Decreased Expression and Activity of P-Glycoprotein in Rat Liver During Acute Inflammation

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Purpose. Drug disposition is often altered in inflammatory disease. Although the influence of inflammation on hepatic drug metabolism and protein binding has been well studied, its impact on drug transport has largely been overlooked. The multidrug resistance (MDR) gene product, P-glycoprotein (P-gp) is involved in the active secretion of a large variety of drugs. Our goal was to ascertain the influence of acute inflammation (AI) on the expression and functional activity of P-gp.

Methods. AI was induced in rats through turpentine or lipopolysaccharide (LPS) administration. Expression of P-gp in liver was detected at the level of protein on Western blots using the monoclonal antibody C-219 and at the level of mRNA using an RNase protection assay. P-gp mediated transport activity was assessed by measuring the verapamil-inhibitable efflux of rhodamine 123 (R123) in freshly isolated hepatocytes.

Results. Turpentine-induced AI significantly decreased the hepatic protein expression of P-gp isoforms by 50–70% and caused a significant 45–65% reduction in the P-gp mediated efflux of R123. Diminished mRNA levels of all three MDR isoforms were seen. LPS-induced AI similarly resulted in significantly reduced levels and activity of P-gp in liver. Although differences in the constitutive levels of P-gp were seen between male and female rats, the influence of AI on P-gp expression and activity was not gender specific.

Conclusions. Experimentally-induced inflammation decreases the *in vivo* expression and activity of P-gp in liver. This is the first evidence that expression of P-gp is modulated in response to experimentally-induced inflammation.

KEY WORDS: multidrug resistance; P-glycoprotein; inflammation; acute phase response; gene regulation; drug disposition.

INTRODUCTION

Pathophysiological changes in humans and in animal models of inflammation are reportedly associated with often dramatic increases in drug plasma concentrations (1–3). Inflammation induced by stimuli such as infection, trauma, malignant growth or ischemia leads to the local and systemic release of hormones and proinflammatory mediators. In particular, a group of these mediators, termed the cytokines, are thought to act upon the liver causing changes in the synthesis of several liver derived proteins (the acute phase proteins) at the level of gene transcription (for review see reference 4). To date, studies examining inflammation-induced changes in drug disposition have mainly focused on alterations to cytochrome P450 metabolic activity and plasma protein binding (2,5,6). Undisputedly, changes in membrane-bound transport proteins, many of which are involved in the absorption or elimination of drugs, could substantially contribute to pathological modifications of drug disposition. However, little is known about the effect of inflammation or cytokines on the expression or activity of drug transporters.

P-glycoprotein (P-gp) is a 170 kDa membrane-bound glycoprotein that functions as an active efflux pump for a number of chemically and physically distinct drugs (for review see references 7–8). P-gp is encoded by the multidrug resistance (MDR) genes, a small gene family, comprising of two members in humans (MDR1 and MDR2) and three in rodents (mdr1a, mdr1b, mdr2). These gene products possess distinct physiological roles; P-gp encoded by the mdr1 genes are associated with drug transport and resistance whereas the mdr2 gene products are not and are thought to play a role in the hepatic transport of phospolipids (9). Although the physiological role of P-gp in normal tissues is unclear, its substrate specificity and localization in epithelial cells of the intestine, liver, kidney and brain suggests that it may play a broad role in the excretion of toxins, hormones and physiological metabolites as well as playing a critical role in the excretion of many drugs and their metabolites. More importantly, the overexpression of P-gp in malignant cells is now felt to be one of the leading causes of drug resistance and therapeutic failure in cancer chemotherapy (7). Many studies have indicated that P-gp expression is regulated in a complex manner; increasing in a tissue or cell specific manner after exposure to xenobiotics, heat shock and UV light (10–12). However, little is known about the *in vivo* expression of P-gp in disease states. Furthermore, the physiological regulation of P-gp *in vivo* remains largely unknown. As the transcription of many liver derived glycoproteins are affected by mediators of inflammation, our objective was to ascertain the influence of inflammation on the *in vivo* expression and regulation of P-gp. In the rat, a rapid and reproducible acute inflammation (AI) is often elucidated locally through a small subcutaneous injection of turpentine oil or systemically by treatment with the bacterial endotoxin, lipopolysaccharide (LPS). These models have been well characterized in regards to cytokine stimulation and influence on the production of the acute phase proteins (4,13,14). Furthermore, although it is believed that different patterns of cytokines are involved in local and systemic inflammation (15), alterations in cytochrome P450 and drug disposition have been reported for both models (2,5). In this study, we used the turpentine and endotoxin models of inflammation to examine the influence of AI on the function and expression of the MDR gene products in rat liver.

