Chronic Doxorubicin Cardiotoxicity Modulates Cardiac Cytochrome P450-Mediated Arachidonic Acid Metabolism in Rats

Abdulaziz M.S. Alsaad, Beshay N.M. Zordoky, Ahmed A. El-Sherbeni, and Ayman O.S. El-Kadi

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2N8.
Running title: Effect of doxorubicin on cytochrome P450 expression

Corresponding Author: Ayman O.S. El-Kadi, PhD

Faculty of Pharmacy & Pharmaceutical Sciences
2142J Katz Group-Rexall Centre for Pharmacy and Health Research
University of Alberta, Edmonton, Alberta
Canada T6G 2E1

Telephone: (780) 492-3071
Fax: (780) 492-1217
Email: aelkadi@pharmacy.ualberta.ca

The number of pages: 40
The number of tables: 1
The number of figures: 9
The number of references: 48
The number of words in Abstract: 250
The number of words in Introduction: 567
The number of words in Discussion: 1464

Abbreviations: AA, arachidonic acid ; ANP, atrial natriuretic peptide ; BNP, brain natriuretic peptide ; DHET, dihydroxyeicosatrienoic acid ; DOX, doxorubicin ; EET, epoxyeicosatrienoic acid ; HETE, hydroxyeicosatetraenoic acid ; P450, cytochrome P450 ; SD, Sprague Dawley ; sEH, soluble epoxide hydrolase
Abstract:

Doxorubicin (DOX, adriamycin) is an effective anticancer agent whose major limiting side effect is cardiotoxicity. This cardiotoxicity is only predicted by the cumulative dose of DOX where the clinical situation involves chronic drug administration. Therefore, we investigate the effect of chronic DOX cardiotoxicity on expression of the cardiac cytochrome P450 (P450) enzymes and arachidonic acid (AA) metabolism in male Sprague Dawley (SD) rats. The chronic toxicity was induced by multiple intraperitoneal injections for a cumulative dose of 15 mg/kg divided into six injections within two weeks. After 14 days of the last injection, the heart, liver, and kidney were harvested and the expression of different genes was determined by real time-PCR. Also, microsomal protein from the heart was prepared and incubated with AA. Thereafter, different AA metabolites were analyzed by LC-ESI-MS. The chronic DOX cardiotoxicity significantly induced gene expression of hypertrophic markers, apoptotic markers, CYP2E1, CYP4A3, CYP4F1, CYP4F5, and soluble epoxide hydrolase enzyme (sEH), which was accompanied by an increase in the activity of P450 ω-hydroxylases and sEH. In addition, both the sEH inhibitor, t-AUCB and the ω-hydroxylase inhibitor, HET0016 significantly prevented the DOX-mediated induction of the hypertrophic markers in the cardiac derived H9c2 cells which further confirm the role of these enzymes in DOX cardiotoxicity. Furthermore, gene expression of P450 and sEH was altered in an organ specific manner. As a result, the chronic DOX administration leads to imbalance between P450-mediated cardiotoxic and cardioprotective pathways. Therefore, P450 ω-hydroxylases and sEH might be considered as novel targets to prevent and/or to treat DOX cardiotoxicity.
Introduction:

Doxorubicin (DOX, adriamycin) is an effective anti-neoplastic agent commonly used to treat different types of cancer such as ovarian, thyroid, gastric, breast, non-Hodgkin’s and Hodgkin’s lymphoma, multiple myeloma, and sarcomas (Weiss, 1992; Arcamone et al., 2000; Cortes-Funes and Coronado, 2007). However, the clinical use of this drug is limited due to cardiotoxicity, which might proceed to irreversible heart failure (Swain et al., 2003; Christiansen and Autschbach, 2006; Outomuro et al., 2007; Carvalho et al., 2009). Although several mechanisms have been proposed to describe the mechanisms by which DOX induces cardiotoxicity, these mechanisms are still not fully understood. These mechanisms include, generation of free radicals/doxorubicinol metabolites, mitochondrial disruption, alteration of cellular energetic, and initiation of apoptotic cascades (Nakamura et al., 2000; Ueno et al., 2006; Takemura and Fujiwara, 2007). Since these cardiotoxic mechanisms are quite different from the anticancer mechanisms, there is still a hope to discover strategies that treat or protect against the DOX-induced cardiotoxicity while maintaining the same anticancer efficacy (Carvalho et al., 2009).

Furthermore, the role of cytochrome P450 (P450) in the pathogenesis of cardiovascular diseases has been extensively studied (Roman, 2002; Elbekai and El-Kadi, 2006). Of interest, the imbalance between P450-mediated arachidonic acid (AA) metabolites epoxyeicosatrienoic acids (EETs) and 20-hydroxyeicosatetraenoic acid (20-HETE) has been linked to several cardiovascular diseases (Zordoky and El-Kadi, 2008b). Generally, EETs, eicosanoids produced by P450 epoxygenases, are considered cardioprotective metabolites, which have beneficial effects on the heart. It has been reported that EETs have a protective effect against cardiac hypertrophy (Xu et al., 2006) and DOX-induced
cardiotoxicity (Zhang et al., 2009). On the other hand, 20-HETE, an eicosanoid produced by P450 ω-hydroxylases, has been reported to have a detrimental effect on the heart (Chabova et al., 2007; Lv et al., 2008; Minuz et al., 2008) leading to cardiac hypertrophy and heart failure (Bao et al., 2011). Mechanistically, these opposing effects of P450-derived AA metabolites are mediated through several intracellular signaling cascades that have been implicated in development and/or progression of cardiovascular toxicity e.g. NF-κB, MAPK, and MMP-9 (Node et al., 1999; Sun et al., 1999; Elbekai and El-Kadi, 2006; Ishizuka et al., 2008; Moshal et al., 2008). As an important player affecting the balance between P450-derived AA metabolites, soluble epoxide hydrolase enzyme (sEH) hydrolyzes the cardioprotective metabolites EETs to biologically less active metabolites, dihydroxyeicosatrienoic acids (DHETs) (Imig et al., 2002). This enzyme is overexpressed in several heart diseases (Monti et al., 2008; Ai et al., 2009).

