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Title: ER Stress due to Altered Cellular Redox Status Positively Regulates Murine Hepatic CYP2A5 Expression

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Abstract

Murine hepatic cytochrome P450 2A5 (CYP2A5) is uniquely induced by a variety of agents that cause liver injury and inflammation, conditions that are typically associated with down-regulation of CYPs. We hypothesized that induction of CYP2A5 occurs in response to hepatocellular damage resulting in endoplasmic reticulum (ER) stress. Treatment of mice *in vivo* and mouse hepatocytes in primary culture with the CYP2A5 inducer pyrazole resulted in overexpression of the ER stress biomarker glucose-regulated protein (GRP) 78. Treatment of primary hepatocytes with ER stress activators thapsigargin, tunicamycin, *trans*-4,5-dihydroxy-1,2-dithiane (DTT_{ox}) and the calcium ionophore A23187 resulted in elevated GRP78 mRNA levels, however, only the reducing agent DTT_{ox} induced levels of CYP2A5 mRNA, protein and coumarin hydroxylase activity. To test the hypothesis that CYP2A5 induction is a due to liver injury resulting from altered cellular redox status we demonstrated that CYP2A5 induction, elevated serum ALT and oxidative protein damage occur concurrently in pyrazole-treated mice. Pyrazole also induced the expression of cytosolic α and μ class glutathione S-transferase expression both *in vivo* and in primary mouse hepatocytes. Moreover, treatment of hepatocytes with the redox cycling quinone menadione resulted in overexpression of CYP2A5 and GSTM1 mRNA. Finally, pretreatment of hepatocytes with the antioxidants N-acetylcysteine and vitamin E attenuated pyrazole-mediated increases in CYP2A5 mRNA levels. These findings clearly indicate that induction of mouse hepatic CYP2A5 during liver injury occurs via a novel mechanism involving ER stress due to altered cellular redox status.

Cytochrome P450 2a5 (CYP2A5) is induced by a diverse array of hepatotoxins including nitrogen heterocycles (Kojo et al., 1998), chlorinated hydrocarbons (Camus-Randon et al., 1996), metal ions (Hahnemann et al., 1992), and porphyrinogenic agents (Donato et al., 2000) which are neither structurally similar nor generally considered to be inducers of CYPs. Moreover, CYP2A5 is induced during viral (Kirby et al., 1994a), fulminant (Chemin et al., 1996), bacterial (Sipowicz et al., 1997), and parasitic hepatitis (Kirby et al., 1994b) pathophysiological conditions that are usually associated with suppression of CYPs. Most studies to date have focused on identifying specific inducers of CYP2A5, however, it is increasingly apparent that induction is not directly related to the nature of the inducing agents but may be an indirect consequence of a specific cellular event associated with the pathogenesis of liver injury (Camus-Randon et al., 1996).

Cell injury initiates a number of stress cascades intended to restore cellular homeostasis. In the ER, folding of nascent polypeptides and processing of proteins is facilitated by elevated Ca^{2+} concentrations, a mild oxidizing environment that allow post-transcriptional modifications of proteins such as disulphide bond formation, and N-linked glycosylation prior to protein exportation out of the ER. Alterations in ER homeostasis trigger the onset of adaptive ER stress responses characterized by induction of glucose-regulated proteins (GRPs) (Little et al., 1994), a class of molecular chaperones with similar functions to their cytosolic counterparts, the heat shock proteins (HSPs). ER stress and GRP induction can be stimulated in cultured cells by a number of different "proteotoxic" conditions such as (a) inhibition of glycosylation (Liu et al., 1997), (b) depletion of ER calcium levels (Lodish and Kong, 1990), (c) disruption of disulfide bond formation by altering redox status of the ER lumen (Benedetti et al., 2000) and (d) inhibition of protein export out of the ER (Price et al., 1992).

We previously demonstrated that of overexpression GRP78 and CYP2A5 colocalizes in injured hepatocytes of mice treated with the hepatotoxin pyrazole suggesting that CYP2A5 induction is associated with organelle-specific damage to the ER (Gilmore et al., 2003). However, it is unclear if ER stress *per se* is responsible for CYP2A5 induction during liver injury. In the present study, we hypothesized that induction of CYP2A5 by pyrazole is a direct consequence of ER damage, dysfunction and stress. Our results indicate that pyrazole has the capacity to cause ER stress in mouse hepatocytes both in culture and *in vivo*. However, of the various agents that elicit ER stress in cultured mouse hepatocytes only the DTT_{ox} caused CYP2A5 induction suggesting that altered cellular redox status is involved in CYP2A5 regulation. This notion was supported by the finding that pyrazole causes protein oxidation and induction of oxidant stress-responsive glutathione S-transferases *in vivo*. Moreover, menadione induces CYP2A5 and the anti-oxidants vitamin E and N-acetylcysteine prevent pyrazole-mediated induction of CYP2A5 further supporting this hypothesis. Thus, the unique induction of CYP2A5 during liver injury is due to a novel regulatory mechanism for CYPs that involves ER stress resulting from altered redox equilibrium. This finding will contribute considerably to our understanding of the role of CYP2A5 in cellular and molecular responses to stress.

Methods

Materials- Hepatocyte-qualified collagenase, and TRIzol™ reagent were obtained from Invitrogen Canada Ltd. (Burlington, ON) and Somnotol® was obtained form MTC Pharmaceuticals Ltd. (Cambridge, ON). Immobilon™-P membrane was obtained from the Millipore Co. (Bedford, MA). Immobiline™, Rediprime II™, Hybond-N™, redivue [α-^{32}P] dCTP, and ECL™ were obtained from Amersham Biosciences Inc. (Baie d'Urfe, QC). All other reagents were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON).

Animal Treatments- Male C57Bl/6 mice approximately 20 g bw were treated with daily intraperitoneal (ip) injections of saline (0.9% sodium chloride) or pyrazole, at 200 mg/kg for 12, 24, 48, and 72 hours for time-dependent experiments, or at 50, 100, and 200 mg/kg for 72 hours in dose-dependent experiments. Mice were euthanized with a single ip injection of 100 mg/kg sodium pentobarbital (0.05 mL/mouse Somnotol®) and serum and liver samples were collected. Serum samples were analyzed for alanine aminotransferase (ALT) levels using an automated serum analyzer (Reflotron®, Boehringer-Mannheim).

Northern Blot Analysis- Total mRNA was isolated from whole liver or hepatocytes using TRIzol™ reagent. A total of 10 µg of RNA was separated on a 0.8% (w/v) agarose-formaldehyde gel, capillary transferred and UV cross-linked onto Hybond-N™ nylon membranes. Membranes were then hybridized with cDNA probes that were labeled with [α-^{32}P] dCTP (specific activity 3000 µCi/mmol) using the Rediprime II™ random primer DNA labeling kit, (Amersham Biosciences Inc.). The following cDNA probes were provided as generous gifts: murine CYP2A5 cDNA was provided by Dr. M. Negishi, (National Institutes of Health, Research Triangle Park, NC), hamster

GRP78 was provided by Dr. A. Lee, (USC School of Medicine/Norris Cancer Centre, Los Angeles, CA), murine GST M1 was provided by Dr. Alan Townsend (Wake Forest University School of Medicine, Winston-Salem, NC), and the internal load control 7S, was provided by Dr. A. Balmain, (Onyx Pharmaceuticals, Richmond, CA). Hybridizing and washing conditions were preformed as previously outlined (Gilmore et al., 2003). Visualization was performed by phosphor-imaging using a GS-250 Molecular Imager® with Molecular Analyst™ software (Bio-Rad Laboratories Inc., Mississauga, ON).