MATERIALS AND METHODS

Animals

AI was induced in male and female Sprague Dawley rats (200–250 g) by two 0.5 mL subcutaneous injections (at 0 h and 24 h) of turpentine (Fine Chem. Ltd., ON) in the dorsolumbar region or by a single 5 mg/kg intraperitoneal injection of lipopolysaccharide (LPS, *E.coli;* Sigma, ON). Reportedly, these

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ABBREVIATIONS: AI, acute inflammation; LPS, lipopolysaccharide; MDR, multidrug resistance; P-gp, P-glycoprotein; P450, cytochrome P450; R123, rhodamine 123; IL, interleukin; INF, interferon; TNF, tumor necrosis factor.

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doses elicit change in the levels of many liver-derived acute phase proteins (2,4,5,13). Control rats received equivalent injections of sterile normal saline. Studies were approved by the University of Toronto animal ethics committee and the research adhered to the "NIH Principles of Laboratory Care".

Control and treated rats were sacrificed at 48 hr (unless otherwise indicated), the liver removed, rapidly frozen in liquid N_2 and stored at −70°C until used for protein and RNA isolation. For R123 efflux studies, the hepatocytes were immediately isolated by two-step collagenase perfusion of the liver (16). Hepatocytes were continuously oxygenated at 37°C and cell viability was assessed by the trypan blue exclusion test.

Immunodetection (Western Blots)

Western blot analysis was performed on protein samples prepared by homogenization of liver (2–3 g) in lysis buffer (0.1 M Tris-HCl, pH 7.5, containing leupeptin 1 μg/ml, pepstatin A 1 μg/ml and phenylmethylsulfonyl fluoride (PMSF) 50 μg/ml; Sigma, ON) using 20 strokes of a Potter-Elvehjem glass tissue homogenizer. Homogenates were centrifuged at 1500 g for 10 min, the supernatant centrifuged at 100,000 g for 30 minutes, and the protein pellet washed and resuspended in a small volume of lysis buffer. Concentrations were measured colorimetrically using the Bio-Rad Protein Assay (Bio-Rad Laboratories, ON). Samples (20 μg) were separated on SDS-PAGE electrophoresis gels and transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered saline (TBS) containing 10% nonfat milk, for 2 h at room temperature and incubated with 2 μg of the P-gp monoclonal antibody, C-219 (Signet, CA) overnight. This antibody (C-219) recognizes an epitope common to each of the P-gp isoforms. The membranes were then incubated for 2 h with the anti-mouse horseradish peroxidase antibody (Bio-Rad Lab., ON) and washed. The bound antibody was visualized using the ECL-Western Blotting Detection Kit (Amersham, ON) and autoradiography. Molecular weight markers were obtained from Bio-Rad. Quantification of autoradiographs was performed by densitometric analysis using the MCID Imaging System (Imaging Research, ON).

RNase Protection Assay

Total cellular RNA was isolated from tissues using the one-step guanidinium-phenol method. The quantity and purity of RNA was estimated spectrophotometrically and the integrity evaluated on 1% agarose denaturing gel. Undegraded RNA samples were analysed by a RNase protection assay as described by Brown *et al.*, (17) using mdr1a, mdr1b, mdr2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes (17) kindly provided by Dr. J. Silverman (NIH, Bethesda, MD). Plasmid templates of the probes were linearized and antisense probes transcribed with T7 polymerase in the presence of [$\alpha-^{32}$P]CTP as described in the Maxiscript kit protocol (Ambion). The [^{32}P]-labelled probes were purified on a 5% polyacrylamide gel. Total RNA (20 μg) was hybridized according to manufacturer protocol with the RPAII kit (Ambion) using 200,000 cpm (mdr1a, mdr1b, mdr2) and 20,000 cpm (GAPDH) of the probes and digested at 30°C for 1 h using 0.1–0.5 U/ml RNase A and 133–150 units/ml RNase T1. The protected fragments were separated on 5% denaturing polyacrylamide gels and visualized by autoradiography. As digestion and running conditions were the same, hybridization and separation of the mdr1b, mdr2 and GAPDH protected fragments were run simultaneously. Mdr1a was hybridized and analysed with GAPDH on separate gels. Reportedly, levels of GAPDH mRNA in rat liver are not significantly influenced by turpentine-induced inflammation (14), thus expression of the MDR gene members were normalized to GAPDH. Quantification of mRNA levels were performed by densitometric analysis with the MCID Imaging System.

