Free Radical Research, April 2010; 44(4): 441–453

Cytochrome P450 induction by phenobarbital exacerbates warm hepatic ischemia-reperfusion injury in rat livers

IMAM H. SHAIK & REZA MEHVAR

Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, Texas, USA

(Received date: 2 October 2009; In revised form date: 22 November 2009)

Abstract

Recent studies have shown that cytochrome P450 inhibitors reduce oxidative stress and injury to the liver following warm ischemia-reperfusion (IR). The aim here was to test the effect of P450 induction by phenobarbital on the IR injury in rat livers. Rats were pre-treated with saline or phenobarbital and subjected to IR or sham operation. IR significantly increased the plasma alanine aminotransferase concentrations. Phenobarbital further exacerbated the injury by an additional 50% increase in the alanine aminotransferase levels. Phenobarbital also caused an~40% increase in the total P450 content of the liver, which was also associated with a 75% increase in the reactive oxygen species (ROS) generation in the IR group. There was a strong correlation between the microsomal ROS generation and total P450 content, CYP3A2 activity or CYP2B1 activity. It is concluded that the induction of P450 by phenobarbital significantly increases hepatic production of ROS, leading to significantly higher hepatic IR injury.

Keywords: *Hepatic ischemia-reperfusion injury, reactive oxygen species, cytochrome P450, enzyme induction, phenobarbital, microsomes*

Introduction

Hepatic ischemia-reperfusion (IR) injury may occur as a normothermic event during liver resection surgery (Pringle's maneouvre), haemorrhagic or septic shock or trauma. Additionally, cold IR injury occurs during liver preservation for transplantation. Although warm and cold IR injuries share some common mechanisms, there are major differences in their pathophysiology [1], with warm IR injury being much more poorly tolerated. Among different known or suspected mediators, reactive oxygen species (ROS) play a major role in the pathophysiology of warm IR injury [1,2]. ROS stimulate the initiation of injury cascades in the absence of effective antioxidant defense and cause enhanced damage not only to the liver itself but also to extrahepatic organs [2–4]. Therefore, strategies to reduce generation of ROS or enhance their removal from the liver are expected to improve the warm hepatic IR injury.

Very recently [5,6], we hypothesized that cytochrome P450 (CYP) enzymes may contribute to the pathophysiology of the warm hepatic IR injury because of their known capacity to produce ROS [7,8]. P450 enzymes are an important class of enzymes that convert various xenobiotics and endogenous substrates to their oxidized forms. Beside their mono-oxygenase activity, various isoforms of P450 also generate different degrees of ROS by uncoupling. Among the P450 enzymes, CYP1A2, CYP2B1, CYP2C11, CYP2E1 and CYP3A2 have been reported as major producers of ROS [7]. Therefore, we attributed improvements observed in the warm hepatic IR injury in our rat models after administration of cimetidine, a CYP2C11 inhibitor, or diallyl sulphide (DAS), a CYP2E1 inhibitor, to the ability of the P450 inhibitors to reduce ROS generation [5,6].

In addition to our studies in the rat liver IR [5,6], other studies have also shown that CYP inhibitors,

Correspondence: Reza Mehvar, PhD, School of Pharmacy, Texas Tech University Health Sciences Center, 1300 S. Coulter, Amarillo, TX 79106, USA. Tel: (806) 356-4015 Ext.337. Fax: (806) 356-4034, Email: reza.mehvar@ttuhsc.edu

ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK Ltd. (Informa Healthcare, Taylor & Francis AS)
DOI: 10.3109/10715761003610729

like cimetidine, chloramphenicol and sulphaphenazole, reduce normothermic IR injury in the heart [9], lung [10] and kidney [11] IR, in both *in vitro* [9–11] and *in vivo* [9,11] models. Collectively, these studies suggest that CYP may play a detrimental role in warm IR injury. However, most of the studied CYP inhibitors exert other, non-specific effects. For example, our recent work [5] demonstrated that DAS, in addition to CYP2E1 inhibition, also causes an induction of heme oxygenase-1 in rats subjected to warm hepatic IR injury. Because heme oxygenase-1 is known to be protective in hepatic IR injury [12], the hepatoprotective effects of DAS may, at least in part, be due to its ability to induce this enzyme. Additionally, chloramphenicol inhibits mitochondrial protein synthesis [13], potentially affecting mitochondrial ROS generation, in addition to CYP inhibition. Therefore, the non-specific effects of P450 inhibitors may have also contributed to their protective effects in warm IR injury. If the protective effects of these inhibitors were indeed due to CYP inhibition, one would expect that CYP induction would have an opposite effect, exacerbating IR injury. However, the effects of CYP induction on the warm hepatic IR injury are not known at this time. Therefore, the objective of the current study was to investigate the effects of CYP induction by phenobarbital on the extent of ROS generation and injury in an established rat model of warm hepatic IR. Our hypothesis was that phenobarbital would increase microsomal ROS generation via an induction of CYP, leading to an increased liver injury after warm IR.

Materials and methods

Chemicals

The following kits and reagents were used in this study: alanine aminotransferase (ALT) assay kit from Teco Diagnostics (Anaheim, CA); 2,3-dihyroxybenzoic acid (2,3-DHB), 2,5-dihydroxybenzoic acid (2,5-DHB), sodium salicylate, monochloroacetic acid, isocitric acid, isocitrate dehydrogenase, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), dichlorofluoroscein (DCF), TRIS, HEPES, glycerol, cytochrome c, tetrahydrofuran, digitonin, 1-aminobenzotriazole (ABT), bovine serum albumin (BSA), sodium phenobarbital and sodium hydrosulphite from Sigma-Aldrich Co. (St. Louis, MO); NADPH from Calzyme Laboratories Inc. (San Luis Obispo, CA); 2,4-dinitrophenylhydrazine from Alfa Aesar (Ward Hill, MA); oxidized glutathione (GSSG), perchloric acid, 1-octanesulphonic acid and potassium periodate from Acros Organics (Morris Plains, NJ); 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT sodium) from Biotium (Hayward, CA); hypoxanthine from Research Organics Inc (Cleveland, OH); xanthine oxidase from MP Biomedicals, Inc. (Solon, OH); rabbit anti-rat primary antibody against cytochrome P450 reductase from Stressgen (Ann Arbor, MI); and HPLC grade acetonitrile from EMD Sciences, Inc. (Gibbstown, NJ). All other reagents were analytical grade and obtained from commercial sources.

Animals

All the procedures involving animals in this study were consistent with the 'Principles of Laboratory Animal Care' (NIH publication #85-23, revised 1985) and approved by our Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats were obtained from Charles River laboratories (Indianapolis, IN) and housed under a 12 h day/night cycle in an institutional animal facility with free access to food and water.

Experimental groups

A total of 28 rats (230–280 g) were divided into two groups of Saline and Pheno. Whereas the Saline group was treated with vehicle, the Pheno group was treated with 80 mg/kg/day phenobarbital at a volume of 1 ml/kg per each i.p. injection for 3 days. Each group was then further divided into two surgical subgroups of sham or IR, creating a total of four groups consisting of Sham-Saline ($n=6$), Sham-Pheno ($n=6$), IR-Saline ($n=8$) and IR-Pheno ($n=8$).

Additionally, in a separate study, specifically designed to test the effects of IR on the ROS generation by mitochondria, eight rats were divided into two equal groups and subjected to either sham or IR procedure.

Surgical procedures

After an overnight fast, vehicle- or phenobarbital-treated rats were anaesthetized with an intramuscular injection of ketamine:xylazine (80:4 mg/kg) and subjected to the IR or sham operation. Ischemia was induced by the well-established partial (70%) ischemia model [14] by occluding the left branches of the portal vein, hepatic artery and bile duct using a microvascular clamp. This model prevents blood flow to the left and median lobes of the liver while allowing uninterrupted blood flow to the right and caudate lobes, preventing intestinal congestion. During the ischemic period, the body temperature of the animals was closely monitored and automatically maintained at 37°C by a combination of a heating plate and a heating lamp connected to a thermo-regulated temperature controller (TCAT-2AC Controller; Physitemp, Clifton, NJ). After 1 h of partial ischemia, the clamp was removed to allow reperfusion. Five millilitres of sterile saline (37°C) was added to the peritoneal

cavity to compensate for volume loss before closure of the abdomen. Sham-operated animals went through an identical procedure except for clamping of the vessels and bile duct. After 3 h of reperfusion, rats were euthanized and samples were collected as explained below.