Recently, we have demonstrated that acute DOX cardiotoxicity alters the expression of P450 and sEH enzymes leading to a decrease in EETs levels and an increase in 20-HETE levels (Zordoky et al., 2010). However, the clinical situation requires chronic administration of the drug where the total cumulative dose is the only currently used predictor of cardiotoxicity (Swain et al., 2003; Carvalho et al., 2009). In the current study, we hypothesized that chronic DOX cardiotoxicity will modulate cardiac expression of P450 and sEH enzymes leading to altered AA metabolism. Therefore, the effect of chronic DOX cardiotoxicity on the cardiac expression of P450 and sEH enzymes was studied. In addition, the effects of sEH inhibitor, trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB) and ω-hydroxylase inhibitor, HET0016 on the hypertrophic markers induced by DOX treatment were determined in
cardiac H9c2 cells. Furthermore, the effect of chronic DOX cardiotoxicity on P450-mediated AA metabolism was investigated. Finally, the gene expression of P450 and sEH enzymes was investigated in the kidney and the liver.
Material and Methods

Materials. Applied Biosystems (Foster City, CA) was the source for High-Capacity cDNA Reverse Transcription Kit, SYBR Green Super Mix, and 96-well optical reaction plates with optical adhesive films. Integrated DNA Technologies Inc. (San Diego, CA) was the synthesizer for real time-PCR primers according to previously published sequences. Sigma-Aldrich (St. Louis, MO) was the source for arachidonic acid, 4-hydroxybenzophenone, and DOX. Cayman Chemical (Ann Arbor, MI) was the provider for 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, 19-HETE, and 20-HETE. Liquid chromatographic-electron spray ionization-mass spectrometry (LC-ESI-MS) reagents were at HPLC-grade. EM Scientific (Gibbstawn, NJ) was the source for acetonitrile and water (HPLC grade). sEH inhibitor (t-AUCB) was a generous gift from Dr. Bruce Hammock (University of California, Davis). ω-hydroxylase inhibitor (HET0016) was obtained from Cayman Chemical (Ann Arbor, MI). Other chemicals were purchased from Fisher Scientific Co. (Toronto, ON, Canada).

Animals. Our experimental procedures and animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Charles River Canada (St. Constant, QC, Canada) was the source for male Sprague Dawley (SD) rats weighing 250-300 g. Multiple intraperitoneal (IP) injections were given to animals for a cumulative dose of 15 mg/kg of DOX divided into six injections within two weeks (n = 7). Control group of same weight received the same volume of normal saline volume (n = 7). A washout period was allowed to elimination of the drug after 14 days of the last injection, animals were euthanized under isoflurane anesthesia. Throughout the treatment period,
all animals were allowed free access to food and water. The hearts, liver, and kidney were harvested and immediately frozen in liquid nitrogen. Samples were stored at -80°C until analysis.

**RNA extraction and cDNA synthesis.** Total RNA was isolated from the frozen tissues (heart, liver, and kidney) using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantification of RNA was determined by measuring the absorbance at 260 nm. The RNA quality was determined by measuring the absorbance at 260/280 ratio. Thereafter, the High-Capacity cDNA reverse transcription kit (Applied Biosystems) was used to synthesize first-strand cDNA according to the manufacturer’s instructions. From each sample, 1.5 μg of total RNA was added to a mix of 2.0 μL 10X RT buffer, 0.8 μL 25X dNTP mix (100mM), 2.0 μL 10X RT random primers, 1.0 μL MultiScribe TM 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.** Specific mRNA expression was quantitatively analyzed by real time-PCR and by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). Reaction mix of 25-μl in volume 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 Master mix, 11.05 μl of nuclease-free water, and 1.25 μl of cDNA sample. The primers were chosen from previously published studies, which are listed in Table 1 (Anwar-mohamed et al., 2010). To test for the contamination of any assay reagents, no-template controls were
incorporated onto the same plate. The plate was sealed with an optical adhesive cover. Thereafter, thermocycler was used and 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. To confirm the specificity of the primers and the purity of the final PCR product, the dissociation curves were performed by the end of each cycle.

**Real time-PCR Data analysis.** The relative gene expression method was used to analyze the real time-PCR data as described in Applied Biosystems User Bulletin No.2. 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 was used.

**Microsomal protein preparation.** Microsomal protein was prepared from the heart tissues as described previously (Aboutabl et al., 2009). In brief, heart 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 was separated by differential ultracentrifugation from the homogenized tissues. Thereafter, the final microsomal pellet was reconstituted in cold sucrose and stored at -80 °C. Lowry method using bovine serum albumin as a standard (Lowry et al., 1951) was used to determine the concentration of heart microsomal protein.