Rhodamine 123 Efflux Assay

Hepatocytes were assayed for P-gp-mediated R123 efflux using the method described by Chieli *et al.* (18). Briefly, freshly isolated hepatocytes (3.0×10^6 cells/ml) were incubated in Krebs-Henseleit bicarbonate buffer containing R123 (1 μg/ml; 2.6 μM) for 30 minutes, washed and the buffer was replaced by R123 free buffer with or without the addition of verapamil (20 μg/ml; 40 μM). Quantification of the intracellular concentration of R123 was performed at 0, 30 min, 1 hr and 2 hr after the removal of R123 from the buffer. Cells were washed 3 times, extracted for 10 minutes with 2 ml n-butanol and the fluorescence intensity measured with a spectrophotofluorimeter at 518/532 nm (excitation/emission). R123 content was corrected on the basis of protein content and cell viability. A constant cell viability of >80% was maintained throughout the experiment.

Data Analysis

Intracellular concentrations of R123 are reported as a percentage of intracellular R123 preloaded at time 0. The P-gp-mediated transport of R123 was estimated as the efflux of R123 which could be inhibited by verapamil (a competitive inhibitor of P-gp, verapamil binds to P-gp inhibiting the efflux of dyes and other P-gp substrates) and was thus calculated as the difference in R123 efflux in the presence and absence of verapamil. Efflux rates from control and inflamed rats were obtained in the first hour after loading.

RESULTS

Protein Expression

Inflammation-induced changes in the expression of the MDR encoded products at the level of protein was assessed on Western blots. Immunodetection of P-gp isoforms in male livers at different times after an injection of turpentine revealed reductions in band intensity from 24 to 72 hours after treatment (Fig. 1). At 48 hours, hepatic expression of the MDR gene products in AI rats were significantly reduced from controls (Fig. 2). This was not gender specific as both male and female groups displayed comparable reductions. However, significantly greater amounts of MDR gene products were detected in female livers as compared to their male counterparts (Fig. 2).

Induction of systemic inflammation through injection of LPS also caused the hepatic expression of the MDR gene products to decrease by more than 80% at 24 hours {Fig. 3, LPS 4.42 ± 0.88 ($n = 4$) *versus* control 7.02 ± 1.2 optical density units ($n = 3$); $p < 0.05$}.

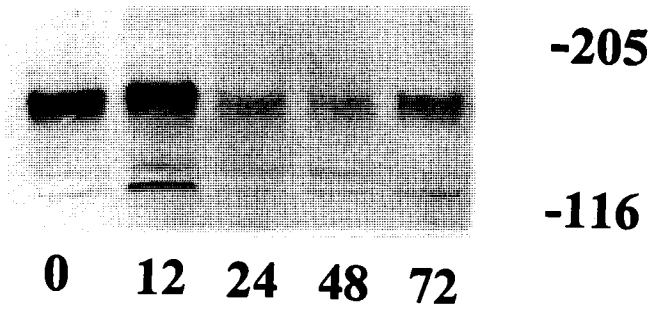

Fig. 1. Western blot analysis demonstrating the reduction of P-glycoprotein expressed in liver after inducing inflammation. Protein (20 μg) isolated from livers of treated males at 0, 12, 24, 48 and 72 hr after a turpentine injection were electrophoresed through a SDS-PAGE gel, transferred to a nitrocellulose membrane and incubated with Mab C-219. Molecular weight markers are in kDa.

