Acute Doxorubicin Toxicity Differentially Alters Cytochrome P450 Expression and Arachidonic Acid Metabolism in Rat Kidney and Liver

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Abbreviations: DOX, doxorubicin, P450, cytochrome P450, SD, Sprague Dawley, EET, epoxyeicosatrienoic acid, DHET, dihydroxyeicosatrienoic acid, HETE, hydroxyeicosatetraenoic acid, sEH, soluble epoxide hydrolase
Abstract

The use of doxorubicin (DOX) is limited by significant cardiotoxicity, nephrotoxicity, and hepatotoxicity. We have previously shown that DOX cardiotoxicity induces several cardiac cytochrome P450 (P450) enzymes with subsequent alteration in P450-mediated arachidonic acid metabolism. Therefore, in the current study, we investigated the effect of acute DOX toxicity on P450 expression and arachidonic acid metabolism in the kidney and liver of male Sprague Dawley rats. Acute DOX toxicity was induced by a single intraperitoneal injection of 15 mg/kg of the drug. After 6 and 24 h, the kidneys and livers were harvested and the expression of several P450 gene and protein expressions were determined by real time-PCR and Western blot analyses, respectively. Kidney and liver microsomal protein from control or DOX treated rats was incubated with arachidonic acid, and its metabolites were determined by liquid chromatography-electron spray ionization-mass spectrometry. Our results showed that acute DOX toxicity caused an induction of CYP1B1 and CYP4A enzymes and an inhibition of CYP2B1 and CYP2C11 in both the kidney and liver. CYP2E1 was induced and soluble epoxide hydrolase (sEH) was inhibited in the kidney only. In addition, DOX toxicity caused a significant increase in the epoxyeicosatrienoic acids formation in the kidney and a significant increase in 20-hydroxyeicosatetraenoic acid formation in both the kidney and the liver. In conclusion, acute DOX toxicity alters the expression of several P450 and sEH enzymes in an organ-specific manner. These changes can be attributed to DOX-induced inflammation and resulted in altered P450-mediated arachidonic acid metabolism.
Introduction

Doxorubicin (DOX) is a potent anthracycline anti-neoplastic drug used to treat a wide variety of malignancies. However, the clinical use of this agent is limited by a significant dose-dependent cardiotoxicity which may progress to end-stage heart failure (Outomuro et al., 2007). In addition to DOX-induced cardiotoxicity, it also causes nephrotoxicity and hepatotoxicity (Injac et al., 2008; Bulucu et al., 2009). DOX-induced nephrotoxicity causes increased capillary permeability and glomerular atrophy (Injac et al., 2008). Although the exact mechanism of DOX-induced nephrotoxicity is not fully elucidated, it is thought to be mediated through DOX-induced oxidative damage (Liu et al., 2007). Similarly, DOX-induced hepatotoxicity is generally mediated through the generation of free radicals (Bulucu et al., 2009). In addition to oxidative damage, DOX toxicity has been shown to induce inflammatory changes in the heart, kidney, and liver tissues of DOX-administered rats (Deepa and Varalakshmi, 2005).

We have previously shown that DOX-induced cardiotoxicity induces soluble epoxide hydrolase (sEH) and several cytochrome P450 (P450) enzymes in the heart of male Sprague Dawley (SD) rats as well as in the heart-derived H9c2 cells with subsequent alteration of P450-mediated arachidonic acid metabolism (Zordoky and El-Kadi, 2008; Zordoky et al., 2010). In addition, it has been recently reported that DOX activates the aryl hydrocarbon receptor (AhR) with a subsequent induction of CYP1A1 (Volkova et al., 2011). On the other hand, administration of DOX to rats and mice has been shown to decrease the catalytic activity of several hepatic P450 enzymes, although it does not cause mechanism-based inactivation of these enzymes (Di Re et al., 1999). Therefore, the effect of DOX on P450 enzymes seems to be tissue and enzyme specific.
P450 enzymes play an important role in arachidonic acid metabolism in addition to the cyclooxygenase and the lipoxygenase pathways (Roman, 2002). Although the role of P450-derived arachidonic acid metabolites in the cardiovascular physiology and pathophysiology grabbed the major scientific attention (Elbekai and El-Kadi, 2006), their roles in the kidney and liver cannot be ignored. Renal and hepatic P450 epoxygenases metabolize arachidonic acid to different epoxyeicosatrienoic acid (EET) regioisomers, while P450 hydroxylases metabolize it to hydroxyeicosatetraenoic acids (HETEs) (Roman, 2002; Sacerdoti et al., 2003). Furthermore, soluble epoxide hydrolase (sEH) enzyme which catalyzes the conversion of EETs to the less biologically active dihydroxyeicosatrienoic acids (DHETs) is also abundantly expressed in the kidney and liver in both human and experimental animals (Enayetallah et al., 2004; Zordoky et al., 2008).

