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

Beshay N. M. Zordoky, Anwar Anwar-Mohamed, Mona E. Aboutabl, and Ayman O. S. El-Kadi

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada

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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 (15 mg/kg) of the drug. After 6 and 24 h, the kidneys and livers were harvested, and several P450 gene and protein expressions were determined by real-time polymerase chain reaction 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 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 antineoplastic 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 (Otomura 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 has not been fully elucidated, it is thought to be mediated through DOX-induced oxidative damage (Liu et al., 2007). Likewise, 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 shown previously 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 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

ABBREVIATIONS: DOX, doxorubicin; sEH, soluble epoxide hydrolase; P450, cytochrome P450; SD, Sprague-Dawley; AhR, aryl hydrocarbon receptor; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; DHET, dihydroxyeicosatetraenoic acid; PCR, polymerase chain reaction; LC-ESI-MS, liquid chromatographic-electron spray ionization-mass spectrometry; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; DANT, 2,3-diaminophthalene; IL-6, interleukin-6; iNOS, inducible nitric-oxide synthase; TNFα, tumor necrosis factor-α.
grabbed the major scientific attention (Elbekai and El-Kadi, 2006), their roles in the kidney and liver cannot be ignored. Renal and hepatic P450 epoxidegenses metabolize arachidonic acid to different epoxyeicosatrienoic acid (EET) regioisomers, whereas P450 hydroxylases metabolize it to hydroxyeicosatetraenoic acids (HETEs) (Roman, 2002; Sacerdoti et al., 2003). Furthermore, 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 (Enayatifallah et al., 2004; Zordoky et al., 2008).

Several investigators have addressed the role of P450-derived arachidonic acid metabolites in renal and hepatic function (Maier and Roman, 2001; Sacerdoti et al., 2003). In general, it has been shown that EETs dilate the precapillary arterioles, whereas 20-HETE elicits a vasoconstrictive effect in these blood vessels (Zhao and Imlig, 2003). However, both EETs and 20-HETE have a diuretic effect through inhibition of 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 portosinusoidal circulation in rat, whereas 20-HETE showed a weaker vasoconstricting effect that was cyclooxygenase-dependent (Sacerdoti et al., 2003). In addition, EETs were shown to be involved in vasopressin-induced glycosylation 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 γ-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-mediated P450-mediated arachidonic acid metabolism.

Materials and Methods

Materials. The 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. (Coralville, IA) according to previously published sequences. Arachidonic acid, 4-hydroxybenzophenone, and DOX were purchased from Sigma-Aldrich (St. Louis, MO). Arachidonic acid metabolite 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 HPLC-grade acetonitrile and water (HPLC-grade) were purchased from EM Scientific (Gibbstown, NJ). Acrylamide, N,N′-bis-methylene-acrylamide, ammonium persulfate, β-mercaptoethanol, glycine, nitrocellulose membrane (0.45 μm), and N,N,N′,N′-tetramethylmethylenediamine were purchased from Bio-Rad Laboratories (Hercules, CA). Chemiluminescence Western blotting detection reagents were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). CYP1B1 rabbit polyclonal primary antibody was purchased from BD Gentest (Woburn, MA). CYP2J2 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 Hambrock (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 Thermo Fisher Scientific (Waltham, MA).

Animals. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male SD rats weighing 250 to 300 g were obtained from Charles River Canada (St. Constant, QC, Canada). Animals were treated intraperitoneally with a single 15 mg/kg dose of DOX (n = 12). Weight-matched controls received the same volume of normal saline (n = 12). Animals were euthanized under isoflurane anesthesia at 6 and 24 h after the treatment. 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, immersed 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, Carlsbad, CA) 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 according to the manufacturer’s instructions. In brief, 1.5 μg of total RNA from each sample was added to a mix of 2.0 μl of 10× reverse transcriptase buffer, 0.8 μl of 25× dNTP mix (100 mM), 2.0 μl of 10× reverse transcriptase random primers, 1.0 μl of MultiScribe reverse transcriptase, and 3.2 μl of nuclelease-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 s, and finally cooled to 4°C.