Sample collection

At the end of the reperfusion period, blood was collected from the abdominal aorta into heparinized syringes and, after centrifugation, plasma was separated. A portion of each plasma samples was stored at 4°C for the analysis of ALT within a week and the remainder of plasma was stored at −80°C. Additionally, the left lobe was used immediately for separation of microsomes. The median lobe was frozen by immersion into liquid nitrogen and stored at −80°C for biochemical analysis. In the mitochondrial study, the livers were used immediately for preparation of mitoplast fractions as explained later in this section.

Measurement of liver injury marker in plasma

Plasma samples were used undiluted or diluted with deionized water (20-fold; IR groups only) and used to quantitate ALT levels spectrometrically.

Microsomal preparation

Microsomes were prepared according to the established ultracentrifugation methods [15], reported previously [5]. The final pellet was dispersed in a storage buffer (50 mM Tris-HCl buffer pH 7.4 containing 150 mM KCl, 1 mM EDTA and 20% glycerol) and stored at −80°C. Protein content in the microsomes was determined by the Bradford [16] assay using bovine serum albumin as standard.

Mitoplast preparation

Mitoplasts are isolated mitochondria without their outer membranes, which have been used for determination of the P450 activity of mitochondria [17,18]. Mitoplasts were prepared according the method reported by Anandatheerthavarada et al. [18]. Briefly, using a motor-driven Potter-Elvehjem glass homogenizer, median lobes of the Sham or IR livers were homogenized (1:9) in a sucrose-mannitol buffer (pH 7.5), containing 20 mM HEPES, 70 mM sucrose, 220 mM mannitol, 2 mM EDTA and 0.5 mg/ml of BSA. The homogenate was centrifuged at 2000 g for 10 min and the supernatant was subsequently centrifuged at 10 000 g for 15 min to isolate the mitochondrial pellet. The crude mitochondria was washed twice and suspended in the same buffer at a concentration of 20–25 mg/ml. To remove the mitochondrial outer membrane, the mitochondrial suspension was further treated with digitonin (100 μg/mg protein) at 4°C. The resultant mitoplasts were washed free of digitonin at 10 000 g and suspended in a buffer containing 0.154 M potassium chloride, 5 mM TRIS, 1 mM EDTA and 20% glycerol (pH 7.4). The protein content of the mitoplast fraction was then determined before storage at −80°C. The 10 000 g (post-mitochondrial) supernatant was then used to isolate microsomes by centrifugation at 100 000 g for 1 h as described above. The degree of contamination of the mitoplast fractions with microsomes was determined by Western blot analysis of the microsomal marker protein cytochrome P450 reductase in the microsomal and mitoplast fractions as described before [19] .

Total CYP and heme content and cytochrome P450 reductase activity in microsomes

Total CYP and heme contents were determined using the method of Omura and Sato [20]. Cytochrome P450 reductase (CPR) activity was measured spectrophotometrically at 550 nm by a kinetic method as reported before [21].

Activity of microsomal CYP isoforms

The CYP isoform activities of CYP3A2 and 2B1, the major CYP isoforms induced by phenobarbital [22], were determined by 6β-hydroxylation of testosterone and dealkylation of benzyloxyresorufin using established methods as reported before [23].

ROS generation using DCFH-DA as a probe

ROS generation by liver microsomes, mitoplasts and whole homogenates were determined by the DCFH-DA assay [24]. In this assay, DCFH-DA is taken up into the hepatocellular organelles and membranes, where it is de-esterified to DCFH. Subsequently, the non-fluorescent DCFH is converted to the fluorescent DCF by ROS. In all the assays described below, we monitored the increase in the fluorescence in a microplate reader at excitation and emission wavelengths of 485 and 525 nm, respectively. The rate of the fluorescence increase was then converted to nmol DCF/mg protein/min, calculated based on a calibration curve using DCF as a standard.

Microsomal ROS. The method used is similar to those reported before [24,25]. Briefly, 20 μl of a 125 μM solution of DCFH-DA in ethanol (2.5 nmol) was dried under nitrogen stream. To each dried tube were added 0.1 mg microsomal protein and a sufficient volume of 50 mM potassium phosphate buffer (pH 7.4)

to bring the total volume to 450 µl. The samples were then incubated at 37°C for 20 min. A 180 µl aliquot of the reaction mixture was then transferred to a microplate at 37°C and 20 µl of a 5 mM NADPH solution was added. The final reaction mixture contained 5 µM DCFH-DA, 0.2 mg/ml microsomal protein and 0.5 mM NADPH. The rate of DCF generation was then monitored for 20 min.

Mitoplast ROS. The method used for the estimation of ROS in mitoplasts was similar to that described above for the microsomes except that the mitoplast ROS generation assay was monitored both in the absence and presence of 1 mM ABT. This concentration of ABT is known to reduce microsomal P450 content by 50% within 10 min [26]. After addition of all the reaction components, the rate of DCF generation was monitored for 40 min and the data during the last 20 min was used for estimation of DCF formation.

Whole liver homogenate ROS The generation of ROS by the whole liver homogenates was studied as reported before [27], with some modifications. Briefly the liver was homogenized (1:9, w/v) in a cold buffer containing 130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 20 mM Tris and 30 mM glucose (pH 7.4). After centrifugation at 1000 g for 10 min to remove cell debris, the homogenate was used in a reaction mixture containing 5 µM DCFH-DA, 0.5 mM NADPH and 1 mg/ml homogenate protein and the rate of DCF generation was monitored at 37°C over 20 min.

Hydroxyl radical generation using salicylate as a probe

We used the generation 2,3-dihyroxybenzoate (2,3-DHB) from salicylate in microsomal preparations as a specific measure of formation of hydroxyl radicals [28]. Briefly, the reaction mixtures (1 ml) contained 1 mg microsomal protein and 1 mM salicylate in a 50 mM potassium phosphate buffer (pH 7.4). The reactions were started at 37°C by the addition of NADPH (0.3 mM) and terminated after 15 min by the addition of 5 µl of perchloric acid. After vortex-mixing and centrifugation at 14 000 rpm for 10 min, a 5 µl aliquot of the supernatant was injected into an HPLC system with an electrochemical detector. The analytes were resolved on a C_{18} microbore column (BAS instrument, Indianapolis, IN), using a mobile phase containing 4 ml acetonitrile, 1 ml tetrahydrofuran and 100 ml buffer. The buffer consisted of 75 mM monochloroacetic acid, 1.5 mM octanesulphonic acid and 0.7 mM EDTA (pH 2.9). The mobile phase flow rate was 0.1 ml/min. The products of salicylate oxidation were detected by an electrochemical detector equipped with a glassy carbon electrode and an Ag/AgCl reference electrode. The detector potential was set to 0.75 V and maximum sensitivity was 5 nA. The formation of 2,3-DHB is reported as nmol/mg microsomal protein/min.

Reduced (GSH) and oxidized (GSSG) glutathione in the liver tissue

The GSH and GSSG contents of liver tissue were estimated using a method recently developed in our laboratory [29]. The assay, which is based on an enzymatic recycling method, measures the concentrations of total glutathione and GSSG directly. The concentration of GSH is then estimated by subtracting the concentration of GSSG from the total glutathione concentration.

Superoxide dismutase and catalase in the liver tissue

The activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase in the liver homogenates were estimated according to the established methods, as described in detail recently [6]. Basically, the SOD activity was estimated as its ability to inhibit the reduction of the tetrazolium dye XTT [30], while catalase activity was determined as its ability to produce formaldehyde from methanol in the presence of optimal concentrations of hydrogen peroxide [31].