Several investigators have addressed the role of P450-derived arachidonic acid metabolites in the renal and hepatic function (Maier and Roman, 2001; Sacerdoti et al., 2003). Generally, it has been shown that EETs dilate the preglomerular arterioles, whereas 20-HETE elicits a vasoconstricting effect on these blood vessels (Zhao and Imig, 2003). However, both EETs and 20-HETE have a diuretic effect through inhibiting sodium reabsorption in the proximal tubule (Moreno et al., 2001). Nevertheless, little information is known about the role of these eicosanoids in the liver (Sacerdoti et al., 2003). In contrast to the kidney, 11,12-EET has been shown to have a vasoconstrictive effect on the porto-sinusoidal circulation in rat, while 20-HETE showed a weaker vasoconstricting effect which was cyclooxygenase dependent (Sacerdoti et al., 2003).
addition, EETs were shown to be involved in vasopressin-induced glycogenolysis in rat hepatocytes (Yoshida et al., 1990).

Several studies have reported the modulation of renal and hepatic P450-mediated arachidonic acid metabolism in several pathophysiological and experimental conditions, most notably fasting, increased dietary gamma-linolenic acid, and inflammation (Qu et al., 1998; Yu et al., 2006; Anwar-mohamed et al., 2010; Theken et al., 2011). However, there is little information about the effect of DOX toxicity on the expression of P450 enzymes in the kidney and liver. Therefore, in the present study, we investigated the effect of acute DOX toxicity on the expression of renal and hepatic P450 and sEH enzymes. In addition, we attempted to explore the mechanism by which acute DOX toxicity could alter P450 expression. Finally, we evaluated the effect of DOX toxicity on P450-mediated arachidonic acid metabolism.
Materials and Methods

Materials. High-Capacity cDNA Reverse Transcription Kit, SYBR Green SuperMix, and 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, CA). Real time-PCR primers were synthesized by Integrated DNA Technologies Inc. (San Diego, CA) according to previously published sequences. Arachidonic acid, 4-hydroxybenzophenone, and DOX were purchased from Sigma-Aldrich (St. Louis, MO). Arachidonic acid metabolites standards 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHET, 8,9-DHET, 11,12-DHET, 14,15-DHET and 20-HETE were obtained from Cayman Chemical (Ann Arbor, MI). Reagents used for liquid chromatographic-electron spray ionization-mass spectrometry (LC-ESI-MS) were at HPLC-grade. Acetonitrile and water (HPLC grade) were purchased from EM Scientific (Gibbstawn, NJ). Acrylamide, N'N'-bis-methylene-acrylamide, ammonium persulphate, β-mercaptoethanol, glycine, nitrocellulose membrane (0.45 µm), and TEMED were purchased from Bio-Rad Laboratories (Hercules, CA). Chemiluminescence Western blotting detection reagents were purchased from GE Healthcare Life Sciences (Piscataway, NJ). CYP1B1 rabbit polyclonal primary antibody was purchased from BD Gentest (Bedford, MA). CYP2J and sEH primary antibodies were obtained as generous gifts from Dr Darryl Zeldin (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC) and Dr Bruce Hammock (Department of Entomology, University of California, Davis, CA), respectively. Other primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemicals were purchased from Fisher Scientific Co. (Toronto, ON, Canada).
Animals. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Sprague Dawley (SD) rats weighing 250-300 g were obtained from Charles River Canada (St. Constant, QC, Canada). Animals were treated intraperitoneally (IP) with a single 15 mg/kg DOX (n = 12). Weight-matched controls received the same volume of normal saline (n = 12). Animals were euthanized at 6 and 24 h following the treatment under isoflurane anesthesia. All animals were allowed free access to food and water throughout the treatment period. The amount of food consumed by each animal was recorded and the animals were weighed before and 24 h after DOX administration. The kidneys and livers were excised, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

RNA extraction and cDNA synthesis. Total RNA from the frozen tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio. Thereafter, first-strand cDNA synthesis was performed by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Briefly, 1.5 µg of total RNA from each sample was added to a mix of 2.0 µl 10X RT buffer, 0.8 µl 25X dNTP mix (100 mM), 2.0 µl 10X RT random primers, 1.0 µl MultiScribe™ reverse transcriptase, and 3.2 µl nuclease-free water. The final reaction mix was kept at 25°C for 10 min, heated to 37°C for 120 min, heated for 85°C for 5 sec, and finally cooled to 4°C.
Quantification by real time-PCR. Quantitative analysis of specific mRNA expression was performed by real time-PCR, by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). 25-µl reaction mix contained 0.1 µl of 10 µM forward primer and 0.1 µl of 10 µM reverse primer, 12.5 µl of SYBR Green Universal Mastermix, 11.05 µl of nuclease-free water, and 1.25 µl of cDNA sample. The primers used in the current study were chosen from previously published studies (Anwar-mohamed et al., 2010) and are listed in Table 1. No-template controls were incorporated onto the same plate to test for the contamination of any assay reagents. An optical adhesive cover was used to seal the plate; thereafter, thermocycling conditions were initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. Dissociation curves were performed by the end of each cycle to confirm the specificity of the primers and the purity of the final PCR product.