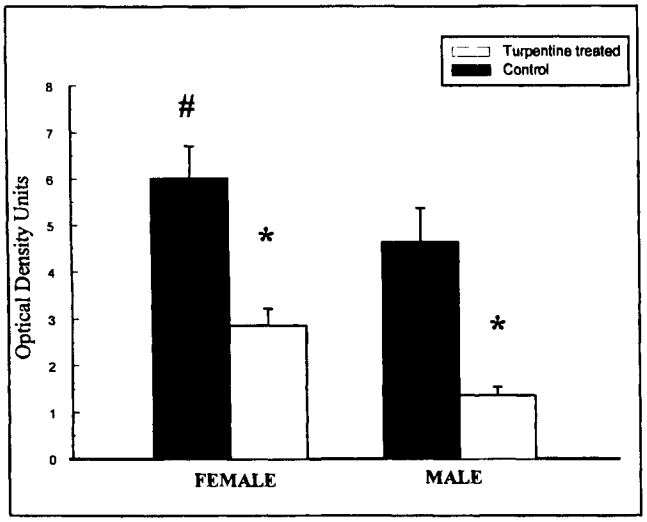

Fig. 2. Effect of turpentine-induced inflammation on the hepatic expression of P-glycoprotein. Livers were isolated at 48 hr from control (■) or turpentine-treated (□) rats. Samples (20 μg) were analysed on western blots as described using the Mab C-219 and the relative content of the MDR gene products were estimated by densitometric analysis. Data is presented as mean ± SEM, n = 12/group. *significantly different from controls; # significantly different from males.

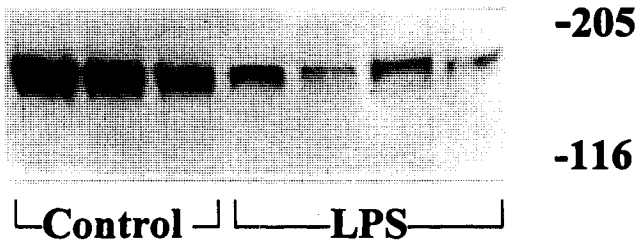

Fig. 3. Western blot analysis demonstrating the reduction of hepatic P-glycoprotein expressed after treatment with endotoxin. 20 μg of protein isolated from livers of endotoxin-treated (LPS) and control males at 24 hr were analyzed on western blots as described. Molecular weight markers are in kDa.

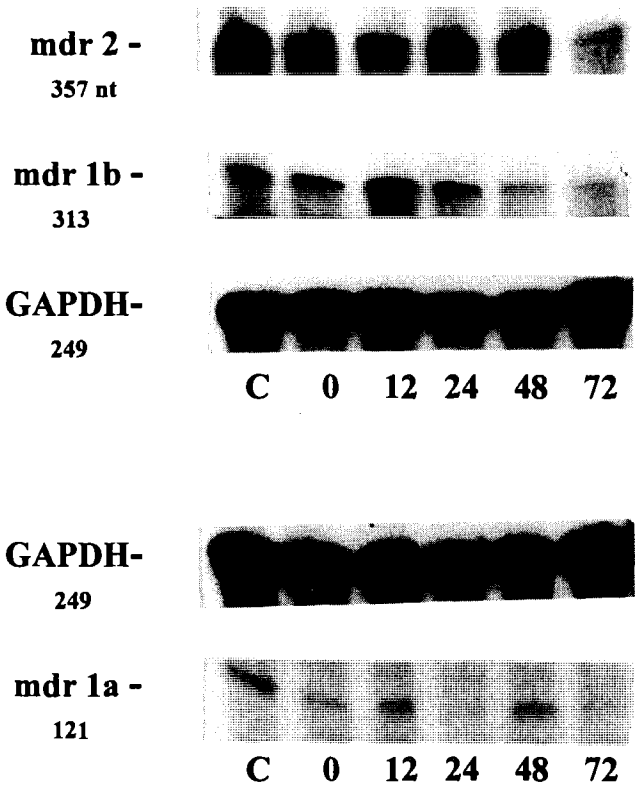

Fig. 4. Time course of MDR downregulation in liver after induction of inflammation with turpentine. Analysis using an RNase protection assay. 20 μg of total RNA isolated from livers of control (C) and treated male rats at 0, 12, 24, 48 and 72 hr after injection, were hybridized to mdr1a, mdr1b, mdr2 and GAPDH ^{32}P- transcribed probes, digested and separated on denaturing polyacrylamide gels. The autoradiographs of protected fragments were obtained 24-48 hour after exposure of the film.

Expression of mRNA

Inflammation-induced changes in the expression of the MDR genes at the level of mRNA was assessed using an RNase protection assay. Similar to changes in protein expression, changes in the MDR mRNA levels of male rats were also seen from 24–72 hour following a turpentine-injection (Fig. 4). Analysis of RNA samples obtained at 48 hour after treatment confirmed that the AI associated reductions in hepatic levels of the MDR genes were significant. As compared to controls, mRNA levels of mdr1a, mdr1b and mdr2 were decreased by 65%, 58% and 38% in male livers and by 70%, 40% and 72% in female livers, respectively (Fig. 5).