**Microsomal incubation and separation of different arachidonic acid metabolites by LC-ESI-MS.** Incubation of heart microsomes (1 mg protein/ml) were performed 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). Five minutes period of pre-equilibration was allowed. After that, 1 mM NADPH was added to initiate the
reaction where the arachidonic acid was added to a final concentration of 50 μM and incubated for 30 min. Termination of the reaction was performed by adding 600 μL of ice-cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. To extract arachidonic acid metabolites, 1 ml ethyl acetate was added twice and dried using speed vacuum (Savant, Farmingdale, NY). Accordingly, the homogenization of heart tissues (1 g) was performed in the incubation buffer and centrifuged at 10,000 g for 30 min. After adding the acetonitrile, the supernatant was extracted twice with 1 ml ethyl acetate and dried using speed vacuum (Savant, Farmingdale, NY). The LC-ESI-MS (Waters Micromass ZQ 4000 spectrometer) method was used to analyze extracted arachidonic acid metabolites as described previously (Aboutabl et al., 2009). In brief, the mass spectrometer was used in negative 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.

**Cytosolic soluble epoxide hydrolase activity assay**

sEH activity was measured using Morisseau and Hammock method with modifications. 14,15-EET was used as the natural substrate (Morisseau C, 2007). Briefly, the cytosolic fraction was diluted to 0.4 mg/mL with sodium phosphate buffer (0.076 M, pH 7.4) supplemented with BSA (2.5 mg/mL). After preincubation for 5 min at 37°C, the assay
was initiated by the addition of 14,15-EET (final concentration of 14,15-EET is 2 μg/mL). The mixture was incubated at 37°C for 5 min. Then the reaction was terminated by the addition of 600 μL ice-cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. 14,15-EET and its corresponding 14,15-DHET were extracted by 1 mL ethyl acetate twice and dried using speed vacuum (Savant, Farmingdale, NY). Extracted 14,15-EET and its metabolite were analyzed using LC-ESI-MS (Waters Micromass ZQ 4000 spectrometer) method as described previously (Anwar-Mohamed et al., 2012).

**Cell culture and treatments**

H9c2 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM, without phenol red, supplemented with 0.45% glucose, 0.15% sodium bicarbonate, 0.11% sodium pyruvate, 10% fetal bovine serum, 20 μM L-glutamine, 100 IU/mL penicillin, 10 μg/mL streptomycin, and 25 ng/mL amphotericin B. Cells were grown in 75-cm² tissue culture flasks at 37°C in a 5% CO2 humidified incubator. For analysis of mRNA, cells were grown at a density of 1–1.5 x 10⁶ cells per well in a 6-well tissue culture plate.

**Treatment of H9c2 cells with soluble epoxide hydrolase inhibitor, t-AUCB and ω-hydroxylase inhibitor, HET0016**

To investigate the cardioprotective effect of sEH and ω-hydroxylase inhibitors, the cardiac-derived H9c2 cells were placed into 6-well cell culture plates and on 60-80% confluency, the cells were treated with either the vehicle, dimethyl sulfoxide (DMSO), or 100 nM t-AUCB for 24 h. Thereafter, the cells were treated with 1 μM DOX in the
presence or absence of t-AUCB for additional 6 h. Similarly, H9c2 cells were treated with either the vehicle (DMSO) or 50 nM HET0016 for 24 h. Thereafter, the cells were treated with 1 μM DOX in the presence or absence of HET0016 for additional 6 h.

**Statistical analysis.** Our data are presented as mean ± standard error of the mean (SEM). Control and DOX-treated measurements were compared using student t-test. In H9c2 experiment, comparison of 4 different groups was performed using one-way ANOVA test. A result was considered statistically significant where p < 0.05.
Results:

Effect of chronic DOX treatment on the apoptotic, inflammatory, and hypertrophic markers

The gene expression of apoptotic, inflammatory, and hypertrophic markers were determined to confirm the occurrence of cardiotoxicity induced by chronic DOX administration in male SD rats. Both gene expression of p53 and Bax were significantly increased in DOX-treated rats to 1.7 and 1.9 fold, respectively as compared to the control group 14 days after last injection of DOX (Fig. 1A). The inflammatory marker IL-6 was also significantly induced to 1.8 fold as compared to control group. Furthermore, to investigate the effect of chronic DOX-induced cardiotoxicity on the hypertrophic markers, gene expression of atrial natriuretic peptide (ANP) and myosin heavy chain beta (β-MHC) were measured relative to control group. The chronic DOX treatment caused a significant induction of ANP and β-MHC to 3.5 and 2.7 fold, respectively. In addition, the ratio of β-MHC to α-MHC was also significantly induced to 1.6 fold of control group (Fig. 1B). However, there was no change in gene expression of brain natriuretic peptide (BNP).