Enzyme Activity Assays- Microsome and cytosolic extracts were prepared by differential centrifugation as previously described (Kirby et al., 1993). Microsomal CYP2A5 activity was determined by assessing 7-hydroxylase activity towards coumarin (100 μM) as originally described by Aitio (Aitio, 1978). Cytosolic GST μ activity towards the substrate DCNB was determined according to the procedure outlined by Habig et al (Habig et al., 1974).

Primary Mouse Hepatocyte Isolation and Culture- Primary mouse hepatocytes were isolated from adult male C57Bl/6 mice by a adaptation of a 2-step retrograde collagenase perfusion technique (Wollenberg et al., 1987). In brief, mice were anaesthetized by an ip injection of 100 mg/kg pentabarbital. The caudal vena cava was catheterized and the liver was then perfused with Hanks' balanced salt solution pH 7.4 (without Ca^{2+}, Mg^{2+}, $HC0_3^-$ and phenol red) containing: 1 mM EGTA, and 10 mM HEPES for approximately 3 minutes and then with hepatocyte-qualified collagenase (0.3 mg/mL) in William's E medium pH 7.4 supplemented with 10 mM HEPES and 0.1 mg/mL albumin for approximately 8 minutes. The liver was removed and the dissociated hepatocytes were rinsed and resuspended in attachment medium that contained: William's E medium pH 7.4, 10 mM

HEPES, 20 Units/L insulin, 2% (v/v) Penicillin/Streptomycin, and 10% (v/v) FBS. Following a two-hour culture period in a humidified atmosphere of 5% CO_2 at 37^{o}C, the media was changed to serum-free William's E medium pH 7.4 supplemented with 10 mM HEPES, 20 Units/L insulin, 2% (v/v) Penicillin/Streptomycin, 10 mM sodium pyruvate, and 0.35 mM L-proline. The media was then supplemented with various treatments as described in the figure legends.

2-Dimensional Gel Electrophoresis- Cytosolic extracts were separated by 2-dimensional (2-D) gel electrophoresis as described previously (Barker et al., 2002). Briefly, 50 µg of cytosolic extracts were separated by isolectric focusing on a 13 cm pre-cast Immobiline™ dry strip with an immobilized linear pH gradient ranging from 3-10 using the IPGphor IEF system (Amersham Biosciences Inc.). Focused strips were separated by SDS-PAGE on a 12% gel and proteins were then visualized by silver staining.

Western blot- Hepatic CYP2A5, GRP78 and GST µ proteins were identified by western blot analysis as previously described (Gilmore et al., 2003). In brief, 50 µg of microsomal protein (CYP2A5, GRP78) or 5 µg of cytosolic protein (GST µ) were separated by SDS-PAGE and transferred to Immobilon™-P membranes. Membranes were blocked in 5% (w/v) powdered low fat skim milk in tris-buffered saline with 0.5% Tween-20 (T-TBS) for 1 hour at room temperature. Membranes were then incubated for 1 hour with either chicken anti-mouse CYP2A5 antibody (1:10000) provided by Dr. H. Raunio (University of Kuopio, Kuopio, Finland), rabbit anti-mouse GRP78 (1:2000) (Stressgen Biotechnologies, Victoria, BC), or rabbit anti-human GST µ (1:2000) antiserum provided by Dr. Theo Bammler (University of Washington, Seattle, WA) in T-TBS. This was followed by a 1 hour incubation with either rabbit anti-chicken (1:10000) or goat anti-rabbit

(1:2000) peroxidase secondary antibodies. Chemiluminescence detection was performed by the ECL™ western blotting method (Amersham Biosciences Inc).

Quantification of Protein Carbonyl Content- Protein carbonyls were quantified using an adaptation of a previously described procedure (Andrus et al., 1998). In brief, proteins from cytosolic extracts (50 µg) were derivatized in 10 mM DNPH and 1 µg of derivatized proteins was blotted onto an Immobilon™-P membrane, using a slot blot apparatus. Membranes were blocked in 3% (w/v) powdered low fat skim milk in T-TBS for 1 hour at room temperature. Membranes were then incubated with rabbit anti-DNP antiserum (1:8000) (Sigma-Aldrich Ltd), followed by a goat anti-rabbit peroxidase IgG secondary antibody (Sigma-Aldrich Ltd.) diluted 1:20000 in T-TBS for 1 hour. Chemiluminescence detection was preformed as described above and protein loading was assessed by amido black staining of membranes (Sigma-Aldrich Ltd.).

Statistical Analysis- Data were presented as means +/- SE and analyzed by one-way analysis of variance (ANOVA). When differences were detected by ANOVA, the means of the groups were then compared by Fisher's LSD test. Results were considered to be significant at $p<0.05$ unless stated otherwise in figure legends.

Results

Pyrazole induces ER stress in mouse liver and in cultured primary mouse hepatocytes

Our previous immunohistochemistry studies suggested that induction of CYP2A5 occurs in hepatocytes during ER stress. To further test this hypothesis we evaluated the capacity of pyrazole to elicit ER stress in mouse liver as well as in mouse hepatocytes in primary culture. Treatment of mice with pyrazole (200 mg/kg) *in vivo* resulted in increased levels of GRP78 mRNA and protein that were 2.0 ($p< 0.05$) and 1.6 ($p< 0.01$) fold higher than controls respectively (Fig. 1). Similarly, exposure of primary mouse hepatocytes to 20 mM pyrazole resulted in GRP78 mRNA and protein levels that were elevated 1.7 ($p< 0.01$) and 1.9 ($p< 0.01$) fold compared to untreated cells.

Stimulation of ER stress by DTT_{ox} induces CYP2A5 expression in primary mouse hepatocytes

To determine whether a direct relationship exists between the development of ER stress and CYP2A5 induction, we hypothesized that CYP2A5 induction occurs in cultured hepatocytes undergoing ER stress stimulated by different mechanisms. Thus, hepatocytes were treated with the following ER stress stimuli: thapsigargin (Ca^{2+} -ATPase inhibitor), tunicamycin (N-glycosidation inhibitor), *trans*-4,5-dihydroxy-1,2-dithiane [DTT_{ox}] (reducing agent) and A23187 (calcium ionophore). All treatments caused ER stress as indicated by increases in GRP78 protein levels (Fig 2A). However, only DTT_{ox} resulted in a statistically significant increase in CYP2A5 expression as indicated by mRNA, protein and COH activity levels that were 1.8 ($p < 0.001$), 2.9 ($p < 0.001$) and 2.6 ($p < 0.001$) fold higher than untreated controls respectively (Fig 2B, C and D). All other ER stress stimuli caused statistically significant suppression of CYP2A5 mRNA levels ($p<0.001$).

