transcripts.

Since As(III)-mediated effects have been linked, at least in part, to activation of the JNK pathway, we next studied phosphorylated JNK levels, known to be correlated with JNK activity (Cavigelli et al., 1996), in As(III)-treated rat hepatocytes. As shown in Fig. 7, As(III) markedly increased phosphorylated JNK amounts; this activation was quite fast, since it was detectable as soon as 30 min following exposure to the metalloid. Cadmium, which failed to up-regulate MRP2 expression, was also found to strongly enhance phosphorylated JNK levels in primary rat hepatocytes (Fig. 7).

Finally, we investigated the effects of the isoflavonoid compound quercetin on As(III)-related MRP2 induction. Quercetin has been previously shown to inhibit As(III)-induced up-regulation of P-glycoprotein expression in human liver cells (Kioka et al., 1992). Figure 8 indicated that treatment by 100 μM quercetin also markedly reduced up-regulation of MRP2 mRNA levels in As(III)-exposed rat hepatocytes; quercetin, however, failed to alter basal MRP2 mRNA levels in control hepatocytes. Because the JNK pathway has been demonstrated to be a target for quercetin (Kobuchi et al., 1999; Uchida et al., 1999), we analyzed its effects on As(III)-medi-
ated induction of JNK activity in primary rat hepatocytes. As indicated in Fig. 9, quercetin did not prevent the increase in phosphorylated JNK levels due to As(III); in fact, quercetin alone was found to activate the JNK pathway in rat hepatocytes. Quercetin also failed to inhibit the increase of c-Jun mRNA amounts that occurred in primary rat hepatocytes exposed to As(III) and that have been postulated to depend, at least in part, on JNK activity (Cavigelli et al., 1996) (Fig. 10). Another cellular property of quercetin is related to antioxidant functions (Musonda and Chipman, 1998; Sestili et al., 1998). In line with this property, we observed that treatment of primary rat hepatocytes by the flavonoid led to a strong induction of cellular levels of GSH, a major endogenous antioxidant compound (Fig. 11). GSH is also supposed to be a target for As(III) and cellular GSH levels decreased in As(III)-treated hepatocytes when compared with their untreated counterparts (Fig. 11). As(III) treatment of quercetin-pretreated rat hepatocytes also reduced GSH amounts; GSH levels found in these cells coexposed to As(III) and quercetin were, however, similar to basal GSH levels found in untreated primary rat hepatocytes (Fig. 11).

Discussion

Arsenic and cadmium have been shown to alter the expression of various liver metabolic pathways, including cytochrome P450s, GSH S-transferases, and P-glycoprotein. The results reported in the present study demonstrate that such regulatory effects of metals can most likely be extended to MRP2, a major liver drug transporter mediating biliary secretion of organic anions. Indeed, exposure of primary rat and human hepatocytes to trivalent arsenical salt was found to increase MRP2 expression at both mRNA and protein level. Trivalent antimonial salts were also shown to augment MRP2 gene expression in rat hepatocytes; however, cadmium was ineffective, indicating that MRP2 gene regulation was not a general feature of such metals. In contrast to MRP2 mRNA levels, those of MRP1 and MRP3 were not altered in As(III)-treated rat hepatocytes, suggesting differential regulation of MRP proteins in response to metalloids and heavy metals.

The molecular basis by which As(III) induced MRP2 expression in primary hepatocytes remains unknown; however, our results demonstrate that As(III)-mediated induction of MRP2 gene expression required active transcription, since it was fully inhibited by actinomycin D. It was also found to be associated with activation of the JNK pathway; indeed, As(III) treatment increased phosphorylated JNK levels and also c-Jun mRNA amounts in primary rat hepatocytes. Interestingly, cadmium and quercetin, which failed to induce MRP2 gene expression, were also demonstrated to activate the JNK pathway. Therefore, the JNK pathway, which appears to play a role in regulation of various genes by As(III) (Cavigelli et al., 1996; Hossain et al., 2000), is unlikely to account alone for MRP2 up-regulation in response to As(III). It is also unlikely that metal-responsive elements, which are known to underlie induction of detoxifying proteins such as metallothioneins in response to some metals (Klaassen et al., 1999), contribute to MRP2 induction in As(III)-treated hepatocytes; indeed, such regulatory elements have not been de-
scribed in the 5'-flanking region of rat and human MRP2 genes (Kauffmann and Schrenk, 1998; Tanaka et al., 1999).