Real time-PCR Data analysis. The real time-PCR data were analyzed using the relative gene expression method as described in Applied Biosystems User Bulletin No.2. Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene (β-actin) and relative to the untreated control of the same time point.

Microsomal protein preparation and Western blot analysis. Microsomal protein was prepared from the kidney and liver tissues as described previously (Aboutabl et al., 2009). Briefly, kidney and liver tissues were washed in ice-cold KCL (1.15% w/v), cut
into pieces, and homogenized separately in cold sucrose solution (1g of tissue in 5 mL of 0.25 M sucrose). Microsomal protein from the homogenized tissues was separated by differential ultracentrifugation. The final microsomal pellet was reconstituted in cold sucrose and stored at -80°C. Kidney and liver microsomal protein concentration was determined by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951). Western blot analysis was performed according to a previously described method (Gharavi and El-Kadi, 2005). Briefly, 2.5 - 40 μg of kidney and liver microsomal protein from each treatment group was separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C in blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (TBS), 5% skim milk, 2% bovine serum albumin, and 0.5% Tween-20. After blocking, the blots were incubated with a primary polyclonal rabbit anti-rat CYP1B1, mouse anti-rat CYP2B1, rabbit anti-rat CYP2C11, rabbit anti-rat CYP2E1, rabbit anti-mouse CYP2J, mouse anti-rat CYP4A, rabbit anti-mouse sEH and rabbit anti-rat actin for 2 h. Incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody for CYP1B1, CYP2C11, CYP2E1, CYP2J, sEH, and actin, or goat anti-mouse IgG secondary antibody for CYP2B1 and CYP4A was carried out for 1 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer’s instructions (GE Healthcare Life Sciences, Piscataway, NJ). The intensity of the protein bands were quantified, relative to the signals obtained for actin, using ImageJ software [National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij].
Microsomal incubation and separation of different arachidonic acid metabolites by LC-ESI-MS. Kidney and liver microsomes (1 mg protein/ml) were incubated in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH=7.4) at 37°C in a shaking water bath (50 rpm). A pre-equilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH. Arachidonic acid was added to a final concentration of 50 µM and incubated for 30 min. The reaction was terminated by the addition of 600 µL ice cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. Arachidonic acid metabolites were extracted by 1 ml ethyl acetate twice and dried using speed vacuum (Savant, Farmingdale, NY). Kidney and liver tissue concentrations of arachidonic acid metabolites were determined according to a previously published method (Poloyac et al., 2004) with some modifications. Briefly, kidney or liver tissues (1 g) were homogenized in the incubation buffer and centrifuged at 10,000g for 30 min. After the addition of acetonitrile, the supernatant was extracted twice with 1 ml ethyl acetate and dried using speed vacuum (Savant, Farmingdale, NY).

Extracted arachidonic acid metabolites were analyzed using LC-ESI-MS (Waters Micromass ZQ 4000 spectrometer) method as described previously (Aboutabl et al., 2009). Briefly, the mass spectrometer was operated in negative ionization mode with single ion recorder acquisition. The nebulizer gas was acquired from an in house high purity nitrogen source. The source temperature was set at 150 °C, and the voltages of the capillary and the cone were 3.51 KV and 25 V, respectively. The samples (10 µL) were separated on reverse phase C18 column (Kromasil, 250 x 3.2 mm) using linear gradient mobile phase system with a mobile phase of water/acetonitrile with 0.005% acetic acid at
flow rate of 0.2 mL/min. The mobile phase system started at 60% acetonitrile, linearly increased to 80% acetonitrile in 30 min, increased to 100% acetonitrile in 5 min, and held for 5 min.

Nitrite assay. Nitrites were measured in serum samples of control and 24 h DOX treated rats as described previously (Nussler et al., 2006). Briefly, 200 μM working nitrite standard was prepared from a 2.0 mM sodium nitrite stock solution in endotoxin-free deionized water, and stored at 4°C until use. A working 2,3-diaminonaphthalene (DAN) solution of 50 μg/mL was prepared by diluting a 20 mg/mL stock solution with 0.62N HCl. All assays were conducted in 96-well plates, with each condition performed in quadruplet. In each well, 20 μL of standard or sample was added to 80 μL of deionized water. Then, 10 μL of working DAN solution was added to each well. The plate was allowed to sit in the dark at room temperature for 10 min. Using the same timing and sequence as for the additions of DAN, 20 μL of 2.8 N NaOH was added to each well, and the plate was gently shaken. The plate then was incubated in the dark for 1 min, following which it was read in a fluorescence plate reader with an excitation of 360 nm and an emission of 430 nm.