Oxidative damage occurs concurrently with pyrazole-mediated CYP2A5 induction in mouse liver

Induction of CYP2A5 by DTT_{ox} suggests that changes in cellular redox equilibrium rather than ER stress *per se* may be a critical regulatory factor in *Cyp2a5* gene regulation. To test this hypothesis we assessed the level of oxidative damage and CYP2A5 expression in pyrazole-treated mouse liver. Predictably, pyrazole resulted in a dose-and time-dependent induction of hepatic CYP2A5 expression that was evident at the level of mRNA, protein and coumarin 7-hydroxylase (COH) activity (Table 1). Increases in CYP2A5 expression were most pronounced following treatment with the highest dose of pyrazole (200 mg/kg) for 72 hours resulting in CYP2A5 mRNA, protein and COH activity levels that were 7.3 ± 0.5, 5.8 ± 0.4, and 13.6 ± 1.5 fold higher than saline-treated controls respectively. Serum ALT levels were increased significantly 18.9 ± 6.9 fold ($p<0.05$) at the highest dose of pyrazole, 72 hours after treatment. To determine whether pyrazole-mediated liver injury is associated with oxidative stress, protein carbonyl levels were measured in liver cytosol as an indicator of ROS-mediated oxidative protein damage (Andrus *et al.*, 1998). The formation of protein carbonyls increased in a time-dependent manner, however, only the highest dose of pyrazole resulted in levels that were statistically higher than controls (1.7 ± 0.1 fold, $p<0.05$).

Pyrazole induces GST expression in mouse liver

To further investigate whether pyrazole elicits oxidative stress in mouse liver, we compared the expression of hepatic cytosolic proteins from untreated and pyrazole-treated mice by 2-D SDS-PAGE. Figure 3 demonstrates that pyrazole produces marked overexpression of two cytosolic proteins with molecular weights ranging from 26 to 29 kDa and isoelectric points ranging from 7.8 to 8.5 pI. One overexpressed protein (denoted by a square) had 100% amino acid sequence

homology with murine GST M1 as determined by N-terminal amino acid sequencing. A second overexpressed protein (denoted by a circle) was identified as GST α by immunoblot analysis (data not shown). We further confirmed pyrazole-mediated GST μ induction by demonstrating time-dependent increases ($p<0.05$) in GSTM1 mRNA, GST μ protein and DCNB activity (Table 2). Dose-response experiments revealed that statistically significant increases of GST mRNA and DCNB activity levels were maximal at a dose of 50 mg/kg pyrazole and remained elevated at higher doses.

Menadione induces CYP2A5 and GSTM1 mRNA levels in cultured primary mouse hepatocytes

The association of CYP2A5 overexpression with oxidative stress lead us to hypothesize that other pro-oxidants are capable of inducing CYP2A5. Treatment of mouse hepatocytes with menadione caused dose-dependent increases in CYP2A5 and GSTM1 transcripts, reaching levels that were 2.0 ± 0.3 ($p < 0.05$) and 2.2 ± 0.1 ($p < 0.05$) fold higher than controls respectively at a dose of 20 μM menadione (Table 3). Moreover, menadione resulted in time-dependent increases in GSTM1 and CYP2A5 mRNA levels that were statistically significant ($p< 0.05$) at 24 and 72 hours respectively (Figure 4A and B).

Antioxidants inhibit pyrazole-mediated induction of CYP2A5 in primary mouse hepatocytes.

To further test the hypothesis that oxidative stress stimulated by pyrazole treatment plays a role in CYP2A5 induction, primary mouse hepatocytes were cultured with two antioxidants N-acetylcysteine and vitamin E prior to pyrazole treatment. While CYP2A5 mRNA levels were increased 1.7 fold when hepatocytes were cultured for 24 hours with 20 mM pyrazole, pre-

treatment with both N-acetylcysteine (4 mM) and vitamin E (25 mM) for 24 hours abrogated the CYP2A5 induction by pyrazole ($p < 0.01$) (Figure 5).

Discussion

Murine hepatic CYP2A5 is uniquely induced by a diverse array of hepatotoxins and infectious agents that cause hepatitis and hepatocellular damage. The molecular mechanisms responsible for up-regulation of *cyp2a5* gene expression have not been characterized, however, our previous studies suggest that induction of CYP2A5 may be a consequence of liver injury targeted towards the ER (Gilmore et al., 2003). The objective of this study was to determine whether ER stress is sufficient to induce CYP2A5 and to identify the specific ER stress stimuli that cause CYP2A5 overexpression. Our results indicate that CYP2A5 is induced during ER stress stimulated by alterations in cellular redox status indicating a novel mechanism in CYP regulation.

The relationship between pyrazole treatment, hepatocellular injury and CYP2A5 induction demonstrated in this study is consistent with observations in other mouse models of hepatotoxicity (Camus-Randon et al., 1996) and hepatitis (Chemin et al., 1996). Several investigators have shown that CYP2A5 induction occurs during the early stages of hepatocellular injury and is not associated with terminal forms of injury such as necrosis (Arvela et al., 1991) or apoptosis (Tetri et al., 2002). This suggests that CYP2A5 induction during hepatocellular injury may be triggered by an early stress stimulus or may be associated with an early adaptive response to cellular stress. Indeed, the fact that pyrazole causes ER stress in mouse liver as well as in primary mouse hepatocytes is consistent with this concept. Previous studies in hepatitis B virus (HBV) transgenic mice also suggest that ER stress plays an important role in CYP2A5 induction. In these mice, long filamentous strands of HBV large surface polypeptide (HBsAg) accumulate within the ER causing a localized chronic inflammatory response that is associated with overexpression of hepatic CYP2A5 (Kirby et al., 1994a). Lineages that accumulate less HbsAg do not develop liver injury and show only minor changes in CYP2A5 levels (Chemin et al., 1996). Moreover, accumulation of

HBsAg within the ER of hepatocytes is associated with the development of an ER stress response (Xu et al., 1997). Collectively, this suggests that CYP2A5 induction may be directly related to either ER damage or to the ER stress-initiating stimulus.

To investigate the role of the ER stress stimulus in *cyp2a5* gene regulation we induced ER stress in primary mouse hepatocytes by different stimulus pathways. Interestingly, of the various agents that stimulated ER stress only DTT_{ox} induced CYP2A5 expression. Conversely, thapsigargin, tunicamycin, and A23187 all resulted in suppression of CYP2A5 mRNA levels. This suggests that ER stress *per se* is not a common pathophysiological factor involved in CYP2A5 regulation. Clearly, marked induction of CYP2A5 by DTT_{ox} suggests that either this compound or the associated cellular responses play a key regulatory role in controlling CYP2A5 expression. The nature of DTT_{ox} metabolism within hepatocytes lead us to believe that changes in cellular redox equilibrium may be of central importance. Disulfide reduction of DTT_{ox} to DTT by GSH and/or by NAD(P)H-dependent oxidoreductase systems drives the redox potential of the ER towards a more reducing environment. This alteration in the ER redox status results in disruption of protein disulfide bond formation and protein folding which in turn induces ER stress (Halleck et al., 1997). DTT_{ox} evidently alters redox status specifically in the ER since only GRP78 is induced and not its cytosolic counterpart HSP70 (Halleck et al., 1997).