Lipid peroxidation in liver

Malondialdehyde (MDA) content of the livers was estimated using a specific HPLC method [5]. The method measures total (protein-bound and free) MDA after alkaline hydrolysis of the protein-bound MDA, followed by derivatization of MDA with 2,4-dinitrophenylhyrazine before analysis by reversed-phase HPLC.

Data analysis

The effects of drug treatment (Saline vs Pheno) and surgical manipulation (Sham vs IR) on different parameters were tested using two-way ANOVA. In the presence of a significant interaction between the two factors (drug treatment and surgical manipulation), Bonferroni's post-test analysis of means was used with a p-value corrected for the number of comparisons. Regression analysis was used to test the relationships between ROS generation by microsomes and CYP content or activities and between plasma ALT levels and CYP activities or ROS generation by microsomes. A p-value of <0.05 or its Bonferroni-adjusted equivalent was considered significant. All statistical tests were carried out using the program

GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The results are presented as mean ± SD.

Results

Plasma levels of liver injury marker enzyme

The plasma concentrations of the hepatic injury marker ALT in different groups are shown in Figure 1. The concentrations of ALT were relatively low in the sham-operated groups (~100 IU/L), regardless of phenobarbital or vehicle pre-treatment. Ischemia-reperfusion surgery caused a substantial injury to the liver as evidenced by 8–10-fold increases in the plasma ALT levels in the Saline and Pheno groups ($p < 0.001$). Pre-treatment of the animals with phenobarbital in the Pheno-IR group significantly ($p < 0.001$) exacerbated the liver injury by a further 50% increase in the plasma concentrations of ALT, compared with the Saline-IR group. However, phenobarbital pre-treatment in the Sham groups did not have any effect on the ALT levels (Figure 1).

Total CYP and heme content and cytochrome P450 reductase activity

The total CYP and heme contents and CPR activities of the microsomes for different groups of animals are presented in Figure 2. Phenobarbital pre-treatment of animals for 3 days caused a 40% increase in the total CYP content of the livers ($p < 0.01$) in both Sham and IR groups (Figure 2A). Additionally, IR caused a modest (20%), but statistically significant ($p < 0.05$), decline in the total CYP content in the microsomes of saline- and phenobarbital-pre-treated groups (Figure 2A). The phenobarbital-induced increase in the total CYP contents was also reflected in the microsomal heme contents that were increased ($p < 0.001$) by 25% and 35% in the Sham and IR groups, respectively (Figure 2B). For CPR, phenobarbital pre-treatment significantly ($p < 0.001$) increased its activity by 51% and 64% in the Sham and IR groups, respectively (Figure 2C). As for the influence of the surgery, IR caused ~20–30% decrease in the CPR activity, when compared with the sham operated rats ($p < 0.001$).

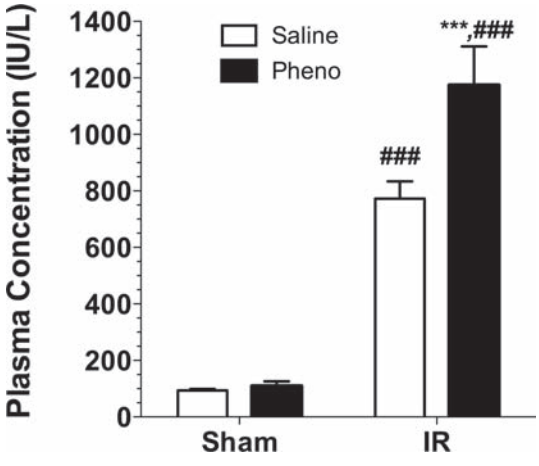

Figure 1. Plasma concentrations of ALT in Saline-Sham, Pheno-Sham, Saline-IR and Pheno-IR groups. Rats were pre-treated with saline or phenobarbital (80 mg/kg/day) for 3 days, followed by 60 min of partial warm hepatic ischemia or sham operation and 3 h of reperfusion. Columns and error bars represent mean and SD, respectively. $^{***}p < 0.001$ vs the respective Saline group; $^{###}p < 0.001$ vs respective Sham group. The statistical analysis is based on Bonferroni's post-test analysis of means because of a significant ($p < 0.001$) interaction between the two factors (drug treatment and surgical manipulation).

CYP3A and 2B activities

The activities of CYP3A, measured by 6β-hydroxylation of testosterone, and CYP2B, measured by dealkylation of benzyloxyresorufin, are presented in Figure 3. Pretreatment of the rats for 3 days with phenobarbital caused 1.8- and 2.2-fold increases ($p < 0.001$) in the microsomal CYP3A2 activities in the Sham and IR surgical groups, respectively (Figure 3, left panel). Additionally, IR caused a 30–40% reduction ($p < 0.01$) in the microsomal CYP3A2 activity, compared with the sham operation (Figure 3, left). As for CYP2B, phenobarbital pre-treatment resulted in 4.6- and 9.2-fold increases ($p < 0.001$) in its activity in the Sham and IR groups, respectively (Figure 3, right). Moreover, the IR procedure caused between 30–60% decreases in the CYP2B1 activity, compared with the Sham group ($p < 0.05$).

ROS generation using DCFH-DA assay as a probe

The results of DCFH-DA oxidation assay, which was used to measure ROS generation by microsomes and liver homogenates, are presented in Figure 4. For microsomes, phenobarbital pre-treatment of the animals caused a significant ($p < 0.001$) increase in the ROS generation capacity in Sham (55% increase) and IR (75% increase) groups, as compared with their respective saline-treated groups (Figure 4, left). Additionally, IR groups produced 20–30% less DCF, compared with the Sham groups ($p < 0.001$).

The DCF generation profiles in the whole liver homogenates (Figure 4, right) were qualitatively very similar to those in the microsomes (Figure 4, left), although the absolute values were 5–10-fold lower in the whole homogenate. Similar to the microsomes, phenobarbital pre-treatment caused a significant ($p < 0.001$) increase (~90%) in the DCF production, whereas the productions of DCF in the IR groups were significantly ($p < 0.001$) lower (~40% decline) than those in their respective Sham groups (Figure 4, right).