Gender differences in MDR mRNA levels were seen in control rats with males expressing more than two fold higher levels ($p < 0.05$) of mdr1b and females expressing higher levels (approximately 35%) of mdr1a and mdr2. Differences were not significant in the inflamed rats.

Functional Activity

The impact of AI-induced changes on P-gp transport activity was examined by measuring efflux of the P-gp substrate, R123, in freshly isolated hepatocytes. Elimination of R123 from preloaded hepatocytes was linear and significantly inhibited by verapamil (Fig. 6). Inflammation induced by LPS or turpentine was found to significantly decrease the inhibitable efflux of R123 (Fig. 7). As compared to controls, the P-gp mediated efflux of R123 was significantly decreased by 65% in female turpentine-treated, 45% in male-turpentine treated and 60% in

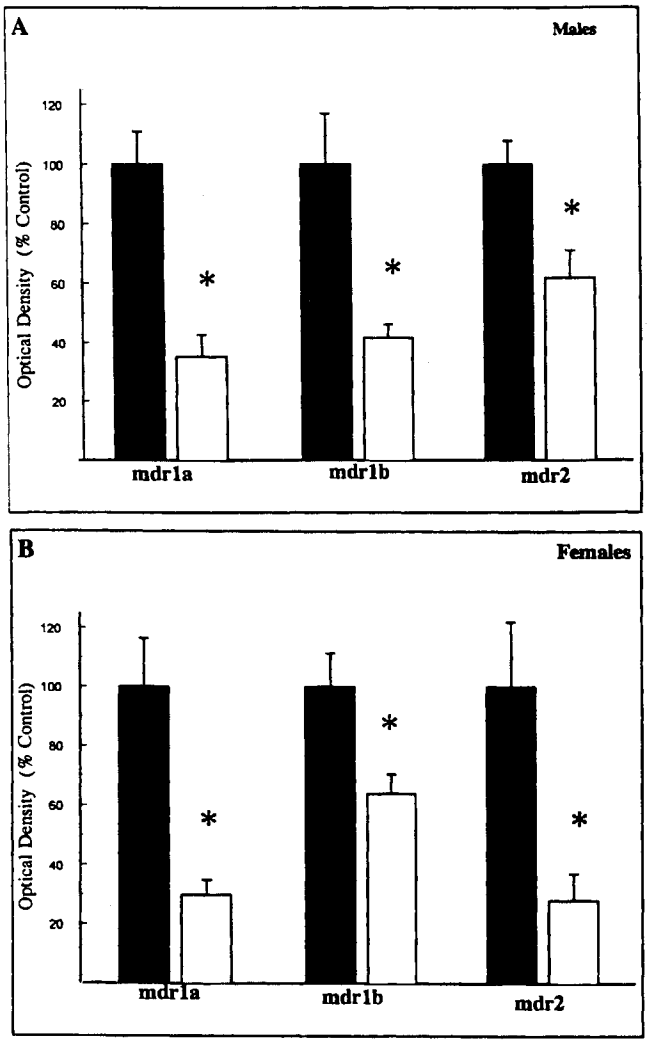

Fig. 5. Effect of experimentally-induced inflammation on MDR mRNA levels. Livers were isolated at 48 hr from control (■) or turpentine-treated (□) male (A) or female (B) rats. Total RNA samples (20 μg) were hybridized to ^{32}P-MDR probes as described. Levels of MDR mRNA were estimated from densitometric analysis of autoradiographs and normalized to GAPDH mRNA. Data (mean ± SEM) are presented as % control, n=5–7. *Significantly different from controls.

male LPS treated rats. Although comparatively depressed by induction of AI, the efflux of R123 was slightly but significantly greater in male as compared to female hepatocytes. Western blot analysis indicated that levels of immunodetectable P-gp were also lower in hepatocytes isolated from treated as compared to control rats (data not shown).

It should be noted that the 45–65% reduction in P-gp activity found in rats treated with turpentine corresponds to the 50–70% decrease in MDR protein expression and the 40–70% decline in MDR1 mRNA levels.