Our data indicate that the relationship between CYP2A5 expression and altered redox status involves both reductive stress and oxidative stress as has been previously reported for *gadd*153 and thioredoxin (TRX2) (Luethy et al., 1990; Trotter and Grant, 2002). This conclusion is based on several lines of evidence: a) DTTox induces CYP2A5 expression in primary mouse hepatocytes. In their studies of ER stress in renal epithelial cells (LLC-PK1), Stevens et al. showed, that the molecular response to DTTox resembles reductive stress induced by DTT (Halleck et al., 1997).

For example, reduction of DTTox in LLC-PK1 cells correlated with induction of grp78 expression. Moreover, DTTox had no effect on overall levels of GSH and NADPH indicating that cells were not undergoing oxidative stress. b) Pyrazole increases protein carbonyl content in mouse liver *in vivo*. During oxidative stress, interaction of ROS and lipid peroxides with proteins results in formation of carbonyl groups on amino acid side chains, a frequently used marker of oxidative stress (Berlett and Stadtman, 1997). While the details of metabolism are unclear, pyrazole like other nitrogen-heterocycles likely undergoes redox cycling causing oxidative stress via depletion of cellular GSH and NAD(P)H levels (Biaglow et al., 1986). c) Pyrazole induces α and μ class GSTs. In response to oxidative stress, induction of GSTs facilitate the nucleophilic addition of GSH to endogenous and exogenous electrophilic compounds including lipid hydroperoxides (Hayes and Pulford, 1995). d) Menadione induces CYP2A5 expression in mouse hepatocytes in primary culture. Menadione is a redox cycling quinone that consumes critical cellular thiols including GSH thereby causing oxidative damage (Bellomo et al., 1987). e) Vitamin E and N-acetyl cysteine abrogate pyrazole-mediated induction of CYP2A5 in primary mouse hepatocytes. The capacity of these antioxidants to protect hepatocytes from pro-oxidant challenges has been well established (Milchak and Douglas Bricker, 2002). These findings support our previous demonstration that pyrazole-mediated induction of CYP2A5 *in vivo* is prevented by vitamin E pretreatment and that reduction of cellular glutathione levels by buthionine sulfoximine also results in CYP2A5 overexpression (Gilmore et al., 2003).

The choice of biomarkers of oxidative stress is difficult as biomarkers may reflect different biological endpoints ranging from changes in gene expression to cellular injury and death. While evaluation of protein carbonyl formation is widely accepted as an indicator of oxidative injury, this may not necessarily reflect the degree of oxidative stress that influences gene expression. It has

previously been shown that changes in levels of protein carbonyl formation are proportional to the dose of pro-oxidant and are detected at tissue sites where free-radial attacks occur (Kohen and Nyska, 2002). Our previous immunohistochemistry studies have shown that induction of hepatic CYP2A5 by pyrazole occurs in a centrilobular distrubution (Gilmore et al., 2003). Evaluation of protein carbonyl levels by Western blot analysis may be less sensitive than by immunohistochemistry as the proportion of hepatocytes affected by oxidative stress is relatively small in relation to the whole liver and affected cells are diluted during the preparation of cytosol (Raina et al., 2000). Moreover, the level of protein carbonyl groups reflects a balance between protein carbonyl formation, protein turnover and repair. Taken together, this may explain differences in the dose and temporal relationships of CYP2A5 expression and protein carbonyl formation.

Studies of mouse models of hepatitis have suggested that *Cyp2a5* overexpression may be related to increased ROS production. For example, in livers of mice infected with *Helicobacter hepaticus* CYP2A5 overexpression colocalizes in hepatocytes with increased superoxide ($O_2^{\cdot-}$) levels as indicated by increased formazan staining (Sipowicz et al., 1997). Similarly, CYP2A5 induction occurs in mouse models of hepatitis that have increased levels of 8-hydroxydeoxyguanosine, a marker of in oxidative DNA damage (Hagen et al., 1994). In addition, the concomitant increases in CYP2A5 and specific GSTs suggest that these enzymes might be regulated under similar conditions of oxidative stress (Fernandes et al., 1996; Sipowicz et al., 1997). In hepatitis B virus (HBV) transgenic mice, induction of μ class GST (GST Yb_1) has been reported as part of a compensatory response to oxidative injury (Fernandes et al., 1996). Furthermore, the pro-oxidant sodium arsenite induces CYP2A5 as well as various antioxidant enzymes such as: NAD(P)H:quininone

oxidoreductases, GST Ya and hemoxygenase-1 (Seubert et al., 2002). It has yet to be determined whether induction of CYP2A5 by other hepatotoxins also involves redox-altering mechanisms.

The molecular mechanisms by which disturabances in cellular redox equilibrium control *cyp2a5* gene expression also warrants further detailed investigation. ROS are known to act as mediators of transcription altering eukaryotic gene expression patterns as an adaptive stress response (Dalton et al., 1999). To date, studies have focused on activation of transcription factors associated with immediate-early gene expression, however, post-transcriptional mechanisms of regulation are equally important during cellular responses to oxidative stress. While the promoter region of *cyp2a5* contains a single NFκB and several AP-1 sites, their importance in controlling CYP2A5 transcription remains unclear. Our preliminary experiments suggest that both transcriptional and post-transcriptional mechanisms are involved in controlling *cyp2a5* gene expression when redox status is changed since DTTox and menadione increase luciferase activities in primary mouse hepatocytes transfected with reporter plasmids containing the luciferase gene flanked with either the 5'-UTR or the 3'-UTR of CYP2A5 (Kirby, unpublished data). Previous studies have shown that regulation of CYP2A5 by pyrazole occurs post-transcriptionally via mRNA stabilization (Hahnemann et al., 1992). Electrophoretic mobility shift assays have shown that pyrazole increases binding of the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) to a *cis*-acting element in the 3'-UTR of CYP2A5 mRNA suggesting a key regulatory role (Raffalli-Mathieu et al., 2002). It is unclear whether these mRNA protein interactions are increased in conditions that alter cellular redox status.