Statistical analysis. Data are presented as mean ± standard error of the mean (SEM). Control and 24 h treatment measurements were compared using student t-test. Whereas, control and treatment measurements at 6 and 24 h were compared using one way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test. A result was considered statistically significant where p < 0.05.
Results

Effect of DOX treatment on food intake and animal body weight.

Taking into account the possible effect of fasting on P450 expression, we investigated the effect of DOX treatment on the food intake and animal body weight. The food intake in DOX-treated animals was significantly reduced by almost 50% from the control. In addition, DOX-treated animals lost an average of 5% body weight as compared to the control.

Effect of DOX treatment on P450 gene expression.

To examine the effect of DOX treatment on the renal and hepatic expression of several P450 genes, total RNA was extracted from the kidney and liver of both control and DOX-treated rats. Thereafter, the expression of different genes was measured using reverse transcription followed by real time-PCR.

Figure 1 shows the effect of DOX-induced toxicity on CYP1 family gene expression at 6 and 24 h after DOX administration. DOX treatment did not cause a statistically significant difference in CYP1A1 gene expression in both the kidney and liver (Fig. 1A). On the other hand, DOX treatment caused a significant induction of CYP1B1 gene expression in the kidney by 100% after 24 h of DOX administration. In addition, DOX caused a significant induction of CYP1B1 in the liver by 250% and 200% at the 6 and 24 h, respectively (Fig. 1B). With regard to CYP2 family, DOX treatment caused a significant inhibition of CYP2C11 gene expression in the kidney and liver by 70% and 50% after 24 h, respectively (Fig. 2A). Similarly, there was a significant inhibition of CYP2B1 gene expression in the kidney and liver by 45% and 80% after 24 h, respectively.
(Fig. 2B). In contrast to CYP2B1 and CYP2C11, DOX treatment for 24 h caused a significant induction of CYP2E1 gene expression by 150% in the kidney only (Fig. 2C). Finally, DOX treatment for 24 h caused a significant inhibition in the gene expression of CYP2J3 by 55% in the liver only (Fig. 2D).

With regard to the major CYP ω-hydrolases, the gene expression of CYP4A and CYP4F was assessed. Figure 3 shows the effect of DOX-induced toxicity on the gene expression of CYP4A at 6 and 24 h after DOX administration. Interestingly, DOX administration caused a significant induction of CYP4A1 in the kidney by 500% at the 24 h time point (Fig. 3A). In a similar manner, DOX-toxicity caused a significant induction of CYP4A2 in the kidney at 24 h and in the liver at 6 h by 100% and 230%, respectively (Fig. 3B). Finally, there was a significant induction of CYP4A3 in the kidney at the 24 h time point; whereas, it was significantly induced in the liver by 220% and 70% at the 6 and 24 h, respectively. In a similar pattern to CYP4A gene expression, acute DOX toxicity caused a significant induction of CYP4F1 by 60% only in the kidney at 24 h (Fig. 4A). On the contrary, acute DOX toxicity caused a 40% inhibition of CYP4F4 gene expression only in the liver at both 6 and 24 h (Fig. 4B). Also, CYP4F5 was significantly inhibited in the kidney of DOX-treated rats at the 6 h time point by about 35% (Fig. 4C). Finally, there was no significant difference in CYP4F6 gene expression in both the kidney and liver at the two time points (Fig. 4D).

Effect of DOX treatment on EPHX2 gene expression.

Because of its important role in converting EETs to the less biologically active DHETs, we investigated the effect of acute DOX toxicity on the expression of the
EPHX2 gene which encodes for the sEH enzyme. In the current study, acute DOX toxicity did not cause any significant change in EPHX2 gene expression in the kidney at both the 6 and 24 h time points. However, there was a significant induction of the EPHX2 gene expression by 160% in the liver only at the 6 h time point but not at the 24 h (Fig. 5).

**Effect of DOX treatment on P450 and sEH protein expression.**

To investigate whether the changes of P450 and EPHX2 gene expression was further translated into protein, microsomal protein was prepared from kidneys and livers of control and rats treated with DOX for 24 h. Thereafter, CYP1B1, CYP2B1, CYP2C11, CYP2E1, CYP2J3, and CYP4A protein levels were determined using Western blot analyses relative to actin as a loading control. In the kidney, DOX treatment caused a significant inhibition of both CYP2B1 and sEH by about 60%; whereas, CYP2E1 and CYP4A protein expression was induced by 90% and 110%, respectively. On the other hand, there was no significant change in the protein expression of CYP1B1, CYP2C11, and CYP2J3 (Fig. 6). In the liver, DOX treatment caused an inhibition of CYP2B1 and CYP2C11 by 74% and 65%, respectively; while, there was a significant induction of CYP4A by 370%. The protein expression of CYP2E1, CYP2J3, and sEH was not significantly altered (Fig. 7). CYP1B1 protein could not be detected in the liver under the current experimental conditions.
Effect of DOX treatment on the inflammatory markers.