Our results also demonstrated that As(III)-induced MRP2 gene expression was strongly inhibited by quercetin. This compound is a widely distributed plant flavonoid that has been found to inhibit As(III)-induced P-glycoprotein expression in human liver cells, probably through reducing the binding of heat shock factors to the heat shock responsive elements present in mdr1 promoter (Kioka et al., 1992). Such a mechanism does not, however, seem to contribute to inhibitory effects of quercetin on As(III)-induced MRP2 gene expression, since heat shock responsive elements have not been reported in the promoters of human and rat MRP2 genes (Kauffmann and Schrenk, 1998; Tanaka et al., 1999). Quercetin is also considered as a potent inhibitor of tyrosine kinases including JNK in various cell types (Kobuchi et al., 1999; Uchida et al., 1999). Quercetin also inhibited the activation of As(III)-treated rat hepatocytes; it also did not block induction of c-Jun mRNAs by As(III). The effect of quercetin on MRP2 up-regulation is therefore probably unrelated to alteration of JNK activity. Another major feature of quercetin lies in its antioxidant functions (Musonda and Chipman, 1998; Sestili et al., 1998). In line with this feature, we found that quercetin significantly increased intracellular levels of the endogenous antioxidant GSH in rat hepatocytes. Similar effects were previously reported in human breast cancer cells and in vivo in mice fed with quercetin (Rodgers and Grant, 1998; Khanduja et al., 1999). The reasons for such an increase of GSH levels remain to be determined. However, it may be hypothesized that quercetin could up-regulate expression of γ-glutamylcysteine synthetase, a key enzyme of GSH synthesis, or, alternatively, could stimulate transport of cysteine, a precursor of GSH. In contrast to quercetin, As(III) down-regulated GSH levels in rat hepatocytes. This may be due to the formation of stable complexes between As(III) and thiol residues of GSH (Scott et al., 1993); such an interaction has been demonstrated to result in a depletion of intracellular GSH pools that may contribute to some arsenic-induced phenotypic effects (Guyton et al., 1996). Interestingly, As(III) also diminished GSH amounts in quercetin-treated hepatocytes; GSH levels in such cells coexposed to quercetin and As(III) remained, however, similar to basal values found in untreated hepatocytes.
whose expression of liver metabolic pathways, including phase III metabolism, is strongly induced in both rat and human primary hepatocytes through a mechanism involving a reactive oxygen species(ROS) response to this metal. Such a hypothesis will probably deserve further studies since arsenic is a common environmental contaminant to which humans are exposed and a substance currently used in the treatment of some human diseases (Perumal et al., 2000). This suggests that human exposure to some metalloids such as arsenical salts will probably result in increased liver expression of MRP2 and consequently, in enhanced biliary secretion of xenobiotics and endogenous compounds handled by MRP2. Similarly, biliary transport of drug substrates for P-glycoprotein may also be augmented, since in agreement with previous studies, we have found an up-regulation of mdr mRNA levels in arsenic-treated hepatocytes. Therefore, an overall stimulation of biliary drug secretion may be observed in response to this metal. Such a hypothesis will probably deserve further studies since arsenic is a common environmental contaminant to which humans are exposed and a substance currently used in the treatment of some human diseases (Perumal et al., 2000).

In summary, our results have demonstrated that arsenical salt treatment strongly induced expression of MRP2 gene in both rat and human primary hepatocytes through a mechanism requiring active transcription. These results further confirm that exposure to metals such as arsenic regulates expression of liver metabolic pathways, including phase III biliary secretion proteins.

References


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