In summary, our results indicate that ER stress is not a requisite condition for CYP2A5 induction but that overexpression of CYP2A5 is related to alterations in cellular redox equilibrium, a novel concept in mammalian CYP regulation. Repression of specific CYPs and CYP-mediated

activities during oxidative stress has previously been reported (Barouki and Morel, 2001). Moreover, CYP-dependent activities are suppressed following menadione treatment, re-emphasizing the unique up-regulation of CYP2A5 under these conditions (Galantai et al., 1992). The functional role of CYP2A5 in the hepatic response to oxidant or reductive stress is unclear. Many of the cytoprotective genes activated during oxidative stress are either antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase, that directly interact with ROS or detoxifying genes such as glutathione S-transferases, heme oxygenase-1, thioredoxin and quinone reductase that minimize the adverse effects of toxic by-products of oxidative damage (Morel and Barouki, 1999). While our results suggest that CYP2A5 may be a component of an adaptive response mechanism to altered cellular redox, a specific cytoprotective role remains to be identified for this CYP isoenzyme.

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Footnotes:

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Legends for Figures

Figure 1. Pyrazole stimulates ER stress in mouse liver and primary hepatocytes. The effect of pyrazole on GRP78 mRNA and protein levels was assessed in mouse livers *in vivo* (200 mg/kg) and mouse hepatocytes in primary culture (20 mM) after 24 hours of treatment. Representative northern blots (A) and western blots (B) and densitometric analysis of four independent experiments are presented. Values represent the mean ± SE relative to control levels. Significantly different from control *$p < 0.05$, **$p < 0.01$.

Figure 2. DTT_{ox} induces CYP2A5 in primary mouse hepatocytes. The effect of various ER stress inducers on CYP2A5 expression was assessed at the mRNA, protein and activity levels. Shown are representative Western blots of GRP78 protein (A), Northern and Western blots of Cyp2a5 mRNA (B) and protein (C) as well as coumarin 7-hydroxylase activity (D) from hepatocytes treated for 48 h with 4 μM thapsigargin (Tg), 1.5 μg/mL tunicamycin (Tu), 10 mM DTT_{ox} (DT), or 1.0 μM A23187 (A2). Densitometric analyses of three independent experiments are expressed relative to untreated (UT) hepatocytes. Values represent the mean ± SE. Significantly different from control *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 3. Pyrazole induces expression of cytosolic glutathione transferases in mouse liver.
Cytosolic proteins from livers of mice treated for 24 hours with saline (A) or 200 mg/kg pyrazole (B) were separated by 2-D gel electrophoresis and silver-stained. Overexpression of murine GST α (circle) and GST M1 (square) were identified by immunoblot analysis and N-terminal amino acid sequencing respectively.

Figure 4: Time-dependent increases of Cyp2a5 (Panel A) and GST M1 (Panel B) mRNA levels by menadione and pyrazole in primary mouse hepatocytes. Hepatocytes were treated with either pyrazole (20 mM) or menadione(20 μM) for the indicated times. CYP2A5 and GSTM1 mRNA levels were determined by Northern blot and densitometric analysis. Values represent the mean ± SE (N=3). *Significantly different from control, $p < 0.05$.

Figure 5. Inhibition of pyrazole-mediated Cyp2a5 mRNA induction by antioxidant pre-treatment of primary mouse hepatocytes. Hepatocytes were pre-treated for 24 hours with either N-acetylcysteine (N-Ac, 4 mM) or Vitamin E (Vit-E, 25 μM) prior treatment with pyrazole (20 mM) for 24 hours. CYP2A5 mRNA levels were determined by Northern blot and densitometric analysis. Values represent the mean ± SE (N=3). **Significantly different from control, $p < 0.01$.

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TABLES

Table 1 Pyrazole increases hepatic CYP2A5 levels, serum ALT and hepatic oxidative damage *in vivo*

Dose* (mg/kg)	CYP2A5 mRNA		CYP2A5 Protein		COH Activity		ALT		Protein Carbonyl Formation	
0	[a]1.0	+/- 0.1	[a]1.0	+/- 0.1	[a]1.0	+/- 0.1	[a]1.0	+/- 0.1	[a]1.0	+/- 0.1
50	[b]3.1	+/- 1.2	[b]3.0	+/- 0.4	[b]5.5	+/- 0.9	[a]2.1	+/- 0.7	[a]0.9	+/- 0.0
100	[c]5.4	+/- 0.8	[c]4.3	+/- 0.4	[b]8.7	+/- 1.0	[a]2.7	+/- 0.5	[a]1.1	+/- 0.3
200	[c]7.3	+/- 0.5	[d]5.8	+/- 0.4	[c]13.6	+/- 1.5	[b]18.9	+/- 6.9	[b]1.7	+/- 0.1
Time+ (h)										
0	[a]1.0	+/- 0.1	[a]1.0	+/- 0.2	[a]1.0	+/- 0.1	[a]1.0	+/- 0.1	[a]1.0	+/- 0.1
12	[a]2.2	+/- 0.7	[a]1.7	+/- 0.6	[b]1.5	+/- 0.4	[a]4.5	+/- 1.6	[a,]1.0	+/- 0.1
24	[b]4.9	+/- 0.7	[b]4.2	+/- 0.6	[c]7.3	+/- 1.1	[a]10.7	+/- 2.8	[a,b,c]1.4	+/- 0.1
48	[b]4.5	+/- 0.9	[b]5.7	+/- 0.7	[c,d]10.6	+/- 2.3	[b]20.3	+/- 6.8	[b,c]1.5	+/- 0.3
72	[c]7.3	+/- 0.5	[c]5.8	+/- 0.4	[d]13.6	+/- 1.5	[b]18.9	+/- 6.9	[c]1.7	+/- 0.1

*Mice were euthanized 72 hours after treatment with the indicated doses of pyrazole. +For time course experiments a dose of 200 mg/kg was utilized. Values represent fold increases in expression relative to untreated controls (N=3). Means that are not significantly different are denoted by a common letter designation whereas means with different letter designations are significantly different ($p<0.05$) as determined by analysis of variance.

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Table 2 Pyrazole induces hepatic GST μ levels *in vivo*

Dose (mg/kg)*	GSTM1 mRNA		GST μ Protein		GST μ Activity (DCNB)	
0	[a]1	+/- 0.1	[a]1.0	+/- 0.4	[a]1.0	+/- 0.1
50	[b]4.3	+/-0.7	[b]2.3	+/- 0.4	[b]2.0	+/- 0.2
100	[b]5.4	+/-1.0	[b,c]3.3	+/- 0.3	[b]2.6	+/- 0.2
200	[b]4.8	+/-0.7	[c]5.2	+/- 1.2	[b]2.3	+/- 0.3
Time (h)+						
0	[a]1.0	+/- 0.0	[a]1.0	+/- 0.3	[a]1.0	+/- 0.1
12	[a]1.9	+/-0.6	[a]1.6	+/- 0.5	[a]0.8	+/- 0.0
24	[a,b] 2.6	+/-0.1	[b]2.1	+/- 0.4	[a]1.0	+/- 0.0
48	[b,c]4.4	+/-1.2	[b]3.0	+/- 0.3	[b]1.6	+/- 0.2
72	[c]4.8	+/- 0.7	[c]5.2	+/- 1.2	[c]2.3	+/- 0.3

*Mice were euthanized 72 hours after treatment with the indicated doses of pyrazole. +For time course experiments a dose of 200 mg/kg was utilized. Values represent fold increases in expression relative to untreated controls (N=3). Means that are not significantly different are denoted by a common letter designation whereas means with different letter designations are significantly different ($p<0.05$) as determined by analysis of variance.