Effect of chronic DOX treatment on P450 gene expression

To investigate the effect of chronic DOX cardiotoxicity on expression of several P450 genes within the heart tissue, total RNA were extracted from the heart of both control and DOX-treated groups. Thereafter, reverse transcription was performed and followed by real-time PCR. The chronic DOX cardiotoxicity significantly induced CYP2E1 gene expression in the heart to 1.8 fold of the control group. On the other hand, there was no
significant change in gene expression of CYP1A1, CYP1B1, CYP2B1, CYP2C11, and CYP2J3 as compared to control group. Figure 2 shows the effect of chronic DOX cardiotoxicity on gene expression of CYP2 family. Furthermore, the chronic DOX cardiotoxicity caused a significant induction in the gene expression of ω-hydroxylase enzymes CYP4A3, CYP4F1, and CYP4F5 to 2, 1.6, and 2 fold, respectively as compared to control group (Fig. 3). However, there was no significant change in gene expression of CYP4A1 and CYP4F4 (Fig. 3).

**Effect of chronic DOX treatment on P450-mediated arachidonic acid metabolism**

The heart microsomes of control and DOX-treated rats were incubated with 50 μM arachidonic acid for 30 min to examine the effect of chronic DOX treatment on the formation of P450-derived AA metabolites. Our results showed that formation of 5,6-, 8,9-, 11,12-, and 14,15-EET were slightly increased as compared to control group (Fig. 4A). In addition, enzymatic conversion of EETs to DHETs was measured. As compared to control group, formation of 11,12-, and 14,15-DHET was significantly increased to 1.9 and 1.3 fold, respectively (Fig. 4B).

To investigate the effect of chronic DOX-induced cardiotoxicity on the total epoxygenase activity, the sum of all the products of epoxygenase enzymes, i.e. the total EETs and DHETs, was measured in the control and DOX–treated rats 14 days after the last injection of DOX. As compared to control group, there was a trend of increase but did not reach the statistical significance change in the total epoxygenase activity in heart microsomes of DOX-treated rats (Fig. 5A). On the other hand, there was a significant increased in 20-HETE formation in the DOX-treated rats as compared to the control.
group (Fig. 5B). Therefore, the ratio of 20-HETE formation to the total EETs was calculated to confirm our finding. Of interest, the ratio was significantly increased to 1.6 fold as compared to the control group (Fig. 5C). With regard to \( \omega-1 \) hydroxylase activity, there was no change in \( \omega-1 \) hydroxylase activity where the formation of 19-HETE was not changed in the DOX-treated rats as compared to the control group (Fig. 5D).

Effect of chronic DOX treatment on \( EPHX2 \) gene expression and sEH activity

The sEH enzyme is an important enzyme involved in conversion of EETs to biologically less active metabolites DHETs. Therefore, the gene expression of \( EPHX2 \) gene, which is the gene encoding sEH, was determined 14 days after the last injection of DOX. Interestingly, chronic DOX cardiotoxicity caused a significant induction in the gene expression of \( EPHX2 \) within the heart tissue to 1.9 fold (Fig. 6A). Similar to the induction seen at the mRNA level, there was also a significant increase in sEH activity in the heart microsomes to 1.6 fold (Fig. 6B). In agreement with the induction of sEH activity in the microsomal fraction, sEH activity was also induced to 1.3 fold in heart cytosols of DOX-treated rats as compared to control group (Fig. 6C).

Effect of soluble epoxide hydrolase inhibitors on DOX-mediated induction of ANP and BNP mRNA in H9c2 cells

To confirm reversal and/or prevention of cardiotoxicity induced by DOX treatment, H9c2 cells were treated with sEH inhibitor (t-AUCB) for 24 hr prior to treatment with DOX. Thereafter, DOX was added for 6 hr and then mRNA was isolated. Our results showed that inhibition of sEH significantly reversed the DOX-induced ANP and BNP mRNA to 0.3 and 0.4 fold, respectively as compared to DOX-treated cells (Fig. 7A).
Effect of ω-hydroxylase inhibitor (HET0016) on DOX-mediated induction of ANP and BNP mRNA in H9c2 cells

To confirm reversal and/or prevention of cardiotoxicity induced by DOX treatment, H9c2 cells were treated with HET0016 for 24 hr prior to treatment with DOX. Thereafter, DOX was added for 6 hr and then mRNA was isolated. Our results showed that inhibition of ω-hydroxylase significantly reverses the DOX-induced ANP and BNP mRNA to 0.5 fold, as compared to DOX-treated cells (Fig. 7B).

Effect of chronic DOX treatment on the expression of P450, sEH, and inflammatory markers genes in the kidney and liver

Total RNA was isolated from the kidney and the liver of DOX-treated and control rats to determine the effect of chronic DOX toxicity on gene expression of P450, sEH, and inflammatory markers in the other tissues. Using the real time-PCR, the expressions of several genes were determined after 14 days of last injection of DOX. Of interest, there was no significant induction gene expression of sEH within the liver or the kidney.

In the liver, the chronic DOX toxicity significantly induced the gene expression of the inflammatory markers TNFα and IL-6 to 2.2 and 4.5 fold, respectively. In addition, there was significant induction in gene expression of CYP1A1, CYP1A2, CYP1B1, and CYP4F5 to 2.8, 1.9, 2.5, and 1.8 fold, respectively. Also, there was a significant inhibition in the gene expression of CYP2B1, CYP2B2, and CYP2C11 to 0.5, 0.5, and 0.7 fold, respectively. However, there was no significant change in gene expression of CYP2E1, CYP2J3, CYP4A1, CYP4A3, CYP4F1, and CYP4F4. Figure 8 shows the effect
of chronic DOX toxicity on gene expression of inflammatory markers, P450, and sEH in the liver.