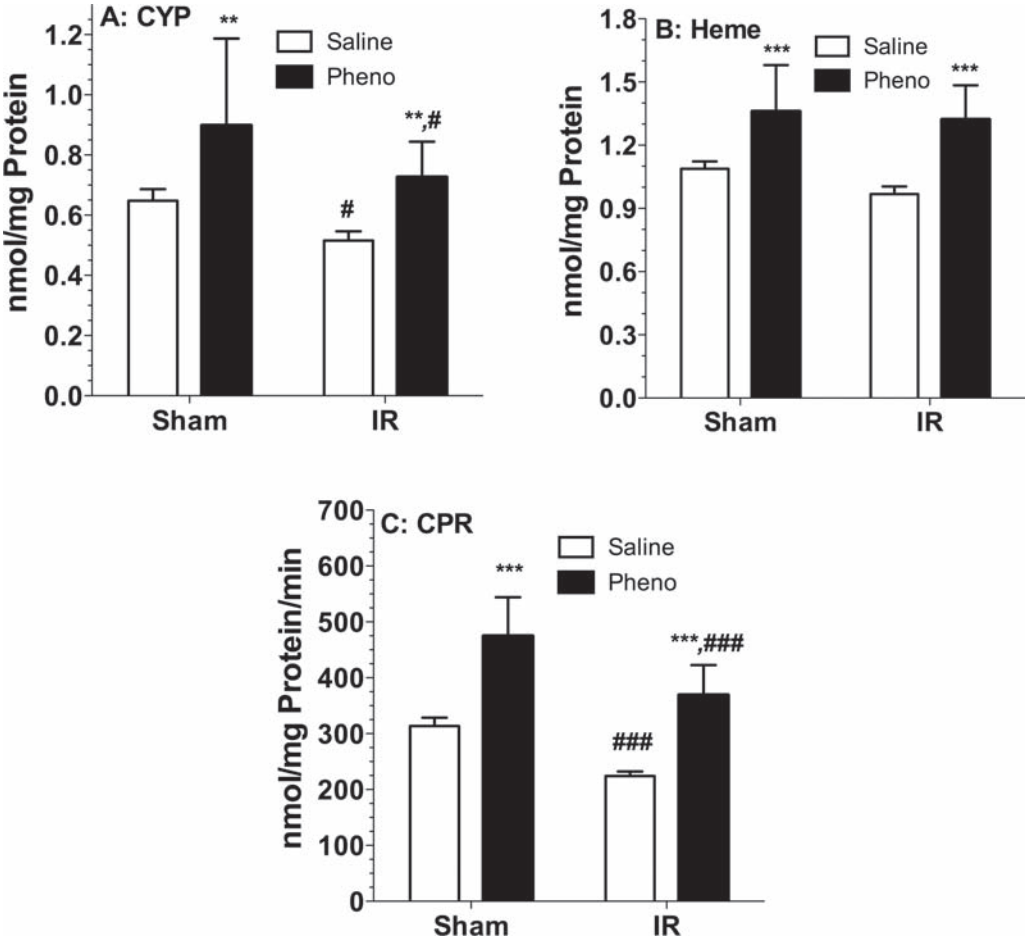

Figure 2. Total CYP (A) and heme (B) contents and cytochrome P450 reductase (CPR) activities (C) of the microsomal fractions of the livers obtained from the Saline-Sham, Pheno-Sham, Saline-IR and Pheno-IR groups. Rats were pre-treated with saline or phenobarbital (80 mg/kg/day) for 3 days, followed by 60 min of partial warm hepatic ischemia or sham operation and 3 h of reperfusion. Columns and error bars represent mean and SD, respectively. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs respective Saline groups; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs respective Sham groups. The statistical analysis is based on two-way ANOVA.

Hydroxyl radical generation using salicylate as a probe

The generation of 2,3-DHB from salicylate in the microsomes, measured by HPLC, is presented in Figure 5. The HPLC method was capable of separating and quantitating 2,5-DHB, 2,3-DHB and salicylate, which were eluted at 3.2, 4.2 and 8.5 min, respectively (chromatograms not shown). Phenobarbital caused 2.6- and 3-fold increases ($p < 0.001$) in the microsomal production of 2,3-DHB in the Sham and IR groups, respectively. Further, IR, by itself, caused 28–36% reduction ($p < 0.05$) in the 2,3-DHB formation.

Relationships between ROS generation and CYP content or activities

The results of regression analyses between microsomal ROS generation by formation of DCF or 2,3-DHB and microsomal total CYP content or activities of CYP3A or CYP2B are presented in Figure 6. All the regression analyses were significant (Figure 6). However, comparing DCF and 2,3-DHB, stronger relationships (higher r^2 values) were obtained for DCF. Additionally, comparing total CYP content, CYP3A activity and CYP2B activity, the strongest correlations were obtained for the total CYP content and the weakest for the CYP2B activities (Figure 6). Overall, the strongest relationship ($p < 0.0001$, $r^2 = 0.85$) was observed between DCF formation as a measure of ROS generation and total CYP content (Figure 6), indicating that, irrespective of the treatment group or surgical procedure, the microsomal formation of DCF is almost exclusively dependent on the total CYP content.

Relationships between ALT and ROS generation

The regression analyses of plasma ALT concentrations vs microsomal ROS generation by conversion of DCFH-DA to DCF or salicylate to 2,3-DHB are presented in Figure 7 for the animals subjected to IR and pre-treated with saline or phenobarbital. A significant relationship was found between ALT and

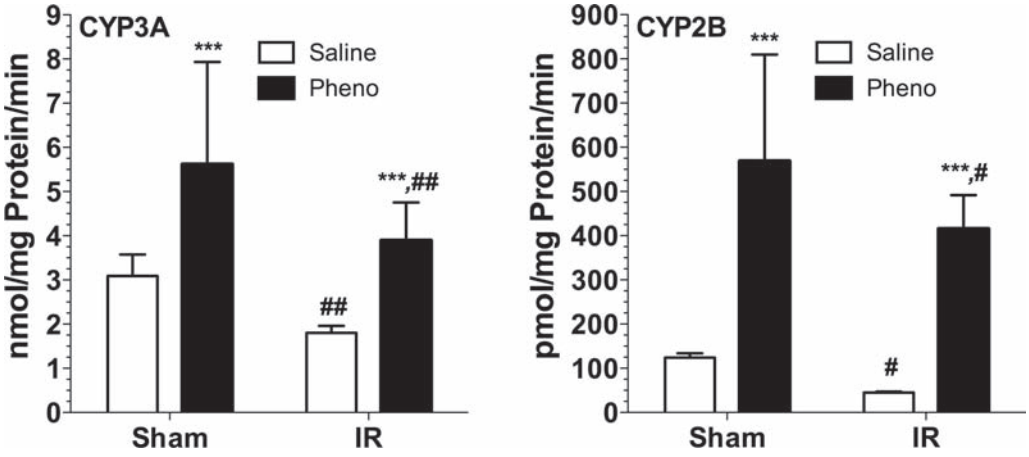

Figure 3. CYP3A (left) and 2B (right) activities in the microsomal fractions of the livers obtained from the Saline-Sham, Pheno-Sham, Saline-IR and Pheno-IR groups. Rats were pre-treated with saline or phenobarbital (80 mg/kg/day) for 3 days, followed by 60 min of partial warm hepatic ischemia or sham operation and 3 h of reperfusion. Columns and error bars represent mean and SD, respectively. $^{***}p < 0.001$ vs respective Saline group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs respective Sham groups. The statistical analysis is based on two-way ANOVA.

DCF or 2,3-DHB within the IR groups. However, the data for the Sham groups were not correlated (data not shown) because, despite an increase in the ROS generation, the ALT remained low in the Sham groups.

Antioxidant capacity of the liver

The liver contents of reduced (GSH) and oxidized (GSSG) glutathione along with the activities of the liver antioxidant enzymes superoxide dismutase (SOD) and catalase are presented in Table I. Although IR did not significantly affect the liver concentrations of GSH, it caused a significant ($p < 0.001$) increase in the levels of GSSG after Pheno pre-treatment (Table I). The effects of IR on the GSSG levels were also reflected in a lower ($p < 0.01$) GSH:GSSG ratio in the IR-Pheno, compared with its Sham counterpart. Interestingly, phenobarbital pre-treatment significantly increased the hepatic levels of GSH ($p < 0.01$) in both Sham and IR groups. Additionally, phenobarbital increased ($p < 0.01$) the hepatic concentrations of GSSG in the IR group only (Table I). However, the increase in the GSSG levels (62%) was higher than that in the GSH concentrations (21%) in the IR group. As for the antioxidant enzymes, whereas both IR and phenobarbital caused significant, but modest, decreases in the hepatic levels of SOD, catalase activity in the liver was not affected by either phenobarbital pre-treatment or ischemia-reperfusion (Table I).

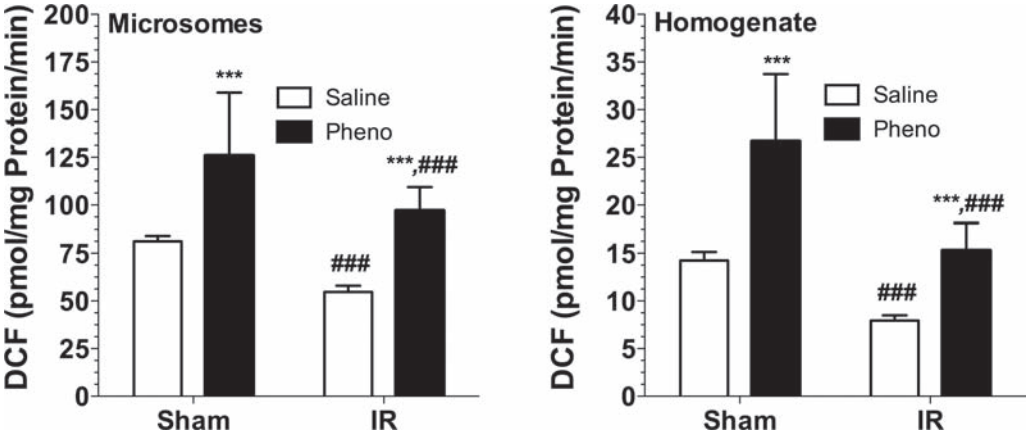

Figure 4. ROS generation capacity of the microsomes (left) and whole homogenates (right) of the livers obtained from the Saline-Sham, Pheno-Sham, Saline-IR and Pheno-IR groups. Rats were pre-treated with saline or phenobarbital (80 mg/kg/day) for 3 days, followed by 60 min of partial warm hepatic ischemia or sham operation and 3 h of reperfusion. Columns and error bars represent mean and SD, respectively. $^{***}p < 0.001$ vs respective Saline group; $^{\#\#\#}p < 0.001$ vs respective Sham group. The statistical analysis is based on two-way ANOVA.