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Table 3 Dose-dependent increases of Cyp2a5 and GST M1 mRNA levels by menadione and pyrazole in primary mouse hepatocytes

	Pyrazole				Menadione			
Dose*	CYP2A5		GSTM1		CYP2A5		GSTM1	
0	[a]1.0	+/- 0.0	[a]1.0	+/- 0.0	[a]1.0	+/- 0.0	[a]1.0	+/- 0.1
5	[b]2.3	+/- 0.1	[b]1.6	+/- 0.1	[b]1.6	+/- 0.0	[b]1.4	+/- 0.1
10	[b]2.5	+/- 0.2	[c]2.0	+/- 0.1	[b]1.5	+/- 0.1	[b]1.8	+/- 0.2
20	[c]3.1	+/- 0.1	[c]2.2	+/- 0.1	[c]2.0	+/- 0.3	[c]2.2	+/- 0.2

*Concentrations of pyrazole and menadione were mM and μM respectively for a period of 72 hours. Values represent fold increases in expression relative to untreated controls (N=3). Means that are not significantly different are denoted by a common letter designation whereas means with different letter designations are significantly different ($p<0.05$) as determined by analysis of variance.

Fig. 1

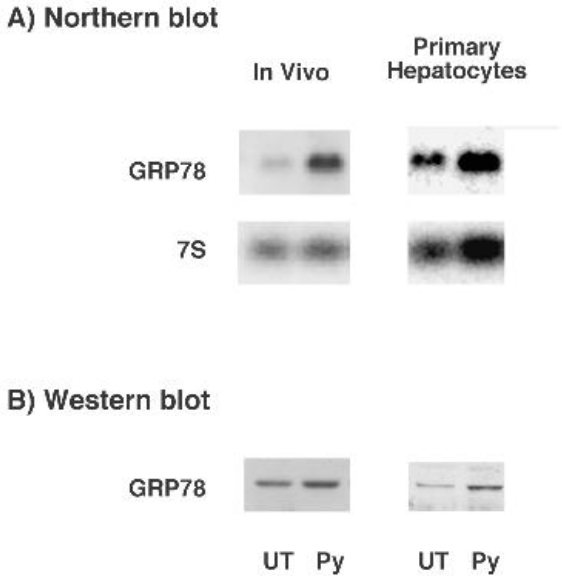

A) Northern blot
In Vivo
Primary Hepatocytes
GRP78
7S
B) Western blot
GRP78
UT
Py
UT
Py
C) Densitometry
mRNA
Protein
Relative Density
3.0
2.5
2.0
1.5
1.0
0.5
0
*
**
**
**
In vivo
Hepatocytes

Fig. 2

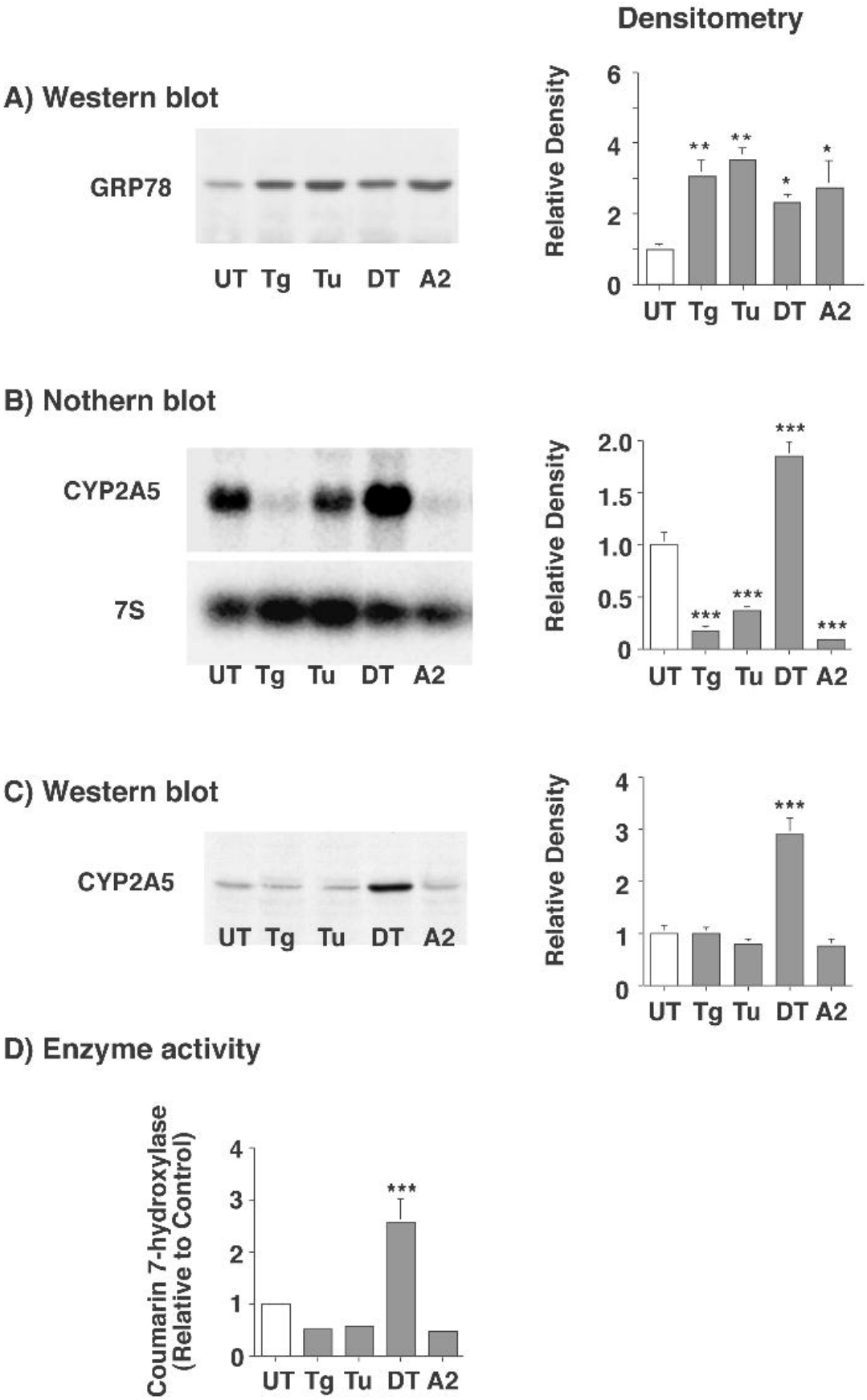

A) Western blot
GRP78
UT Tg Tu DT A2
Densitometry
Relative Density
6
4
2
0
**
**
*
*
UT Tg Tu DT A2
B) Nothern blot
CYP2A5
7S
UT Tg Tu DT A2
Relative Density
2.0
1.5
1.0
0.5
0
***
***
***
***
UT Tg Tu DT A2
C) Western blot
CYP2A5
UT Tg Tu DT A2
Relative Density
4
3
2
1
0
***
UT Tg Tu DT A2
D) Enzyme activity
Coumarin 7-hydroxylase (Relative to Control)
4
3
2
1
0
***
UT Tg Tu DT A2