DISCUSSION

This study demonstrates that experimentally-induced inflammation stimulates a reduction in the *in vivo* expression of P-gp in rat liver, both at protein and mRNA levels. Furthermore, this inflammation-induced decline in P-gp expression corresponds to a decrease in the functional activity of the protein

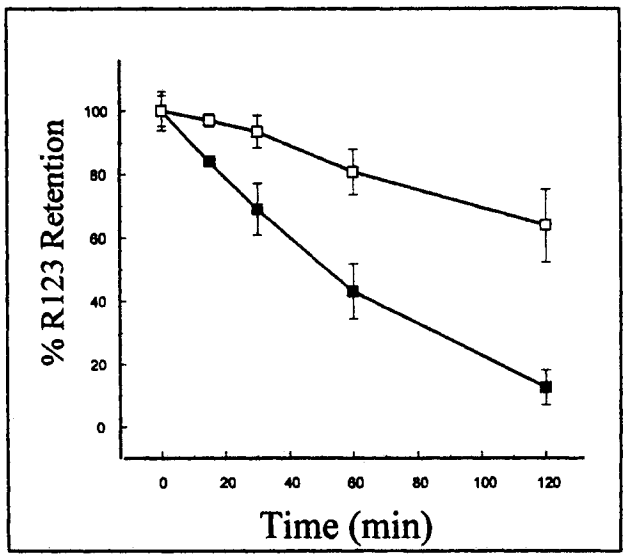

Fig. 6. Rhodamine 123 efflux from isolated hepatocytes. Efflux of R123 was measured from male control hepatocytes that were preloaded for 30 minutes with the fluorescent dye and thereafter incubated in dye-free buffer in the presence (–□–) or absence (–■–) of verapamil (20 μg/ml). Data (Mean ± SD, n=3) are expressed as percentage of values obtained immediately after preloading R123 (time 0).

in liver. This novel finding of an *in vivo* down regulation of P-gp is important in two distinctly different ways. First, it indicates that during inflammation, changes in membrane bound transporters such as P-gp may play a role or contribute to reported inflammation-induced alterations in drug disposition. Indeed, mdr1 knockout mice display increased brain penetration and reduced elimination of many P-gp substrates such as

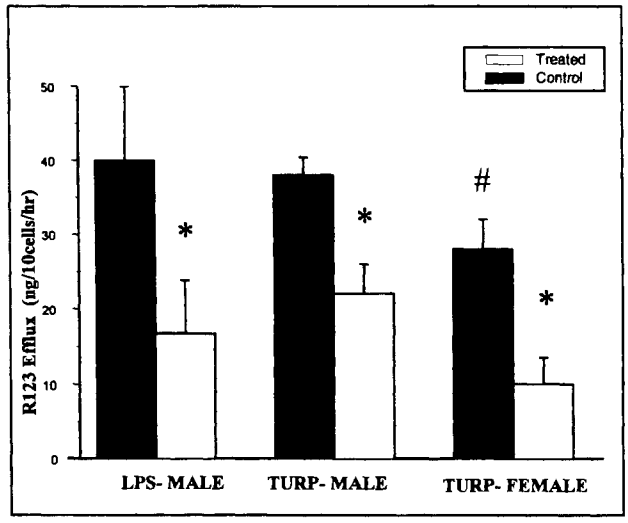

Fig. 7. Influence of inflammation on the P-glycoprotein mediated efflux of rhodamine-123 (R123). The 1hr efflux of R123 was measured in the presence and absence of verapamil in hepatocytes isolated from endotoxin (LPS)-treated, turpentine-treated and control rats at 48 hr. The P-gp mediated efflux of R123 is estimated as the component of efflux which could be inhibited by verapamil. Values (mean±SD) were obtained in triplicate from 3 separate experiments for each group of rats. *Significantly different from controls, # significantly different from males.

digoxin, vinblastin and cyclosporin A (19). Second, the finding that endogenous mediators can cause a prolonged suppression of P-gp protein and MDR mRNA levels has potential usefulness in controlling P-gp-mediated drug resistance of tumor cells.