In an attempt to investigate the mechanism by which DOX causes the aforementioned changes in P450 and sEH expression, the gene expression of three major inflammatory markers, IL-6, iNOS, and TNFα were assessed in the liver of DOX treated rats as compared to the control. Interestingly, there was a significant induction of the three inflammatory markers IL-6, iNOS, and TNFα by 2700%, 225%, 100%, respectively after 6 h of DOX administration. Moreover, there was a higher induction of the IL-6, iNOS, and TNFα by 5700%, 1500%, 260%, respectively after 24 h of DOX administration (Fig. 8A). In order to investigate whether DOX caused a systemic inflammation, we measured the serum nitrite level in the control and rats treated with DOX for 24 h. Consistent with the gene expression data, there was a significant increase of nitrite in the sera of DOX treated rats as compared to the control by about 110% (Fig. 8B).

Effect of DOX treatment on P450-mediated arachidonic acid metabolism.

To investigate the effect of DOX treatment on the formation of P450-derived arachidonic acid metabolites, kidney and liver microsomes of either control or 24 h DOX-treated rats were incubated with 50 µM arachidonic acid for 30 min. Thereafter, arachidonic acid metabolites were measured using LC-ESI-MS. In kidney microsomes of DOX-treated rats, the formation of 8,9-, 11,12- and 14,15-EET were significantly higher than the control values by about 165%, 240%, and 250%, respectively (Fig. 9A). We also measured the levels of enzymatic hydration products of EETs, the DHETs. As shown in
In order to investigate the effect of DOX on the total epoxygenase activity, we calculated the sum of all the products of epoxygenase enzymes, the total EETs and DHETs, in control and rats treated with DOX for 24 h. The total epoxygenase activity was not significantly changed in the kidney microsomes of DOX-treated rats as compared to the control (Fig. 10A). In accordance with the inhibition of sEH protein expression, DOX treatment caused a significant 45% inhibition of the sEH activity (calculated as the total DHETs / total EETs) (Fig. 10B). On the other hand, to determine the effect of DOX treatment on P450 \( \omega \)-hydroxylases activity, we determined the rate of 20-HETE formation in the kidney microsomes from control and DOX-treated rats. DOX treatment significantly increased the 20-HETE formation by 120% in comparison to the control group (Fig. 10C).

In liver microsomes of DOX-treated rats, there was no significant change in the formation rate of all the EETs and DHETs regioisomers compared to the control (Fig. 11). Similarly, both the total epoxygenase activity and sEH activity were not significantly changed in the liver microsomes of DOX-treated rats as compared to the control (Fig. 12A and B). On the contrary, DOX treatment significantly increased the 20-HETE formation by 40% in comparison to the control group (Fig. 12C).

**Effect of acute DOX toxicity on endogenous arachidonic acid metabolites concentrations.** To investigate the effect of the altered P450 activity on the endogenous arachidonic acid metabolite concentration, kidney and liver tissues were extracted to
measure the endogenous concentrations of these metabolites. Out of the 9 metabolites under investigation, we were able to quantify 8,9-DHET, 11,12-DHET, 14,15-DHET, and 20-HETE. The other metabolites were not detected or below the limit of quantification. In the kidney, there was a significant decrease in the endogenous concentration of the DHETs, whereas there was no significant change in the 20-HETE concentration (Fig. 13A). On the other hand, there was no significant change in the DHETs concentrations in the liver, whereas there was a significant 2-fold increase in the 20-HETE concentration (Fig 13B).
Discussion

The current study demonstrates for the first time the effect of DOX toxicity on the expression of renal and hepatic P450 enzymes and P450-mediated arachidonic acid metabolism. In the present study, acute DOX toxicity has been induced by a single IP injection of 15 mg/kg of the drug in male SD rats. This dose has been shown previously to induce cardiotoxicity, nephrotoxicity, and hepatotoxicity (Injac et al., 2008; Zordoky et al., 2010). In the current study, acute DOX toxicity caused a significant induction of the inflammatory markers IL-6, iNOS, and TNFα in the liver as early as 6 h after DOX administration preceding most of the changes in P450 enzymes expression. Although DOX toxicity has been shown previously to increase the biochemical markers of inflammation such as C-reactive protein and fibrinogen (Deepa and Varalakshmi, 2005), this is the first time to demonstrate the effect of DOX toxicity on the gene expression of inflammatory markers.