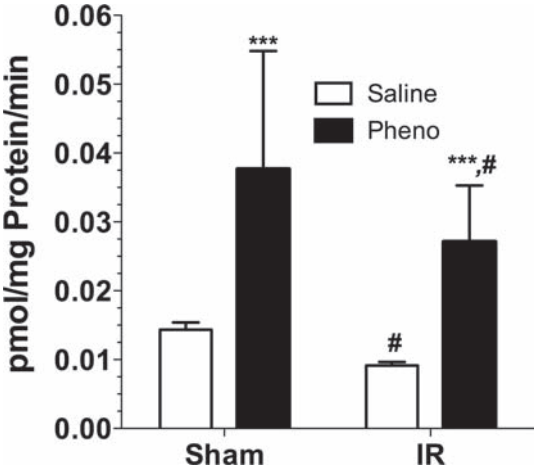

Figure 5. The rate of 2,3–dihydroxybenzoate formation in the microsomes obtained from the Saline-Sham, Pheno-Sham, Saline-IR and Pheno-IR groups. Rats were pre-treated with saline or phenobarbital (80 mg/kg/day) for 3 days, followed by 60 min of partial warm hepatic ischemia or sham operation and 3 h of reperfusion. Columns and error bars represent mean and SD, respectively. ***$p < 0.001$ vs respective Saline group; $^{\#}p < 0.05$ vs respective Sham group. The statistical analysis is based on two-way ANOVA.

Lipid peroxidation

The hepatic concentrations of the lipid peroxidation marker MDA were 13.6 ± 1.6, 15.0 ± 3.6, 16.1 ± 2.4 and 14.7 ± 1.4, nmol/g liver in the Sham-Saline, Sham-Pheno, Saline-IR and Pheno-IR, groups, respectively. The hepatic MDA levels were not affected by either IR or phenobarbital pre-treatment.

ROS generation from mitoplasts

As stated in the methods, the ROS formation in the mitoplasts was investigated in a separate study, comprising Sham and IR groups. The results of DCFH oxidation by mitoplasts in the absence and presence of ABT are presented in Figure 8. Also presented in Figure 8 are representative Western blot data for CPR that is used as a measure of mitoplast contamination by microsomes. The CPR contents of mitoplasts were ~10% of that in the microsomes, which is in agreement with the previously reported studies [19], indicating that mitoplasts prepared from fresh livers in this study had minimal microsomal contamination. Interestingly, in contrast to the microsomal data (Figure 4, left), DCF formation by IR mitoplasts was significantly ($p < 0.05$) higher than that in the Sham mitoplasts by 47% or 43% in the absence or presence of ABT, respectively (Figure 8). Additionally, ABT significantly ($p < 0.001$) reduced DCF formation by 65.5% and 66.8% in the Sham and IR mitoplasts, respectively (Figure 8).

Discussion

Recent studies have shown that oxidative stress, which is one of the major concerns in various pathophysiological states, is aggravated by phenobarbital pre-treatment [32,33]. For instance, Minamiyama et al. [32] showed that the induction of CYP enzymes by phenobarbital followed by lipopolysaccharide (LPS) exposure aggravated the LPS-induced injury to the rat liver [32]. Additionally, Dostalek et al. [33] reported that phenobarbital pre-treatment of rats induced oxidative stress, manifested by increased generation of hydrogen peroxide and lipid peroxidation markers, such as F2-isoprostanes. These changes were prevented when the animals were also treated with ABT, suggesting that the oxidative stress-inducing effects of phenobarbital were related to the induction of P450. Our data in the current study are in agreement with these reports in that liver injury induced by warm IR was exacerbated by phenobarbital pre-treatment, as evidenced by a further 50% increase in the plasma concentrations of ALT (Figure 1).

Phenobarbital, an anti-epileptic drug, is one of the most extensively studied inducers of the P450 enzymes, which elicits its effect by regulation of genes responsible for the expression of these enzymes. The two major P450 enzymes induced by phenobarbital, CYP2B and CYP3A [34,35], are regulated by different pathways. Whereas CYP2B enzymes are induced by transcriptional activation of androstane receptor, a nuclear orphan receptor, CYP3A genes are induced by activation of pregnane X receptor [36,37]. Phenobarbital not only induces specific isoforms of P450, but also results in an increase in total CYP as well as heme contents of microsomes. We also observed a 40% increase in the total P450 and heme contents in the liver microsomes (Figures 2A and B). However, ischemia reperfusion *per se* resulted in a ~20% decline in both total CYP and heme contents in microsomes, which is in agreement with previous reports from our own laboratory [5,6] and others [38,39].

It is well known that the degree of uncoupling and ROS generation is dependent on the CYP isoforms and presence or absence of substrates [7,8,40]. The two P450 sub-families induced by phenobarbital (CYP3A and CYP2B) have been particularly implicated in the generation of ROS by uncoupling [7]. Although CYP3A is constitutively expressed at relatively high levels, the constitutive expression of the CYP2B sub-family is very low [41]. Therefore, contribution of CYP2B enzymes to ROS generation is expected to be minimal under basal uninduced conditions. However, phenobarbital pre-treatment caused a more significant increase in the activity of CYP2B (5–9-fold) than that of CYP3A (~2-fold) (Figure 3). Therefore, the increase in microsomal ROS generation after phenobarbital pre-treatment (Figure 4) is most likely due to the contribution of both P450 sub-families.

It is widely believed that warm hepatic IR injury is associated with increased ROS production and oxidative stress [2]. However, this belief is mostly based on