The mechanism by which experimentally-induced inflammation acts to reduce the expression of P-gp likely occurs through cytokine-mediated pathways. Systemic inflammation perpetrated by LPS injection is associated with the induction of interleukin (IL)-1, IL-6, tumor necrosis factor (TNF) and interferon (INF)-γ whereas turpentine is thought to selectively induce IL-1β and IL-6 (15). The subsequent induction of IL-6 in the liver is thought to mediate transcriptional regulation and altered hepatic protein synthesis of the acute phase proteins in both models of inflammation (4). It has been well demonstrated that downregulation of the cytochrome P450 (P450) genes, seen during inflammation, is primarily mediated by IL-6 and IL-1 (4,5,20). Interestingly, in addition to extensive similarities in substrate specificities, coordinate increases in the expression of the P-gp and P450 gene families are often seen after drug exposure (10,28). Hence it is quite possible that P-gp and P450 have overlapping regulatory mechanisms. Accordingly, IL-6 and/or IL-1 may thus play a role in the regulation of MDR gene expression during turpentine and LPS-induced inflammation. Recently Trauner *et al.* (1997) found that injection of LPS derived from *S. typhimurium* to male Sprague Dawley rats resulted in a downregulation in the hepatic expression of the multidrug resistance protein, Mrp2, but not P-gp (21). As patterns of cytokine induction and endotoxic properties often differ after treatment with LPS obtained from alternate bacterial strains (22) this may, in part, be responsible for the discrepancy between our findings. Nevertheless, a cytokine-mediated downregulation of MDR/P-gp expression is supported by *in vitro* studies which demonstrate a reduction in the mdr1 gene expression of human colon carcinoma cells after incubation with INF-γ and TNF-α (23). Furthermore, involvement of P-gp in the cellular secretion of several of the proinflammatory cytokines has been recently demonstrated (27).

Inflammation-induced alterations in the expression of the MDR gene products were found to occur at the level of mRNA (figure 5). As AI-induced changes in hepatic synthesis of the acute phase proteins is primarily mediated via altered gene transcription (4,13), our data may suggest a reduction in transcription of the MDR gene family. Recently, potential binding sites for members of the C/EBP family of transcription factors have been identified on the untranslated promoter regions of the MDR genes in human, mice and hamsters (24,25). The C\EBP family of transcription factors are involved in the regulation of the acute phase proteins after exposure to inflammatory stimuli (4). Thus transcription control through the C\EBP family may provide a possible cellular signalling pathway by which inflammatory mediators suppress P-gp expression. On the other hand, diminished mRNA levels could also result from a reduction in mRNA stability. Lee *et al.* (26) have demonstrated that enhanced mRNA stability is an important factor contributing to the xenobiotic-stimulated overexpression of P-gp in cultured rat hepatocytes. However, whether this mechanism is involved in MDR downregulation has not been established.

The susceptibility to inflammation induced changes in P-gp expression and activity was not found to be gender specific. Furthermore, the overall changes in protein and mRNA expression of the MDR genes were relatively predictive of associated changes in transport function. However, minor differences in the extent of reduction in P-gp/MDR activity and expression were observed between turpentine-treated male and female rats. Hence, the possibility of post-translational changes in P-gp expression during AI exists. However, it is important to note that immunodetection with C-219 measures both the mdr1 (P-gp) and mdr2 (phospholipid transporter) gene products and levels of both gene products may be altered during an AI. Indeed, our data indicates that mRNA levels of mdr2 are reduced after turpentine-treatment, particularly in female livers. Furthermore, similar to previous reports (29), females were found to express higher levels of mdr2 mRNA than males in the liver. Thus, minor differences in the gender response to AI likely stems from both constitutive differences in MDR/P-gp expression and cross-reactivity of immunodetection methods.

In conclusion, our data demonstrates that induction of AI in rats reduces the hepatic expression and activity of P-gp *in vivo* at the level of mRNA. This may indicate that endogenous mediators released in response to inflammatory stimuli are capable of suppressing MDR expression and activity. P-gp confers an important role in drug therapy in two ways: 1) transport activity in healthy tissues plays an important role in drug secretion and elimination and 2) the over expression of P-gp in cancer cells confers drug resistance. Although these studies have been conducted in the liver, it will be of interest to examine if other tissues are similarily affected. Nevertheless, data demonstrating reductions in P-gp/ MDR expression have seldom been reported. Clearly, characterization of the molecular mechanisms and cell signalling pathways for MDR gene suppression is essential for our understanding of these proteins. Hence, acute inflammation may be useful as a model system to dissect these mechanisms. The importance of further investigations are also emphasized as exploitation of down regulatory pathways could become useful in overcoming P-gp mediated drug resistance.

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