In the current study, acute DOX toxicity significantly increased CYP1B1 gene expression in both the kidney and liver. Interestingly, DOX has been shown to activate the AhR in the cardiac derived H9c2 cells and in the heart of male SD rats (Volkova et al., 2011). Similarly, we have previously shown that DOX induces the AhR-regulated genes, CYP1A1 and CYP1B1, in H9c2 cells as well as the rat heart (Zordoky and El-Kadi, 2008; Zordoky et al., 2010). However, the induction of CYP1B1 gene expression in the kidney and liver by DOX cannot be simply attributed to AhR activation because CYP1A1 which is also regulated through the AhR was not induced. DOX-induced inflammation may be a more relevant explanation for CYP1B1 induction, as it has been previously reported that LPS-induced inflammation causes a significant induction of CYP1B1 in the
liver of male SD rats (Anwar-mohamed et al., 2010). Mechanistically, it was postulated that inflammation induces \textit{CYP1B1} gene expression through the hormonal pathway that also regulates CYP1B1 (Malaplate-Armand et al., 2003). Finally it is still to be determined whether the effect of DOX to activate the AhR is heart specific or it is ubiquitous.

On the contrary to \textit{CYP1B1}, DOX toxicity caused a significant inhibition of both \textit{CYP2B1} and \textit{CYP2C11} in the kidney and liver, whereas there was a significant inhibition of \textit{CYP2J3} gene expression only in the liver. CYP2B1, CYP2C11, and CYP2J3 are important epoxygenase enzymes that metabolize arachidonic acid to several EETs regioisomers (Kroetz and Zeldin, 2002). Several investigators have also reported the down-regulation of these enzymes in different models of inflammation (Iber et al., 2001; Li-Masters and Morgan, 2001; Anwar-mohamed et al., 2010). Therefore, we can attribute the inhibition of CYP2B1 and CYP2C11 to DOX-induced inflammation. Interestingly, CYP2C9 activity was decreased by 315\% in breast cancer patients receiving doxorubicin/cyclophosphamide chemotherapy (Elkiran et al., 2007). On the other hand, DOX-induced toxicity caused a significant induction of CYP2E1 in the kidney at both the mRNA and the protein level, whereas there was no significant change in the liver. Similar results have been reported previously in models of irritant-induced inflammation (Iber et al., 1999). It is important to mention that \(\omega-1\) hydroxylation and 19-HETE formation were not changed in the kidney by CYP2E1 induction most probably due to the low basal expression of CYP2E1 as compared to the total P450 content in the kidney (Poloyac et al., 2004).
With regard to P450 \( \omega \)-hydroxylase enzymes, DOX-induced toxicity caused a significant induction of CYP4A enzymes in both the kidney and the liver of male SD rats. In agreement with these results, we have previously shown that DOX-induced cardiotoxicity causes significant induction of CYP4A enzymes in the heart of male SD rats (Zordoky et al., 2010). In addition, we and other investigators have also demonstrated that CYP4A enzymes are induced in the kidney and liver in several models of inflammation (Mitchell et al., 2001; Anwar-mohamed et al., 2010; Theken et al., 2011). Moreover, it is well established that fasting can induce CYP4A enzymes (Kroetz et al., 1998). Therefore, we can attribute CYP4A induction to be mediated, at least in part, by DOX-induced inflammation and/or reduction in food consumption. Nevertheless, the increased 20-HETE formation may worsen the inflammatory condition by activating NF-\( \kappa \)B and increasing the generation of inflammatory cytokines in a positive feedback circuit (Ishizuka et al., 2008). On the contrary to CYP4A enzymes, the effect of DOX on CYP4F enzymes was isoform specific. DOX caused a significant induction of CYP4F1 in the kidney and an inhibition of CYP4F4 and CYP4F5 in the liver and kidney, respectively. While, there was no change in CYP4F6 expression in the kidney and liver. Both CYP4F1 and CYP4F4 have been shown to be inhibited in a rat model of LPS-induced inflammation (Anwar-mohamed et al., 2010). Therefore, the observed effect of DOX toxicity on \( CYP4F \) gene expression is less likely to be attributed to DOX-induced inflammation.

We have previously reported that DOX induces sEH in H9c2 cells and the rat heart (Zordoky et al., 2010). Therefore, it was important to investigate the effect of DOX on the expression of sEH in organs other than the heart to examine whether sEH
induction is heart specific or not. In the present study, DOX caused a significant induction of \textit{EPHX2} gene only in the liver after 6 h of DOX administration; however, there was no change in sEH protein expression in the liver. In the kidney, there was a significant inhibition in sEH protein expression through a post-transcriptional mechanism, because DOX did not change the \textit{EPHX2} gene expression. These results demonstrate that DOX effect on sEH is tissue-specific and DOX-induced sEH protein expression and activity is specific to the heart which further confirms its role in DOX specific cardiotoxicity.