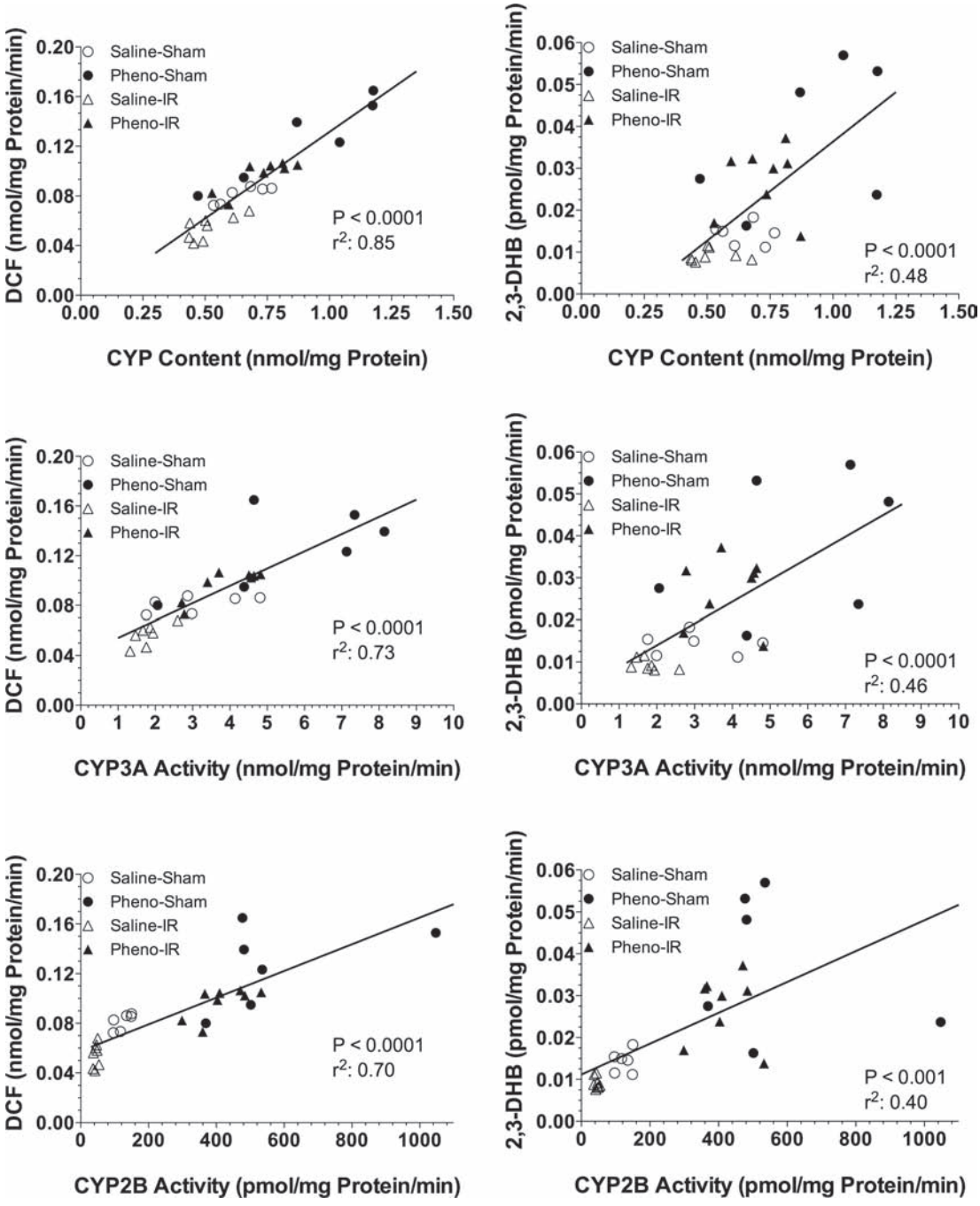

Figure 6. The relationship between microsomal ROS generation via formation of DCF from DCFH-DA (left panels) or formation of 2,3-DHB from salicylate (right panels) and total CYP content (top), CYP3A activity (middle) or CYP2B activity (bottom). Symbols and lines represent individual rats and the regression lines, respectively.

indirect evidence of IR-induced increases in lipid peroxidation [42] and the hepatoprotective effects of antioxidants administered to animals before IR [43,44]. The most direct evidence has only been provided in an *ex vivo* isolated perfused rat liver model of warm IR, which showed increased ROS appearance in the perfusate shortly after reperfusion, measured by electron spin resonance spectroscopy [45]. However, we are not aware of any studies reporting the effects of IR on the ROS generation in microsomes. When we studied microsomal ROS generation by DCFH-DA, as expected, phenobarbital pre-treatment of the rats resulted in 55% and 75% increases in ROS generation in the sham and IR groups, respectively, in comparison with their respective vehicle treated rats (Figure 4, left). However, we also observed a small (20–30%), but significant, decline in the microsomal ROS generation in the IR groups as compared with their respective sham controls. To confirm these findings, we tested microsomal ROS generation using a different marker, salicylate, which is a specific probe for hydroxyl radical formation [46].

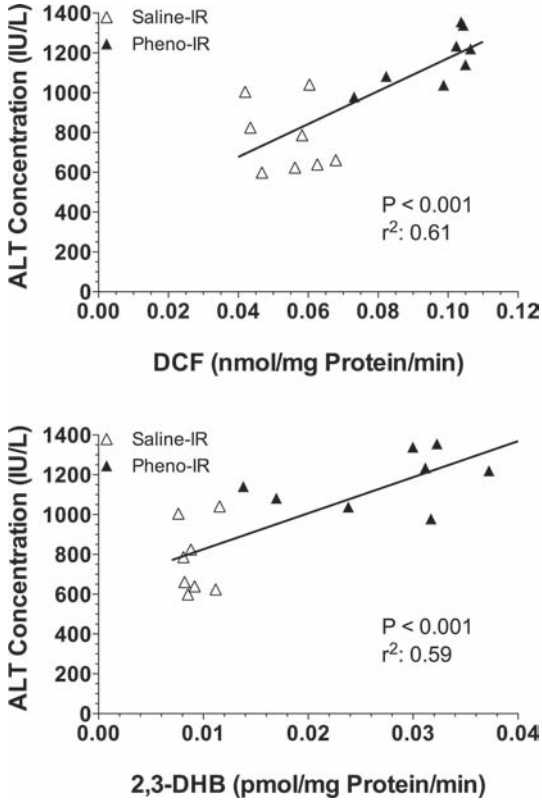

Figure 7. The relationship between plasma ALT concentrations and microsomal ROS generation via formation of DCF from DCFH-DA (top panel) or formation of 2,3-DHB from salicylate (bottom panel) in rats subjected to IR. Symbols and lines represent individual rats and the regression lines, respectively.

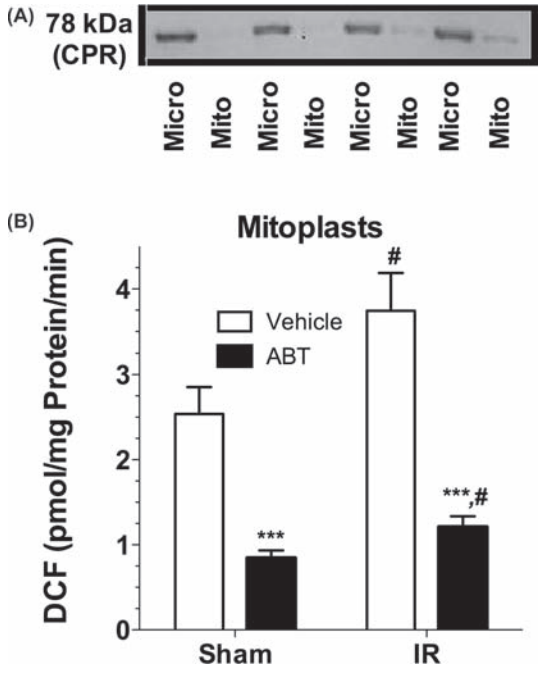

Figure 8. Representative blots of cytochrome P450 reductase (A) in microsomes (Micro) and mitoplasts (Mito) and ROS generation capacity of the mitoplasts of the livers (B) obtained from the Saline-Sham and Saline-IR groups in the absence (Vehicle) and presence (ABT) of 1 mM 1-aminobenzotriazole. Rats underwent 60 min of partial warm hepatic ischemia or sham operation followed by 3 h of reperfusion. Columns and error bars represent mean and SD, respectively. $^{***}p < 0.001$ vs respective Vehicle group; $^{\#}p < 0.05$ vs respective Sham group. The statistical analysis is based on two-way ANOVA.

The studies with salicylate confirmed the phenobarbital-induced increase and IR-induced decrease in the microsomal ROS generation (Figure 5), similar to that observed with DCFH-DA. Furthermore, when the ROS generation was determined in the whole liver homogenate using DCFH-DA, even though the absolute values were ~5–10-fold lower, the pattern in the liver homogenate was qualitatively similar to that in the microsomes (Figure 4). Overall, these data clearly show that phenobarbital increases ROS generation in both microsomes and whole liver homogenates. However, microsomal generation of ROS after IR is lower than that after sham operation. Therefore, the previously-reported increase in the ROS generation as a result of IR by the intact liver [45] is not due to an increase in the microsomal generation of ROS by uncoupling of P450 and cannot be replicated in the whole liver homogenate.

To identify the reasons for the discrepancy between the reported increase in ROS generation in the intact liver after IR and the observed decline in ROS gen-

Table I. Hepatic concentrations (mean ± SD) of the markers of the antioxidant capacity of the liver in Saline-Sham, Pheno-Sham, Saline-IR and Pheno-IR groups. Rats were pre-treated with saline or phenobarbital (80 mg/kg/day) for 3 days, followed by 60 min of partial warm hepatic ischemia or sham operation and 3 h of reperfusion.