In the kidney, there was induction in gene expression of TNFα and IL-6 to 2.9 and 4.7 fold, respectively as compared to the control group. Also, there was a significant induction in the gene expression of CYP1B1, CYP2J3, and CYP4F5 to 1.8, 1.6, and 1.7 fold, respectively. Also, there was a significant inhibition in gene expression of CYP2B2 to 0.74 fold as compared to the control. However, there was no significant change in the gene expression of CYP1A1, CYP1A2, CYP2B1, CYP2C11, CYP2E1, CYP4A1, CYP4A3, CYP4F1, and CYP4F4. Figure 9 shows the effect of chronic DOX toxicity on gene expression of inflammatory markers, P450, and sEH in the kidney.
Discussion

Recently, we have demonstrated that acute DOX cardiotoxicity induces the cardiac expression of P450 and sEH enzymes \textit{in vivo} and \textit{in vitro} (Zordoky and El-Kadi, 2008a; Zordoky et al., 2010). In the current work, we investigate the effects of chronic DOX cardiotoxicity on the expression of P450, sEH, and P450-mediated AA metabolism in male SD rats. The chronic DOX cardiotoxicity was induced by six intraperitoneal injections of 15 mg/kg cumulative dose within two weeks. A washout period of 14 days was allowed to ensure that the observed effects are related to the chronic rather than the acute effect of DOX treatment. Thereafter, several apoptotic, inflammatory, and hypertrophic markers have been used to confirm the occurrence of chronic DOX cardiotoxicity. Interestingly, our results showed that there was a significant induction in gene expression of hypertrophic markers ANP and $\beta$-MHC. Furthermore, the ratio of $\beta$-MHC:$\alpha$-MHC was significantly induced. Several other studies have supported our finding where DOX cardiotoxicity selectively induces ANP in different animal model such as dogs (Chen et al., 1999; Rahman et al., 2001) and in the cardiac cells line H9c2 (Zordoky and El-Kadi, 2008a). However, in contrary to the chronic model, those hypertrophic markers were inhibited in acute DOX cardiotoxicity or \textit{in vitro} studies using the neonatal cardiomyocytes (Rahman et al., 2001; Zordoky et al., 2010). These differences might be attributed to the differences in model, dose, and duration of treatment.

Regarding cardiac gene expression of P450, there was a quite difference in their expression from the acute DOX model. In contrary to the acute DOX model, our current
results show that there is a significant induction in the cardiac gene expression of CYP2E1 with no change in gene expression of CYP1A1, CYP1B1, CYP2C11, and CYP2J3. These P450 enzymes are involved in the formation of P450-mediated AA metabolites to different extents. CYP1A1 is involved in formation of HETE metabolites while CYP1B1 is involved in formation of EETs and HETEs (Choudhary et al., 2004). Also, EETs can be produced by major P450 epoxygenases such as CYP2B1, CYP2J3, and CYP2C11 (Laethem et al., 1994; Ng et al., 2007). However, CYP2E1 is involved in formation of 19-HETE (Laethem et al., 1993). In agreement with our results, there was no change in gene expression of CYP2B1 in H9c2 cells and in the acute DOX model. Furthermore, the cardiac gene expression of P450 ω-hydroxylases CYP4A3 and CYP4F1 were significantly induced similar to the acute model. These ω-hydroxylases are mainly involved in the formation of 20-HETE. However, there was no change in gene expression of CYP4A1 and CYP4F4 that was induced in the acute DOX model (Zordoky et al., 2010). In contrary to the acute DOX model, our results showed that cardiac gene expression of CYP4F5 was significantly induced.

To investigate the effect of the aforementioned changes in P450 gene expression on P450-mediated AA metabolism, AA was incubated with microsomes extracted from the heart tissue and the P450-derived AA metabolites were analyzed by LC-MS. Of importance, our findings showed that there is no significant change in formation of 5,6-, 8,9-, 11,12-, and 14,15-EET relative to the control group. In addition, there was no significant increase in epoxygenase activity, which was estimated by calculating the sum of total EETs and DHETs. However, there was a significant increase in formation rates of 14,15-, and 11,12- DHETs. Therefore, it was necessary to investigate the effects of
chronic DOX cardiotoxicity on expression and activity of sEH enzyme which is responsible for the enzymatic conversion of EETs to less biologically active metabolites DHETs (Imig et al., 2002). Of interest, our results showed that chronic DOX cardiotoxicity significantly induced the gene expression of *EPHX2*. This finding was translated to a significant increase in the sEH activity within the heart microsomal and cytosolic fractions as compared to control group, which resulted in a higher formation rate of DHETs. In agreement with these findings, we have previously shown that acute DOX cardiotoxicity induced cardiac gene expression of *EPHX2* both *in vivo* and *in vitro* (Zordoky et al., 2010).