In the current study, our results show that DOX administration caused a significant increase in the 8,9-, 11,12 and 14,15-EETs formation in the kidney, although it did not change the total epoxygenase activity. Therefore, the increase in the EETs formation can be attributed to the inhibition of sEH activity which in turn is explained by the inhibition of sEH protein expression in the kidney of DOX-treated rats. Although there was no significant change in the DHETs formation rate in the \textit{in vitro} microsomal incubation experiment, there was a significant decrease in the endogenous concentration of DHETs in the kidney tissue which may be attributed to the inhibition of sEH expression. Unfortunately, we could not detect the endogenous EETs in the kidney tissues to estimate sEH activity. Generally, EETs are considered beneficial endogenous compounds, because they have vasodilator, anti-inflammatory, anti-apoptotic, and natriuretic effects (Elbekai and El-Kadi, 2006). Therefore, we can assume that the inhibition of sEH expression and activity in the kidney with the subsequent increase in EETs formation is an adaptive response to protect the kidney against DOX toxicity. Despite the inhibition of some epoxygenase enzymes in both the kidney and liver, there
was no significant change in the total epoxygenase activity. This could be explained by the fact that the induced CYP4A enzymes have also some epoxygenase activity (Nguyen et al., 1999) which may compensate for the decrease in other enzymes activity. Similar to the heart, DOX causes a significant increase in the renal and hepatic ω-hydroxylase activity which is attributed to CYP4A induction. CYP4A enzymes are the major ω-hydroxylase enzymes which metabolize arachidonic acid to 20-HETE (Roman, 2002). Parallel to the observed increase in the 20-HETE formation rate in the liver microsomes, there was a significant increase in the endogenous 20-HETE concentration in the liver tissue. However, there was no change in the endogenous 20-HETE concentration in the kidney tissue despite the increased 20-HETE formation rate in the kidney microsomes. This discrepancy could be attributed to an altered availability of the free arachidonic acid substrate in the kidney tissue, competition from other enzymatic pathways, and/or altered secondary metabolism of the formed 20-HETE. Interestingly, 20-HETE has been shown to mediate cytotoxicity and apoptosis in ischemic kidney epithelial cells and its inhibition protects the kidney from ischemia/reperfusion injury (Nilakantan et al., 2008; Hoff et al., 2011). Therefore, we can conclude that the induction of CYP4A enzymes and the subsequent increase in 20-HETE formation is a maladaptive response to DOX toxicity that will participate, at least in part, in the deterioration of the renal and hepatic function.

In conclusion, acute DOX toxicity alters the expression of several P450 and sEH enzymes in an organ-specific manner. Although the exact mechanism is not fully elucidated yet, DOX-induced inflammation contributes, at least in part, in the alteration of P450 enzymes by DOX. The changes in P450 and sEH expression resulted in altered arachidonic acid metabolism as the 20-HETE formation was increased in both the kidney
and the liver; whereas, EETs formation was increased only in the kidney. Taking into account the physiological functions of these metabolites, the increase in the EETs and 20-HETE formation can be considered an adaptive and a maladaptive response, respectively.
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Authorship Contributions

Participated in research design: Zordoky and El-Kadi.

Conducted experiments: Zordoky, Anwar-Mohamed, and Aboutabl.

Performed data analysis: Zordoky and Anwar-Mohamed.

Wrote or contributed to the writing of the manuscript: Zordoky and El-Kadi.
References:


Footnotes:

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Figure legends

**Fig. 1. Effect of DOX toxicity on CYP1 family gene expression.** Total RNA was isolated from the kidneys and livers of control and animals treated with DOX after 6 and 24 h. CYP1A1 (A) and CYP1B1 (B) gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 6). * P < 0.05 compared to control.

**Fig. 2. Effect of DOX toxicity on CYP2 family gene expression.** Total RNA was isolated from the kidneys and livers of control and animals treated with DOX after 6 and 24 h. CYP2B1 (A), CYP2C11 (B), CYP2E1 (C), and CYP2J3 (D) gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 6). * P < 0.05 compared to control.

**Fig. 3. Effect of DOX toxicity on CYP4A subfamily gene expression.** Total RNA was isolated from the kidneys and livers of control and animals treated with DOX after 6 and 24 h. CYP4A1 (A), CYP4A2 (B), and CYP4A3 (C) gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 6). * P < 0.05 compared to control.

**Fig. 4. Effect of DOX toxicity on CYP4F subfamily gene expression.** Total RNA was isolated from the kidneys and livers of control and animals treated with DOX after 6 and 24 h. CYP4F1 (A), CYP4F4 (B), CYP4F5 (C), and CYP4F6 (D) gene expressions were
Fig. 5. Effect of DOX toxicity on *EPHX2* gene expression. Total RNA was isolated from the kidneys and hearts of control and animals treated with DOX after 6 and 24 h. *EPHX2* gene expression was determined by real-time PCR. Results are presented as mean ± SE (n = 6). * P < 0.05 compared to control.