	Sham		IR	
Parameter	Saline	Pheno	Saline	Pheno
GSH (mM)[a]	3.70 ± 0.57	4.39 ± 0.83**	4.10 ± 0.74	4.96 ± 0.60**
GSSG (mM)[b]	0.151 ± 0.025	0.150 ± 0.037	0.193 ± 0.056	0.312 ± 0.108**,###
GSH:GSSG[b]	24.8 ± 4.58	29.8 ± 5.65	23.1 ± 8.55	17.2 ± 4.6##
SOD (IU/mg protein)[a]	38.6 ± 3.1	32.8 ± 6.2*	29.6 ± 3.8##	26.8 ± 7.3*,##
Catalase (IU/mg protein)[a]	1350 ± 149	1350 ± 119	1270 ± 157	1280 ± 167

$^{*}p < 0.05$ and $^{**}p < 0.01$ vs respective Saline groups; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs respective Sham groups.
[a] The statistical analysis is based on two-way ANOVA. [b] The statistical analysis is based on Bonferroni's post-test analysis of means because of significant interactions between the two factors (drug treatment and surgical manipulation).

eration in both the microsomes and whole liver homogenates in our study after IR, we decided to test the role of mitochondrial P450 in ROS generation. A number of P450 enzymes have been reported to be localized in mitochondria, Golgi apparatus, plasma membrane, lysosomes and peroxisomes [47]. Among the P450 enzymes located outside microsomes, mitochondrial P450 have been studied most extensively, indicating the presence of CYP1A1/2, 2B1/2, 2E1 and 3A1/2 enzymes in this organelle [18]. In our initial attempts, mitochondria were isolated from frozen livers used in this study. However, the mitochondrial fractions were substantially (up to 50%) contaminated by microsomes, as demonstrated by the presence of microsomal cytochrome P450 reductase, most likely because of the use of frozen livers [48]. Therefore, a pilot study was conducted with additional Sham and IR groups ($n=4$/group), and the mitochondrial fractions were isolated from fresh livers and used for preparation of mitoplasts. As expected, these fractions prepared from fresh livers only had ~6–10% microsomal contamination, which is in agreement with the earlier reports [18]. Interestingly, in contrast to microsomes (Figure 4, left), ROS generation by mitoplasts, measured by DCFH-DA oxidation, showed a 50% increase as a result of IR (Figure 8). Additionally, the ROS generation by mitoplasts was substantially (~65%) inhibited by the P450 inhibitor, ABT, suggesting that the DCF formation by mitoplasts is P450-dependent. It should be noted that ROS generation by mitoplasts (Figure 8) was substantially lower than that by the microsomes (Figure 4, left), on a per mg protein basis. However, because mitochondria are the important site for initiating and propagating cellular damages such as apoptosis and necrosis, the IR-induced increase in ROS generation locally in mitochondria may be of importance. Overall, these preliminary results suggest that mitochondrial P450 enzymes may play a role in the IR-induced ROS generation and liver injury. However, detailed future studies are needed to test this postulate.

Another mechanism by which phenobarbital may have contributed to the higher IR injury is via its effect on CPR (Figure 2C). In addition to being the obligate electron donor for P450 function, CPR generates ROS directly by donation of electron to oxygen molecules, leading to production of ROS [49]. In our studies, phenobarbital increased microsomal CPR activities by 50–60% (Figure 2C). This is in agreement with other studies showing that phenobarbital increases microsomal CPR activity [33]. Therefore, the phenobarbital-induced higher microsomal ROS generation observed in our studies (Figures 4 and 5) may be due, in part, to its ability to induce CPR.

Although the correlation between microsomal ROS generation and the activities of CYP3A or CYP2B were significant, the strongest correlation was found between the ROS generation and total CYP content (Figure 6). This data, therefore, may suggest that P450 enzymes other than CYP3A and CYP2B significantly contribute to the microsomal ROS generation. Indeed, previous studies [7] have shown that P450 enzymes such as CYP2E1, CYP1A and CYP2C are also very leaky, producing substantial amounts of superoxide and hydrogen peroxide.

In conclusion, our current study shows that phenobarbital significantly induces P450 enzymes, such as CYP3A and CYP2B, leading to a higher production of microsomal ROS and liver injury after warm IR in rats. When combined with our recent studies [5,6] showing improvements in the hepatic IR injury after inhibition of P450, these data suggest that P450 enzymes may play a detrimental role in hepatic IR injury. Additionally, our limited studies using mitochondrial fractions suggest that mitochondrial P450 may be involved in the effects of P450 on the injury. Further investigations are needed to determine the mechanisms by which P450 enzymes impact warm hepatic ischemia reperfusion injury.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Lichtman SN, Lemasters JJ. Role of cytokines and cytokine-producing cells in reperfusion injury to the liver. Semin Liver Dis 1999;19:171–187.

[2] Jaeschke H. Role of reactive oxygen species in hepatic ischemia-reperfusion injury and preconditioning. J Invest Surg 2003;16:127–140.

[3] Galaris D, Barbouti A, Korantzopoulos P. Oxidative stress in hepatic ischemia-reperfusion injury: the role of antioxidants and iron chelating compounds. Curr Pharm Des 2006;12: 2875–2890.

[4] Glantzounis GK, Salacinski HJ, Yang W, Davidson BR, Seifalian AM. The contemporary role of antioxidant therapy in attenuating liver ischemia-reperfusion injury: a review. Liver Transpl 2005;11:1031–1047.

[5] Shaik IH, George JM, Thekkumkara TJ, Mehvar R. Protective effects of diallyl sulfide, a garlic constituent, on the warm hepatic ischemia-reperfusion injury in a rat model. Pharm Res 2008;25:2231–2242.

[6] Shaik IH, Mehvar R. Effects of cytochrome P450 inhibition by cimetidine on the warm hepatic ischemia-reperfusion injury in rats. J Surg Res 2009. Epub ahead of print 16 October 2008.

[7] Zangar RC, Davydov DR, Verma S. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. Toxicol Appl Pharmacol 2004;199:316–331.

[8] Davydov DR. Microsomal monooxygenase in apoptosis: another target for cytochrome c signaling? Trends Biochem Sci 2001;26:155–160.

[9] Granville DJ, Tashakkor B, Takeuchi C, Gustafsson AB, Huang C, Sayen MR, Wentworth P, Jr, Yeager M, Gottlieb RA. Reduction of ischemia and reperfusion-induced myocardial damage by cytochrome P450 inhibitors. Proc Natl Acad Sci USA 2004;101:1321–1326.