Fig. 3

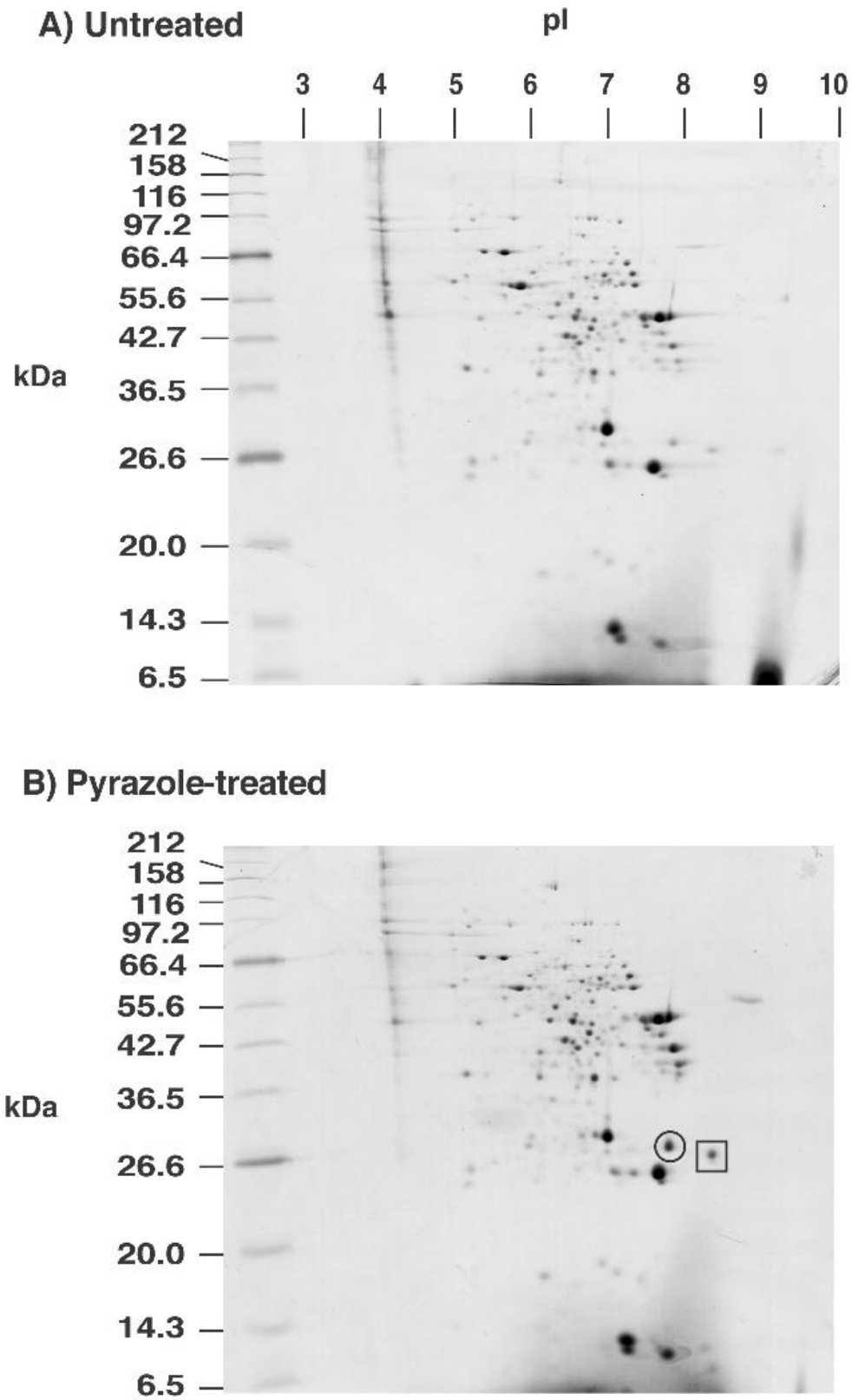

A) Untreated
pI
3
4
5
6
7
8
9
10
212
158
116
97.2
66.4
55.6
42.7
kDa
36.5
26.6
20.0
14.3
6.5
B) Pyrazole-treated
212
158
116
97.2
66.4
55.6
42.7
kDa
36.5
26.6
20.0
14.3
6.5