Fig. 6. Effect of DOX toxicity on P450 and sEH protein expression in the kidney. Kidney microsomal protein was isolated from the kidney of control and animals treated with DOX for 24 h. Microsomal protein was separated on a 10% SDS-PAGE. CYP1B1, CYP2B1, CYP2C11, CYP2E1, CYP2J, CYP4A, sEH, and Actin proteins were detected by the enhance chemiluminescence method. The graph represents the relative amount of protein normalized to the loading control (mean ± SE, n = 3), and the results are expressed as percentage of the control values taken as 100%. * P < 0.05 compared with control.

Fig. 7. Effect of DOX toxicity on P450 and sEH protein expression in the liver. Liver microsomal protein was isolated from the kidney of control and animals treated with DOX for 24 h. Microsomal protein was separated on a 10% SDS-PAGE. CYP2B1, CYP2C11, CYP2E1, CYP2J, CYP4A, sEH, and Actin proteins were detected by the enhance chemiluminescence method. The graph represents the relative amount of protein...
normalized to the loading control (mean ± SE, n = 3), and the results are expressed as percentage of the control values taken as 100%. * P < 0.05 compared with control.

**Fig. 8. Effect of DOX toxicity on inflammatory markers gene expression (A) and serum nitrite level (B).** (A) Total RNA was isolated from the livers of control and animals treated with DOX after 6 and 24 h. IL-6, iNOS, and TNFα gene expressions were determined by real-time PCR. (B) Nitrite level was determined in the sera of control and animals treated with DOX for 24 h as described under materials and methods. Results are presented as mean ± SE (n = 6). * P < 0.05 compared to control.

**Fig. 9. Effect of DOX toxicity on EETs (A) and DHETs formation (B) in the kidney.** Kidney microsomes of control or animals treated with DOX for 24 h were incubated with 50 μM arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. EETs and DHETs were extracted twice by 1 ml of ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into LC-ESI-MS for metabolite determination. Results are presented as mean ± SE (n = 6). * P < 0.05 compared with control.

**Fig. 10. Effect of DOX toxicity on the total epoxygenase (A), sEH (B), and ω-hydroxylase activity (C) in the kidney.** (A) The total epoxygenase activity was determined from the sum of EETs and DHETs formation. (B) sEH activity was determined by dividing the total DHETs over the total EETs. (C) ω-hydroxylase activity
was determined from the 20-HETE formation. Kidney microsomes of control or animals treated with DOX for 24 h were incubated with 50 μM arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. 20-HETE was extracted twice by 1 ml of ethyl acetate and dried using speed vacuum. Reconstituted metabolite was injected into LC-ESI-MS for metabolite determination. Results are presented as mean ± SE (n = 6). * P < 0.05 compared with control.

Fig. 11. Effect of DOX toxicity on EETs (A) and DHETs formation (B) in the liver. Liver microsomes of control or animals treated with DOX for 24 h were incubated with 50 μM arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. EETs and DHETs were extracted twice by 1 ml of ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into LC-ESI-MS for metabolite determination. Results are presented as mean ± SE (n = 6). * P < 0.05 compared with control.

Fig. 12. Effect of DOX toxicity on the total epoxygenase (A), sEH (B), and ω-hydroxylase activity (C) in the liver. (A) The total epoxygenase activity was determined from the sum of EETs and DHETs formation. (B) sEH activity was determined by dividing the total DHETs over the total EETs. (C) ω-hydroxylase activity was determined from the 20-HETE formation. Liver microsomes of control or animals treated with DOX for 24 h were incubated with 50 μM arachidonic acid. The reaction
was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. 20-HETE was extracted twice by 1 ml of ethyl acetate and dried using speed vacuum. Reconstituted metabolite was injected into LC-ESI-MS for metabolite determination. Results are presented as mean ± SE (n = 6). * P < 0.05 compared with control.

Fig. 13. Effect of acute DOX toxicity on endogenous arachidonic acid metabolites concentrations in the kidney (A) and Liver (B). After homogenization and centrifugation, endogenous arachidonic acid metabolites were extracted twice from kidney and liver tissues by 1 ml of ethyl acetate and dried using speed vacuum. Reconstituted metabolite was injected into LC-ESI-MS for metabolite determination. Results are presented as mean ± SE (n = 6). * P < 0.05 compared with control.
Table 1. Primers sequences used for real time-PCR reactions

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<th>Gene</th>
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<th>Reverse Primer</th>
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Fig. 5

![Graph showing the expression of EPHX2 in kidney and liver tissues with different treatments.](image-url)
Fig. 8

(A) Log fold of induction (relative to β-actin)

(B) Serum Nitrite Level (μM)
Fig. 13

(A) Metabolite Conc. (pmol/g tissue) for 8,9-DHET, 11,12-DHET, 14,15-DHET, and 20-HETE under Control and DOX conditions. The bars show significant differences (*).

(B) Metabolite Conc. (pmol/g tissue) for 8,9-DHET, 11,12-DHET, 14,15-DHET, and 20-HETE under Control and DOX conditions. The bar for 20-HETE under DOX shows a significant increase (*).