[10] Bysani GK, Kennedy TP, Ky N, Rao NV, Blaze CA, Hoidal JR. Role of cytochrome P-450 in reperfusion injury of the rabbit lung. J Clin Invest 1990;86:1434–1441.
[11] Paller MS, Jacob HS. Cytochrome P-450 mediates tissue-damaging hydroxyl radical formation during reoxygenation of the kidney. Proc Natl Acad Sci USA 1994;91: 7002–7006.
[12] Kato H, Amersi F, Buelow R, Melinek J, Coito AJ, Ke B, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 overexpression protects rat livers from ischemia/reperfusion injury with extended cold preservation. Am J Transplant 2001;1:121–128.
[13] He H, Chen M, Scheffler NK, Gibson BW, Spremulli LL, Gottlieb RA. Phosphorylation of mitochondrial elongation factor Tu in ischemic myocardium: basis for chloramphenicol-mediated cardioprotection. Circ Res 2001;89:461–467.
[14] Spiegel HU, Bahde R. Experimental models of temporary normothermic liver ischemia. J Invest Surg 2006;19: 113–123.
[15] Lake BG. Preparation and characterisation of microsomal fractions for studies on xenobiotic metabolism. In: Snell K, Mullock B, editors. Biochemical toxicology, a practical approach. Oxford, UK: IRL Press; 1987. p. 183–215.
[16] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
[17] Niranjan BG, Wilson NM, Jefcoate CR, Avadhani NG. Hepatic mitochondrial cytochrome P-450 system. Distinctive features of cytochrome P-450 involved in the activation of aflatoxin B1 and benzo(a)pyrene. J Biol Chem 1984;259: 12495–12501.
[18] Anandatheerthavarada HK, Addya S, Dwivedi RS, Biswas G, Mullick J, Avadhani NG. Localization of multiple forms of inducible cytochromes P450 in rat liver mitochondria: immunological characteristics and patterns of xenobiotic substrate metabolism. Arch Biochem Biophys 1997;339:136–150.
[19] Bhagwat SV, Mullick J, Raza H, Avadhani NG. Constitutive and inducible cytochromes P450 in rat lung mitochondria: xenobiotic induction, relative abundance, and catalytic properties. Toxicol Appl Pharmacol 1999;156:231–240.
[20] Omura T, Sato R. The carbon monoxide-binding pigment of the liver microsomes. Evidence for its hemoprotein nature. J Biol Chem 1964;239:2370–2378.
[21] Vuppugalla R, Mehvar R. Short-term inhibitory effects of nitric oxide on cytochrome P450-mediated drug metabolism: time dependency and reversibility profiles in isolated perfused rat livers. Drug Metab Dispos 2004;32:1446–1454.
[22] Buhler R, Lindros KO, Nordling A, Johansson I, Ingelman-Sundberg M. Zonation of cytochrome P450 isozyme expression and induction in rat liver. Eur J Biochem 1992;204: 407–412.
[23] Vuppugalla R, Shah RB, Chimalakonda AP, Fisher CW, Mehvar R. Microsomal cytochrome P450 levels and activities of isolated rat livers perfused with albumin. Pharm Res 2003;20:81–88.
[24] Bondy SC, Naderi S. Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. Biochem Pharmacol 1994;48:155–159.
[25] Puntarulo S, Cederbaum AI. Production of reactive oxygen species by microsomes enriched in specific human cytochrome P450 enzymes. Free Radic Biol Med 1998; 24:1324–1330.
[26] Mugford CA, Mortillo M, Mico BA, Tarloff JB. 1-Aminobenzotriazole-induced destruction of hepatic and renal cytochromes P450 in male Sprague-Dawley rats. Fundam Appl Toxicol 1992;19:43–49.
[27] Fu Y, Ji LL. Chronic ginseng consu-mption attenuates age-associated oxidative stress in rats. J Nutr 2003;133:3603–3609.
[28] Ingelman-Sundberg M, Kaur H, Terelius Y, Persson JO, Halliwell B. Hydroxylation of salicylate by microsomal fractions and cytochrome P-450. Lack of production of 2,3-dihydroxybenzoate unless hydroxyl radical formation is permitted. Biochem J 1991;276:753–757.
[29] Shaik IH, Mehvar R. Rapid determination of reduced and oxidized glutathione levels using a new thiol-masking reagent and the enzymatic recycling method: application to the rat liver and bile samples. Anal Bioanal Chem 2006;385:105–113.
[30] Ukeda H, Maeda S, Ishii T, Sawamura M. Spectrophotometric assay for superoxide dismutase based on tetrazolium salt 3'–1–(phenylamino)-carbonyl–3, 4-tetrazolium]-bis (4-methoxy 6-nitro)benzenesulfonic acid hydrate reduction by xanthine-xanthine oxidase. Anal Biochem 1997;251:206–209.
[31] Johansson LH, Borg LA. A spectrophotometric method for determination of catalase activity in small tissue samples. Anal Biochem 1988;174:331–336.
[32] Minamiyama Y, Takemura S, Toyokuni S, Imaoka S, Funae Y, Hirohashi K, Yoshikawa T, Okada S. CYP3A induction aggravates endotoxemic liver injury via reactive oxygen species in male rats. Free Radic Biol Med 2004;37:703–712.
[33] Dostalek M, Brooks JD, Hardy KD, Milne GL, Moore MM, Sharma S, Morrow JD, Guengerich FP. *In vivo* oxidative damage in rats is associated with barbiturate response but not other cytochrome P450 inducers. Mol Pharmacol 2007;72: 1419–1424.
[34] Venditti P, Daniele CM, De Leo T, Di Meo S. Effect of phenobarbital treatment on characteristics determining susceptibility to oxidants of homogenates, mitochondria and microsomes from rat liver. Cell Physiol Biochem 1998;8:328–338.
[35] Shephard EA, Phillips IR, Pike SF, Ashworth A, Rabin BR. Differential effect of phenobarbital and beta-naphthoflavone on the mRNAs coding for cytochrome P450 and NADPH cytochrome P450 reductase. FEBS Lett 1982;150:375–380.
[36] Czekaj P. Phenobarbital-induced expression of cytochrome P450 genes. Acta Biochim Pol 2000;47:1093–1105.
[37] Corcos L, Lagadic-Gossmann D. Gene induction by phenobarbital: an update on an old question that receives key novel answers. Pharmacol Toxicol 2001;89:113–122.
[38] Jung JY, Lee SM. The roles of Kupffer cells in hepatic dysfunction induced by ischemia/reperfusion in rats. Arch Pharm Res 2005;28:1386–1391.
[39] Eum HA, Lee SM. Effects of Trolox on the activity and gene expression of cytochrome P450 in hepatic ischemia/reperfusion. Br J Pharmacol 2004;142:35–42.
[40] Cederbaum AI, Wu D, Mari M, Bai J. CYP2E1-dependent toxicity and oxidative stress in HepG2 cells. Free Radic Biol Med 2001;31:1539–1543.
[41] Brady JF, Wang MH, Hong JY, Xiao F, Li Y, Yoo JS, Ning SM, Lee MJ, Fukuto JM, Gapac JM, Yang CS. Modulation of rat hepatic microsomal monooxygenase enzymes and cytotoxicity by diallyl sulfide. Toxicol Appl Pharmacol 1991;108:342–354.
[42] Fukai M, Hayashi T, Yokota R, Shimamura T, Suzuki T, Taniguchi M, Matsushita M, Furukawa H, Todo S. Lipid peroxidation during ischemia depends on ischemia time in warm ischemia and reperfusion of rat liver. Free Radic Biol Med 2005;38:1372–1381.
[43] Schauer RJ, Gerbes AL, Vonier D, Meissner H, Michl P, Leiderer R, Schildberg FW, Messmer K, Bilzer M. Glutathione protects the rat liver against reperfusion injury after prolonged warm ischemia. Ann Surg 2004;239:220–231.
[44] De Tata V, Brizzi S, Saviozzi M, Lazzarotti A, Fierabracci V, Malvaldi G, Casini A. Protective role of dehydroascorbate in rat liver ischemia-reperfusion injury. J Surg Res 2005;123: 215–221.
[45] Togashi H, Shinzawa H, Yong H, Takahashi T, Noda H, Oikawa K, Kamada H. Ascorbic acid radical, superoxide, and hydroxyl radical are detected in reperfusion injury of rat liver using electron spin resonance spectroscopy. Arch Biochem Biophys 1994;308:1–7.

[46] Grootveld M, Halliwell B. Aromatic hydroxylation as a potential measure of hydroxyl-radical formation *in vivo*. Identification of hydroxylated derivatives of salicylate in human body fluids. Biochem J 1986;237:499–504.
[47] Neve EP, Ingelman-Sundberg M. Intracellular transport and localization of microsomal cytochrome P450. Anal Bioanal Chem 2008;392:1075–1084.
[48] Pon LA. Mitochondria from rat liver. In: Schon EA, editor. Methods in cell biology: Mitochondria. San Diego: Elsevier; 2007. p. 17–22.
[49] Morehouse LA, Thomas CE, Aust SD. Superoxide generation by NADPH-cytochrome P-450 reductase: the effect of iron chelators and the role of superoxide in microsomal lipid peroxidation. Arch Biochem Biophys 1984;232:366–377.

This paper was first published online on Early Online on 12 February 2010.