Quantification by Real-Time PCR. Quantitative analysis of specific mRNA expression was performed with real-time PCR, by subjecting the resultant cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). The 25-μl reaction mix contained 0.1 μl of 10× M forward primer and 0.1 μl of 10× M reverse primer, 12.5 μl of SYBR Green Universal MasterMix, 11.05 μl of nuclelease-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 s 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. In brief, 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 kidney and liver tissues as described previously (Aboutabl et al., 2009). In brief, kidney and liver tissues were washed in ice-cold KCl (1.15% w/v), cut into pieces, and homogenized separately in cold sucrose solution (1 g 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. The kidney and liver microsomal protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Western blot analysis was performed according to a method described previously (Gharaei and El-Kadi, 2005). In brief, 2.5 to 40 μg of kidney and liver microsomal protein from each treatment group was separated by 10% SDS-polyacrylamide gel (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 (Tris-buffered saline), 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 CYP2J2, 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, CYP2J2, sEH, and actin
Arachidonic acid was added to a final concentration of 50 μM. Metabolites by LC-ESI-MS. 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 of ice-cold acetoni-trile followed by the internal standard, 4-hydroxybenzophenone. Arachidonic acid metabolites were extracted by 1 ml of ethyl acetate and dried using a speed vacuum (Thermo Fisher Scientific). Kidney and liver tissue concentrations of arachidonic acid metabolites were determined according to a previously published method (Poloyac et al., 2004) with some modifications. In brief, kidney or liver tissues (1 g) were homogenized in the incubation buffer and centrifuged at 10,000g for 30 min. After the addition of acetoni-trile, the supernatant was extracted twice with 1 ml of ethyl acetate and dried using a speed vacuum (Thermo Fisher Scientific).

Extracted arachidonic acid metabolites were analyzed using the LC-ESI-MS (Micromass QZ 4000 spectrometer; Waters, Milford, MA) method as described previously (Aboutabl et al., 2009). In brief, 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 mass spectrometer was operated in negative ionization mode with single ion recorder acquisition. The samples (10 μl) were injected into the mass spectrometer and were monitored for 1 min, after which it was read in a fluorescence plate reader with an excitation of 360 nm and an emission of 430 nm. A result was considered statistically significant when \( P < 0.05 \).

### 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 compared with the controls.

#### 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 either the kidney or 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 6 and 24 h, respectively (Fig. 1B). With regard to the 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). Likewise, 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 P450 ω-hydroxylases, 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. Of interest, 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 6 and 24 h, respectively. In a pattern similar to that for 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). In addition, CYP4F5 was significantly inhibited in the kidney of DOX-treated rats at the 6-h time point by approximately 35% (Fig. 4C). Finally, there was no significant difference in CYP4F6 gene expression in either the kidney or 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 either the 6- or 24-h time points. However, there was a significant induction of EPHX2 gene expression by 160% in the liver only at the 6-h time point but not at the 24-h time point (Fig. 5).

**Effect of DOX Treatment on P450 and sEH Protein Expression.** To investigate whether the changes in P450 and EPHX2 gene expression were 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 approximately 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

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FIG. 1. Effect of DOX toxicity on CYP1 family gene expression. Total RNA was isolated from the kidneys and livers of controls 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 means ± S.E. (n = 6). *, P < 0.05 compared with control.

FIG. 2. Effect of DOX toxicity on CYP2 family gene expression. Total RNA was isolated from the kidneys and livers of controls 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 means ± S.E. (n = 6). *, P < 0.05 compared with control.
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 was induced by a single intraperitoneal 15 mg/kg injection 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 expression data, there was a significant increase in nitrite in the sera of DOX-treated rats compared with the control rats by approximately 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 rates of 8,9-, 11,12-, and 14,15-EET were significantly higher than the control values by approximately 165, 240, and 250%, respectively (Fig. 9A). We also measured the levels of enzymatic hydration products of EETs, the DHETs. As shown in Fig. 9B, there was no significant change in the rate of DHETs formation compared with the control.

To investigate the effect of DOX on 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 compared with the control rats (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 ω-hydroxylase activity, we determined the rate of 20-HETE formation in the kidney microsomes from control and DOX-treated rats. DOX treatment significantly increased 20-HETE formation by 120% in comparison with the control group (Fig. 10C).

In liver microsomes of DOX-treated rats, there was no significant change in the formation rate of all the EET and DHET regioisomers compared with rates in the control rats (Fig. 11). Likewise, neither the total epoxygenase activity or sEH activity was significantly changed in the liver microsomes of DOX-treated rats compared with the control rats (Fig. 12, A and B). On the contrary, DOX treatment significantly increased 20-HETE formation by 70% in comparison with that in the control group (Fig. 12C).

**Effect of Acute DOX Toxicity on Endogenous Arachidonic Acid Metabolite 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. Of the nine 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 were 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 was induced by a single intraperitoneal 15 mg/kg injection 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 an inhibition of CYP2B1 and CYP2C11 by 74 and 65%, respectively, whereas there was a significant induction of CYP4A by 370%. The protein expression of CYP2E1, CYP2J3, and sEH was not significantly altered (Fig. 7). CYP2B1 protein could not be detected in the liver under the current experimental conditions.