Similarly, our findings showed that P450 ω-hydroxylase activity was increased after 14 days of the last DOX injection as compared to the control group. This finding is matching the increase in cardiac gene expression of major P450 ω-hydroxylase enzymes i.e. CYP4A3, CYP4F1, and CYP4F5. Also, it was accompanied by a significant increase in the formation of cardiotoxic metabolite 20-HETE. Despite the induction of the ω-1 hydroxylase *CYP2E1* gene expression, there was no significant change in 19-HETE formation in the heart of DOX-treated group. This discrepancy between the gene expression and the enzymatic activity of CYP2E1 may be attributed to the regulation of CYP2E1 by different post-transcriptional mechanisms and to the relatively low basal levels of CYP2E1 in the heart. Similar to our finding, several studies have reported that the increase in CYP2E1 expression did not correlate with the rate of 19-HETE formation (Amet et al., 1997; Poloyac et al., 2004). Of importance, overproduction of 20-HETE has been extensively implicated in development and/or progression of several cardiovascular diseases (Certikova Chabova et al., 2010; Bao et al., 2011). Therefore, the elevation in
20-HETE levels might be another potential mechanism by which the chronic DOX treatment causes the progressive cardiotoxicity.

On the other hand, several studies have demonstrated that cardioprotective metabolites EETs have opposing effects to those mediated by cardiotoxic metabolite 20-HETE (Roman, 2002; Elbekai and El-Kadi, 2006). Therefore, the ratio of 20-HETE formation to the total EETs formation was calculated. Of interest, this ratio was significantly induced by chronic DOX cardiotoxicity. Indeed, this finding suggests that the imbalance between 20-HETE and EETs might be involved in the pathogenesis of chronic DOX cardiotoxicity. Mechanistically, the opposing effects of 20-HETE and EETs are mediated through different signaling cascades that have been linked to development and/or progression of cardiovascular toxicity such as NF-κB, oxidative stress, apoptosis, and inflammatory cytokines (Elbekai and El-Kadi, 2006; Ishizuka et al., 2008). To confirm the involvement of P450-mediated AA metabolites in DOX-induced cardiotoxicity, we used the H9c2 cell line which is derived from embryonic rat heart ventricles (Kimes and Brandt, 1976). Of interest, our results demonstrated that the sEH inhibitor, t-AUCB and the ω-hydroxylase inhibitor, HET0016 significantly reversed the DOX-mediated induction of the hypertrophic markers ANP and BNP in H9c2 cells. The modest increase in BNP gene expression after treatment with t-AUCB may be attributed to the cardioprotective effect of EETs, which is mediated through BNP (Chaudhary et al., 2009). Therefore, these findings confirm the involvement of sEH and ω-hydroxylase enzymes in DOX-induced cardiotoxicity.
In addition, to determine to which extent those changes in gene expression of P450 and sEH enzymes are specific to the heart, gene expression of these enzymes was determined in the kidney and the liver. With regard to P450 enzymes, CYP1B1 was induced in the liver and kidney, which is in agreement with the acute DOX model (Zordoky et al., 2011). This induction could be attributed to the activation of the AhR and to the DOX-induced inflammation. In agreement with acute model, there was inhibition of CYP2B1 and CYP2C11 gene expression in the liver. This inhibition is attributed to inflammation where several studies have shown that inflammation is involved in down-regulation of those enzymes (Iber et al., 1999; Li-Masters and Morgan, 2001). In contrary to the acute model, our results showed that there was a significant induction in gene expression of CYP2J3 with no significant change in CYP2B1 and CYP2C11 in the kidney. Also, in contrary to acute study, there was no significant change in the gene expression of CYP2E1 in the liver and kidney. With regard to major P450 ω-hydroxylases, our results showed that there was no significant change in CYP4A1, CYP4A3, CYP4F1, and CYP4F4 in the liver and kidney. However, there was a significant induction in the gene expression of CYP4F5 in the liver and kidney. Regarding sEH gene expression, there was no significant change within the liver and kidney. Therefore, the chronic DOX toxicity specifically modulated gene expression of P450 and sEH enzymes in an organ and enzyme specific manner. Interestingly, several recent studies have demonstrated the role of EETs in tumor growth and metastasis (Panigrahy et al., 2011). Therefore, it is important to investigate whether DOX induces sEH and decreases EETs levels within the tumor tissues or not.
In conclusion, the chronic DOX administration significantly modulates cardiac expression of P450 and sEH enzymes and their activity leading to imbalance between P450-mediated cardiotoxic and cardioprotective pathways. This imbalance was accompanied by a significant production of DHETs and 20-HETE where sEH and ω-hydroxylase enzymes demonstrate pivotal roles in DOX cardiotoxicity suggesting another mechanism by which DOX causes progressive cardiotoxicity. Inhibition of these enzymes conferred protection against DOX toxicity in the cardiac H9c2 cells. Therefore, sEH and ω-hydroxylase enzymes might be considered as novel targets to treat and/or to protect against chronic DOX cardiotoxicity.
Acknowledgements

The authors are grateful to Dr. Vishwa Somayaji for his excellent technical assistance with liquid chromatography-electron spray ionization-mass spectrometry.
Authorship Contributions

*Participated in research design*: Abdulaziz Alsaad, Beshay Zordoky, and Ayman El-Kadi

*Conducted experiments*: Abdulaziz Alsaad, Beshay Zordoky, and Ahmed El-Sherbeni

*Performed data analysis*: Abdulaziz Alsaad and Beshay Zordoky

*Wrote or contributed to the writing of the manuscript*: Abdulaziz Alsaad, Beshay Zordoky, and Ayman El-Kadi
References:


Footnotes:

This work was supported by a grant from the Canadian Institutes of Health Research [Grant 106665]. A.M.S.A. is teaching assistant at King Saud University (KSU) and the recipient of the active scholarship from the ministry of higher education and KSU, Riyadh, Saudi Arabia.
Figure legends

**Fig. 1. Effect of chronic DOX cardiotoxicity on the apoptotic (A) and hypertrophic markers (B).** Total RNA was isolated from the heart of control and DOX-treated rats after 14 days of last injection of DOX. Apoptotic and inflammatory markers (Bax, p53, and IL-6) (A) and hypertrophic markers (ANP and β-MHC) (B) gene expressions were determined by real-time PCR. The β-MHC:α-MHC ratio was calculated (A). Results are presented as mean ± SE (n = 7). * P < 0.05 compared to control.