Fig. 4A

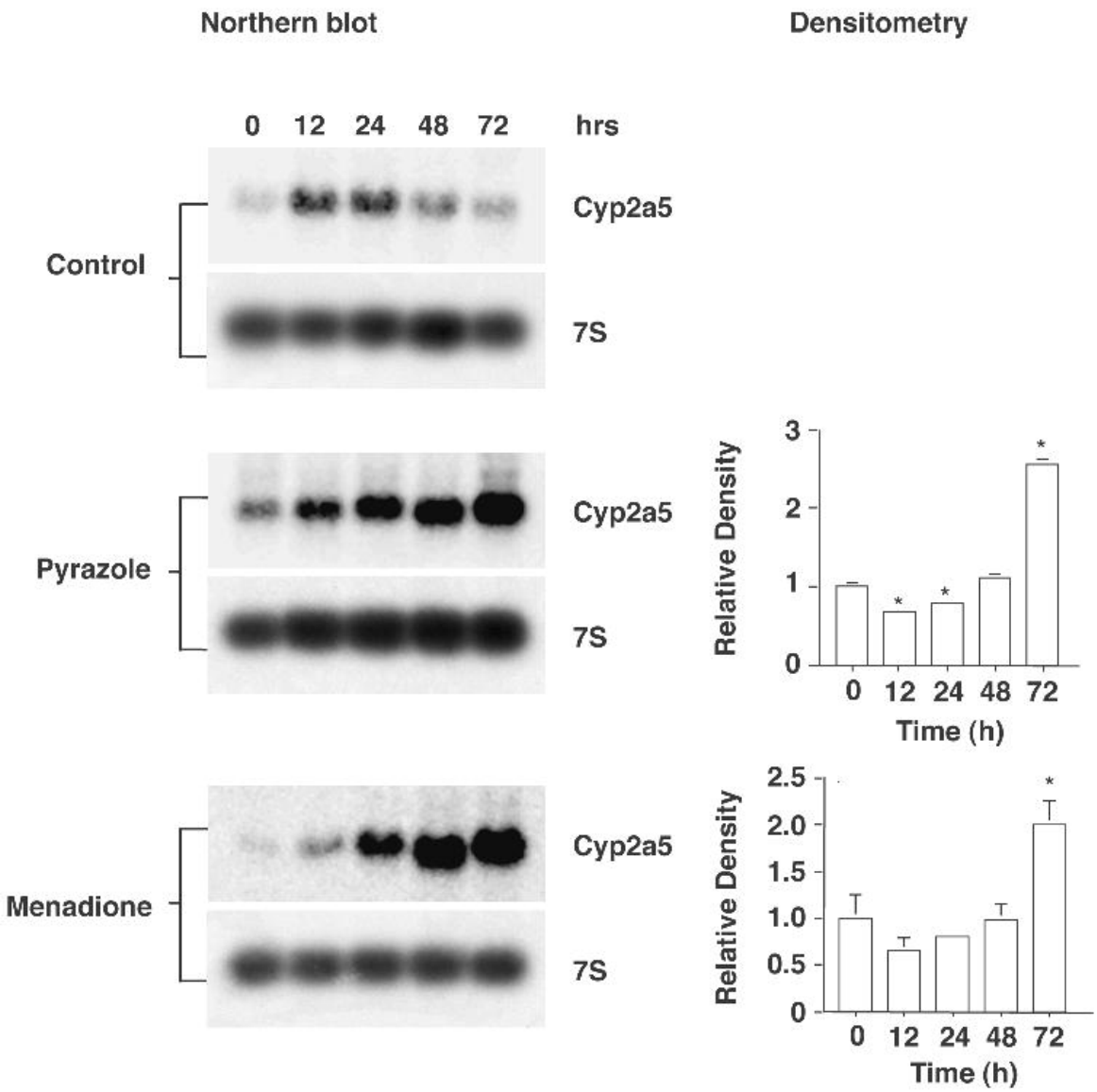

Northern blot
Densitometry
0 12 24 48 72 hrs
Control
Cyp2a5
7S
Pyrazole
Cyp2a5
7S
Menadione
Cyp2a5
7S
Relative Density
3
2
1
0
0 12 24 48 72
Time (h)
Relative Density
2.5
2.0
1.5
1.0
0.5
0
0 12 24 48 72
Time (h)

Fig. 4B

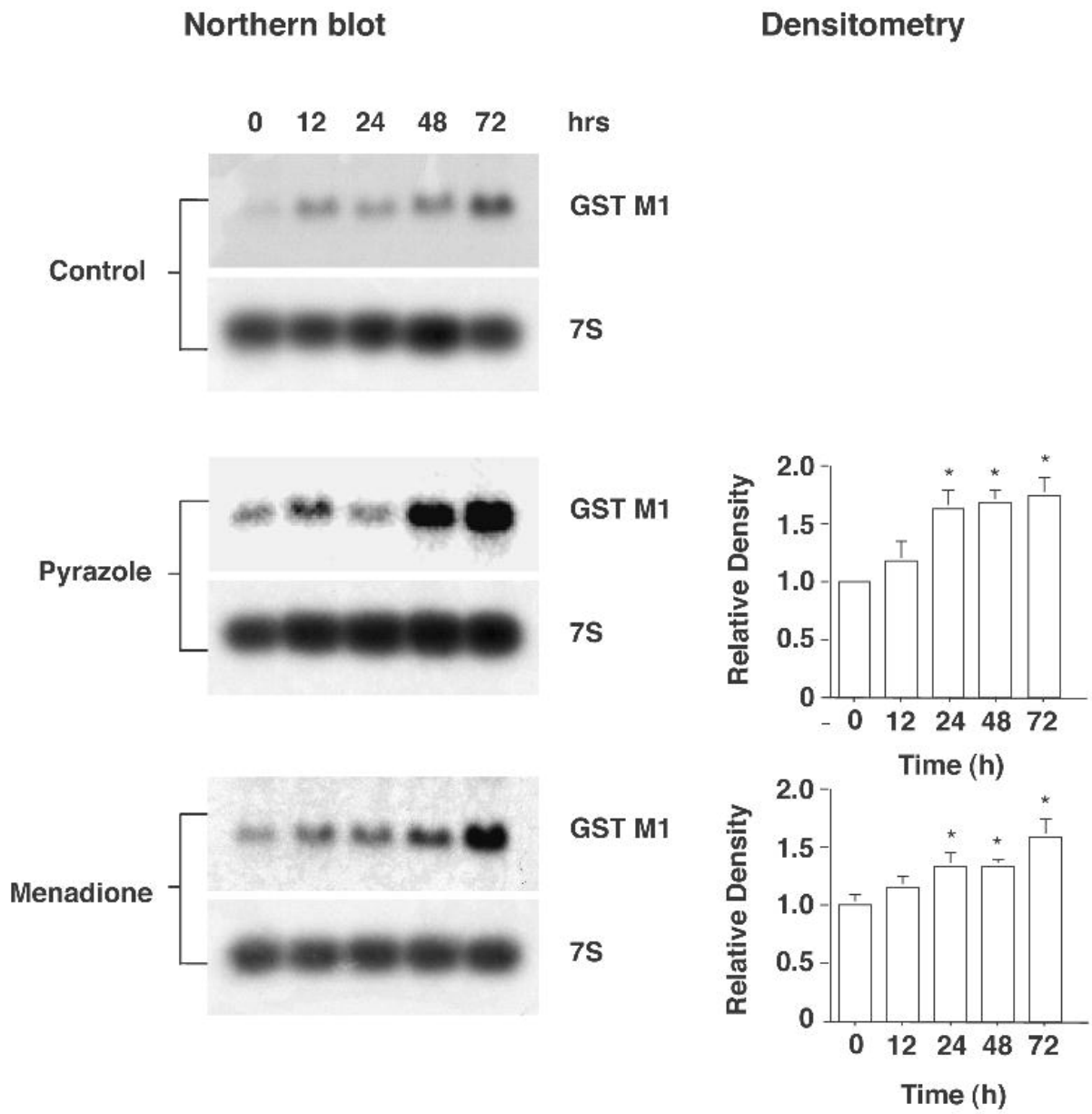
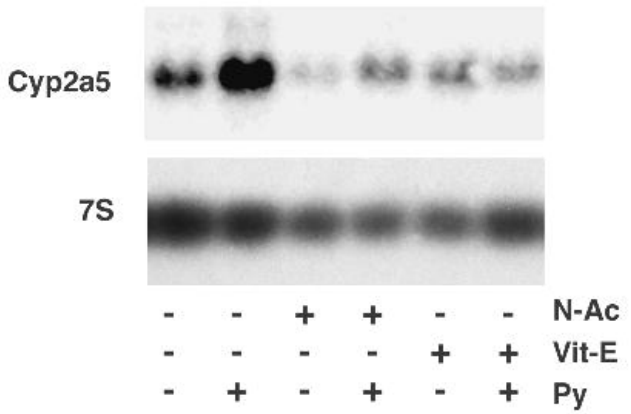

Northern blot
Densitometry
0 12 24 48 72 hrs
Control
GST M1
7S
Pyrazole
GST M1
7S
Menadione
GST M1
7S
Relative Density
2.0
1.5
1.0
0.5
0
0 12 24 48 72
Time (h)
Relative Density
2.0
1.5
1.0
0.5
0
0 12 24 48 72
Time (h)

Fig. 5

A) Northern blot



B) Densitometry