**Effect of DOX Treatment on 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 compared with control rats. Interestingly, there was a significant induction of the three inflammatory markers IL-6, iNOS, and TNFα by 2700, 225, and 100%, respectively, after 6 h of DOX administration. Moreover, there was a higher induction of IL-6, iNOS, and TNFα by 5700, 1500, and 260%, respectively, after 24 h of DOX administration (Fig. 8A). To investigate whether DOX caused a systemic inflammation, we measured the serum nitrite level in the control rats and rats treated with DOX for 24 h. Consistent with the gene expression data, there was a significant increase in nitrite in the sera of DOX-treated rats compared with the control rats by approximately 110% (Fig. 8B).
early as 6 h after DOX administration, preceding most of the changes in P450 enzyme expression. Although DOX toxicity was shown previously to increase the biochemical markers of inflammation such as C-reactive protein and fibrinogen (Deepa and Varalakshmi, 2005), this is the first demonstration of 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. Of interest, DOX has been shown to activate the AhR in cardiac-derived H9c2 cells and in the heart of male SD rats (Volkova et al., 2011). Likewise, we have previously shown that DOX induces AhR-regulated genes, CYP1A1 and CYP1B1, in H9c2 cells as well as in 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, because it has been reported previously that lipopolysaccharide-induced inflammation causes a significant induction of CYP1B1 in the liver of male SD rats (Anwar-Mohamed et al., 2010). It was postulated mechanistically that inflammation induces 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 DOX activation of the AhR is heart-specific or ubiquitous.

Contrary to the effect on CYP1B1, DOX toxicity caused a significant inhibition of both CYP2B1 and CYP2C11 in the kidney and liver, whereas there was a significant inhibition of CYP2J3 gene expression only in the liver. CYP2B1, CYP2C11, and CYP2J3 are important epoxygenase enzymes that metabolize arachidonic acid to several EET 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. Of interest, CYP2C9 activity was decreased by 315% in patients with breast cancer who...
were 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 protein levels, whereas there was no significant change in the liver. Similar results were reported previously in models of irritant-induced inflammation (Iber et al., 1999). It is important to mention that ω-1 hydroxylation and 19-HETE formation were not changed in the kidney by CYP2E1 induction most probably because of the low basal expression of CYP2E1 compared with the total P450 content in the kidney (Poloyac et al., 2004).

With regard to P450 ω-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, CYP4A induction seems to be mediated, at least in part, by DOX-induced inflammation and/or a reduction in food consumption. Nevertheless, the increased 20-HETE formation may worsen the inflammatory condition by activating nuclear factor-κB and increasing the generation of inflammatory cytokines in a positive feedback circuit (Ishizuka et al., 2008). Contrary to the effect on 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, whereas 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 lipopolysaccharide-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 reported previously 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 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 of sEH protein expression through a post-transcriptional mechanism, because DOX did not change EPHX2 gene expression. These results demonstrate that the 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-EET formation in the kidney, although it did not change the total epoxygenase activity. Therefore, the increase in 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 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. In general, EETs are considered beneficial endogenous compounds, because they have vasodilator, anti-inflammatory, antiapoptotic, 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 EET 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 finding 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 its effect in the heart, DOX causes a significant increase in 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 liver microsomes, there was a significant increase in the endogenous 20-HETE concentration in the liver tissue. However, there

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 10.** Effect of DOX toxicity on the total epoxygenase, sEH, and ω-hydroxylase activity in the kidney. A, 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 20-HETE formation. Kidney microsomes of controls 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 metabolites were subjected to LC-ESI-MS for metabolite determination. Results are presented as means ± S.E. (n = 6). *, P < 0.05 compared with control.

![Graph A](image4.png)

![Graph B](image5.png)

**Fig. 11.** Effect of DOX toxicity on EETs (A) and DHETs formation (B) in the liver. Liver microsomes of controls 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 a speed vacuum. Reconstituted metabolites were subjected to LC-ESI-MS for metabolite determination. Results are presented as means ± S.E. (n = 6). *, P < 0.05 compared with control.
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 20-HETE formed. Of interest, 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 with 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 has not yet been fully elucidated, DOX-induced inflammation contributes, at least in part, to the alteration of P450 enzymes by DOX. The changes in P450 and sEH expression resulted in altered arachidonic acid metabolism as 20-HETE formation was increased in both the kidney and the liver, whereas EETs formation was increased only in the kidney. If we take into account the physiological functions of these metabolites, the increase in the EET 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

Address correspondence to: Dr. Ayman O. S. El-Kadi, Faculty of Pharmacy and Pharmaceutical Sciences, 3126 Dentistry/Pharmacy Centre, University of Alberta, Edmonton, AB, Canada T6G 2N8. E-mail: aelkadi@pharmacy.ualberta.ca