**Fig. 2. Effect of chronic DOX cardiotoxicity on CYP2 family gene expression.** Total RNA was isolated from the heart of control and DOX-treated rats after 14 days of last injection of DOX. CYP2E1, CYP2C11, and CYP2J3 gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 7). * P < 0.05 compared to control.

**Fig. 3. Effect of chronic DOX cardiotoxicity on CYP4 family gene expression.** Total RNA was isolated from the heart of control and DOX-treated after 14 days of last injection of DOX. CYP4A3, CYP4F1, and CYP4F5 gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 7). * P < 0.05 compared to control.

**Fig. 4. Effect of chronic DOX cardiotoxicity on EETs (A) and DHETs formation (B).** Heart microsomes of control and DOX-treated rats were isolated after 14 days of last injection of DOX and 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 1ml 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 = 7). * P < 0.05 compared to control.

Fig. 5. Effect of chronic DOX cardiotoxicity on epoxygenase (A), ω-hydroxylase activity (B), ratio of 20-HETE formation to total EETs (C), and ω-1 hydroxylase activity (D). (A) Epoxygenase activity was determined from the sum of EETs and DHETs formation. (B) ω-hydroxylase activity was determined from the 20-HETE formation. (C) The ratio of 20-HETE:total EETs. (D) ω-1 hydroxylase activity was determined from the 19-HETE formation. 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 1ml 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 = 7). * P < 0.05 compared to control.

Fig. 6. Effect of chronic DOX cardiotoxicity on EPHX2 gene expression (A), sEH activity (microsomes) (B), and sEH activity (cytosols) (C). (A) Total RNA was isolated from the hearts of control and DOX-treated rats after 14 days of last injection of DOX. EPHX2 gene expression was determined by real-time PCR (n = 7). (B) Heart microsomes of control and DOX-treated rats were isolated after 14 days of last injection of DOX and 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 1ml of ethyl acetate and dried
using speed vacuum. Reconstituted metabolites were injected into LC-ESI-MS for metabolite determination. sEH activity was calculated as the ratio of total DHETs/ total EETs. (C) Heart cytosols of control and DOX-treated rats were isolated after 14 days of last injection of DOX and sEH activity was measured by the addition of 14,15-EET (final concentration of 14,15-EET is 2 μg/mL) to the cytosolic fractions. After incubation for 5 min at 37°C, the reaction was terminated by the addition of ice-cold acetonitrile. The corresponding 14,15-DHET were extracted by ethyl acetate, dried and reconstituted by acetonitrile and measured using LC-ESI-MS. Results are presented as mean ± SE (n = 7) * P < 0.05 compared with control.

Fig. 7. Effect of soluble epoxide hydrolase inhibitor, t-AUCB (A) and ω-hydroxylase inhibitor, HET0016 (B) on DOX-mediated induction of ANP and BNP mRNA in H9c2 cells. Cells were treated with t-AUCB or HET0016 for 24 h. Thereafter, cells were treated with DOX for 6 h and then total RNA was isolated from the H9c2 cells of control and DOX-treated cells. Hypertrophic markers (ANP and BNP) gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 4). * P < 0.05 compared to control.

Fig. 8. Effect of chronic DOX toxicity on gene expression of inflammatory markers, P450, and sEH in the liver. Total RNA was isolated from the kidney of control and DOX-treated rats after 14 days of last injection of DOX. TNFα, IL-6, CYP1A1, CYP1A2, CYP1B1, CYP2B1, CYP2B2, CYP2C11, CYP2E1, CYP2J3, CYP4A1, CYP4A3, CYP4F1, CYP4F5, and sEH gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 7). * P < 0.05 compared to control.
Fig. 9. Effect of chronic DOX toxicity on gene expression of inflammatory markers, P450, and sEH in the kidney. Total RNA was isolated from the liver of control and DOX-treated rats after 14 days of last injection of DOX. TNFα, IL-6, CYP1A1, CYP1A2, CYP1B1, CYP2B1, CYP2B2, CYP2C11, CYP2E1, CYP2J3, CYP4A1, CYP4A3, CYP4F1, CYP4F5, and sEH gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 7). * P < 0.05 compared to control.
Table 1. Primers sequences used for real time-PCR reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>CCAACGAGTTCCGGCCT</td>
<td>TGCCCCAAACCAAGAGAAATGA</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>GCTTTACTGTGCAAAGGAGAGA</td>
<td>GGAAGGGAGATTCAAGTCAGGA</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>AACCCTTGATGACCGCAGTAAA</td>
<td>TGTGGTACTCCAATAGGGACAAGATC</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>CACCAGCTATCAGTGATGGATGGTG</td>
<td>GTCTGCCCTTTGCACAGGAA</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>AAGCGGTGTGTGTGTGTTGAGAA</td>
<td>AGAGACTTCAGGTAAATAATGCTGCA</td>
</tr>
<tr>
<td>CYP2J3</td>
<td>CATTGAGCTCAACAAGTGCTTTT</td>
<td>CAATTTCTAGGGCTGTGATGTCG</td>
</tr>
<tr>
<td>CYP4A1</td>
<td>TTGAGCTACGCTGACAGATCCAC</td>
<td>CCCATTTCGACTTCAGACACA</td>
</tr>
<tr>
<td>CYP4A2</td>
<td>CTCGCCCATAGGGAAGCTTATC</td>
<td>CCTTCAGCTCATATGCGGAATTT</td>
</tr>
<tr>
<td>CYP4A3</td>
<td>CTCGCCCATAGCCATGCTTTATC</td>
<td>CCTTCAGCTCATATGCGGAATTT</td>
</tr>
<tr>
<td>CYP4F1</td>
<td>CCCCAAGGGCTTTTTGATG</td>
<td>GAGCGCAACCGCAGCT</td>
</tr>
<tr>
<td>CYP4F4</td>
<td>CAGGTCTGGAAGCAGGTAAGTAAA</td>
<td>CCTTCAGGTCGGGACAGGAG</td>
</tr>
<tr>
<td>CYP4F5</td>
<td>AGGATGCGTGCTGCTACTG</td>
<td>GGCTCAAAGCAGCACAGAA</td>
</tr>
<tr>
<td>ANP</td>
<td>GGAGCCTGCGGAAAGGATCAA</td>
<td>TATCTTCGCTACCAGGGAAGCTG</td>
</tr>
<tr>
<td>BNP</td>
<td>CAGAAGGCTCTGGAGCTGATAAG</td>
<td>TGTAGGGGCTTGGCTTTG</td>
</tr>
<tr>
<td>Bax</td>
<td>CCCACCAGCTCTGCAACAGTTC</td>
<td>GTGTCTCCCAGCCATCCT</td>
</tr>
<tr>
<td>β-MHC</td>
<td>CGCTCAATCGATGCGCGGAT</td>
<td>GCCCCAAATGCGACAGCAT</td>
</tr>
<tr>
<td>EPHX2</td>
<td>CACATCCAAGCCACCAAGCC</td>
<td>CAGGCCTCACATCCTCAG</td>
</tr>
<tr>
<td>IL-6</td>
<td>ATATGTCTCAGGGAGATCTTGGGAA</td>
<td>GTGCATCATCGCTGTTTAC</td>
</tr>
<tr>
<td>iNOS</td>
<td>GTGCTAAATGCGGAAAGGTCATG</td>
<td>GCTTCCGACTTTCTGTCTCAGTA</td>
</tr>
<tr>
<td>p53</td>
<td>CAGGCTTGAGGTTGCTGTTTGT</td>
<td>ATGCTCTTCTTGTGTCGGGAA</td>
</tr>
<tr>
<td>TNF</td>
<td>CAAGGCTACCCATGCAAAAATTGT</td>
<td>GGGCATCCACAGTCCTTTC</td>
</tr>
<tr>
<td>β-actin</td>
<td>CCAGATCATGTTGAGACCTTCAA</td>
<td>GTGCTACGACCAGGAGCATACA</td>
</tr>
</tbody>
</table>
Figure 1

(A) Fold change (relative to β-actin)

- **Bax**
- **p53**
- **IL-6**

(B) Fold change (relative to β-actin)

- **ANP**
- **Beta-MHC**
- **Beta-MHC : Alpha-MHC**
Figure 2

(A) Fold change (relative to β-actin) for CYP1A1 and CYP1B1.

(B) Fold change (relative to β-actin) for CYP2B1 and CYP2C11.

(C) Fold change (relative to β-actin) for CYP2E1 and CYP2J3.

* Indicates significance.
Figure 4

(A)

EETs formation (pmol/min/mg protein)

14,15 EET  11,12 EET  8,9 EET  5,6 EET

(B)

DHETs formation (pmol/min/mg protein)

14,15 DHET  11,12 DHET  8,9 DHET  5,6 DHET

Legend:
- **Control**
- **DOX**

*Statistically significant difference
Figure 5

(A) Total epoxygenase activity (pmol/min/mg protein) for Control and DOX groups.

(B) ω-1-Hydroxylase activity for 20-HETE formation (pmol/min/mg protein) for Control and DOX groups.

(C) Microsomal 20-HETE : Total EETs ratio for Control and DOX groups.

(D) ω-1-Hydroxylase activity for 19-HETE formation (pmol/min/mg protein) for Control and DOX groups.
Figure 6

(A) Fold change (relative to β-actin)

(B) sEH Activity (microsomes) (Total DHETs/Total EETs)

(C) sEH Activity (cytosol) (pmol/min/mg protein)
Figure 8

(A) Fold change (relative to β-actin) for sEH, TNF, and IL-6.

(B) Fold change (relative to β-actin) for CYP1A1, CYP1A2, and CYP1B1.

(C) Fold change (relative to β-actin) for CYP2B1, CYP2B2, and CYP2E1.

(D) Fold change (relative to β-actin) for CYP2Cl1 and CYP2J3.

(E) Fold change (relative to β-actin) for CYP4A1 and CYP4A3.

(F) Fold change (relative to β-actin) for CYP4F1, CYP4F4, and CYP4F5.