Introduction

Arsenic is ubiquitous in the environment, and a considerable percentage of the global population is suffering from arsenic related organ dysfunctions because of this unavoidable exposure (Ghosh et al. 2009). The absorption of both inorganic trivalent (arsenite, As(III)) and pentavalent (arsenate, As(V)) in the gastrointestinal tract is high in both humans and experimental animals (Anwar-Mohamed and El-Kadi 2010). However, it has been well documented that As(III) toxicity was induced by a single intraperitoneal injection of 12.5 mg/kg of As(III). Our results showed that As(III) treatment caused a significant induction of the cardiac hypertrophic markers in addition to Cyp1b1, Cyp2b, Cyp2c, Cyp4f, and sEH gene expression in mice hearts. Furthermore, As(III) increased sEH protein expression and activity in hearts with a consequent decrease in 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs) formation. Whereas the formation of 8,9-, 11,12-, 14,15-dihydroxyeicosatrienoic acids (DHEs) was significantly increased. As(III) also increased sEH mRNA and protein expression levels in addition to the hypertrophic markers which was reversed by knockdown of sEH in H9c2 cells. In conclusion, acute As(III) toxicity alters the expression of several P450s and sEH enzymes with a consequent decrease in the cardioprotective EETs which may represent a novel mechanism by which As(III) causes progressive cardiotoxicity. Furthermore, inhibiting sEH might represent a novel therapeutic approach to prevent As(III)-induced hypertrophy.

Keywords: Cytochrome P450s, arachidonic acid metabolism, arsenic, soluble epoxide hydrolase, cardiac hypertrophy, epoxyeicosatrienoic acids, dihydroxyeicosatrienoic acids, epoxygenases, hydroxylases

Acute arsenic toxicity alters cytochrome P450 and soluble epoxide hydrolase and their associated arachidonic acid metabolism in C57Bl/6 mouse heart

Anwar Anwar-Mohamed, Ahmed A. El-Sherbeni, Seok H. Kim, Hassan N. Althurwi, Beshay N. M. Zordoky, and Ayman O. S. El-Kadi

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

Abstract

Acute arsenic (As(III)) exposure has been reported to cause cardiac toxicity. However, this toxicity was never linked to the disturbance in cytochrome P450 (P450)-mediated arachidonic acid metabolism. Therefore, we investigated the effect of acute As(III) toxicity on the expression of P450 and soluble epoxide hydrolase (sEH) and their associated arachidonic acid metabolism in mice hearts. As(III) toxicity was induced by a single intraperitoneal injection of 12.5 mg/kg of As(III). Our results showed that As(III) treatment caused a significant induction of the cardiac hypertrophic markers in addition to Cyp1b1, Cyp2b, Cyp2c, Cyp4f, and sEH gene expression in mice hearts. Furthermore, As(III) increased sEH protein expression and activity in hearts with a consequent decrease in 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs) formation. Whereas the formation of 8,9-, 11,12-, 14,15-dihydroxyeicosatrienoic acids (DHEs) was significantly increased. As(III) also increased sEH mRNA and protein expression levels in addition to the hypertrophic markers which was reversed by knockdown of sEH in H9c2 cells. In conclusion, acute As(III) toxicity alters the expression of several P450s and sEH enzymes with a consequent decrease in the cardioprotective EETs which may represent a novel mechanism by which As(III) causes progressive cardiotoxicity. Furthermore, inhibiting sEH might represent a novel therapeutic approach to prevent As(III)-induced hypertrophy.

Keywords: Cytochrome P450s, arachidonic acid metabolism, arsenic, soluble epoxide hydrolase, cardiac hypertrophy, epoxyeicosatrienoic acids, dihydroxyeicosatrienoic acids, epoxygenases, hydroxylases
mediated arachidonic acid metabolism in the heart. The importance of P450 enzymes in the cardiovascular physiology emerges from their ability to metabolize arachidonic acid to epoxygenesatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs) (Roman 2002). The cardioprotective effects of EETs has been demonstrated in ischemia-reperfusion injury (Seubert et al. 2007), cardiac hypertrophy (Xu et al. 2006), and recently in As(III)-induced cardiotoxicity (Liu et al. 2011). On the other hand, 20-HETE is known to have detrimental effects in many cardiovascular diseases (Chabova et al. 2007; Lv et al. 2008; Minuz et al., 2008). Therefore, intricate homeostatic mechanisms are needed to keep the balance between these metabolites. In this context, previous reports from our lab have shown that isoproterenol-induced hypertrophy and doxorubicin-induced cardiotoxicity disturb this balance with decreased formation of the cardioprotective EETs (Zordoky et al. 2008; Zordoky et al. 2010). Therefore, it is plausible to expect that As(III)-induced cardiotoxicity is associated with a similar disturbance to the P450-mediated arachidonic acid metabolism.

In addition to P450 enzymes, soluble epoxide hydrolase (sEH) is another major player in determining the level of EETs. The cardioprotective EETs are hydrolyzed by sEH to the less biologically active dihydroxyeicosatrienoic acids (DHETs) (Imig et al. 2002). EPHX2, the gene encoding sEH, has been found to be a susceptibility factor for heart failure (Monti et al. 2008). In addition, EPHX2 gene expression has been reported to increase in animal models of angiotensin II- and isoproterenol-induced cardiac hypertrophy (Ai et al. 2009; Zordoky et al. 2008). Moreover, sEH inhibitors have been shown to prevent and/or reverse the development of cardiac hypertrophy in several models (Xu et al. 2006; Loch et al. 2007; Ai et al. 2009).

Therefore, in the current study, we hypothesize that acute As(III) cardiotoxicity alters the expression of several P450 and sEH enzymes in the heart of male C57Bl/6 mice. Therefore, we investigated the effect of acute As(III) cardiotoxicity on the expression of several P450 and sEH enzymes in addition to the formation of arachidonic acid metabolites to determine whether the changes in P450 and sEH expression have led to changes in P450-mediated arachidonic acid metabolites. Our findings show that As(III)-induced cardiotoxicity causes induction of several P450 and EPHX2 gene expressions. In addition, our results provide the first evidence that As(III)-induced cardiotoxicity is associated with alteration in cardiac 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, rat sEH (EPHX2) Silencer® Select Pre-designed and validated siRNA, and Silencer® Select Negative Control #2 siRNA 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 sodium arsenite 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, 19-HETE, 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). 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 C57Bl/6 mice weighing 25–30 g were obtained from Charles River Canada (St. Constant, QC, Canada). Animals were treated intraperitoneally (IP) with a single 12.5 mg/kg As(III) (n = 12). Weight-matched controls received the same volume of normal saline (n = 12). Animals were euthanized 6 and 24 h following the injection under isoflurane anesthesia. All animals were allowed free access to food and water throughout the treatment period. Heart tissues 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, Carlsbad, CA) according to the manufacturer’s instructions, and quantified by measuring the absorbance at 260 nm. RNA purity was determined by measuring the 260/280 ratio. Therewith, first-strand cDNA synthesis was performed by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) 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 s, 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, Foster City, CA). 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 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. 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.

Preparation of microsomal and cytosolic protein fractions
Heart microsomes were prepared by differential centrifugation of homogenized tissues as previously described (Lin et al., 1991; Barakat et al. 2001). Briefly, individual heart tissues were rapidly removed and washed in ice-cold potassium chloride [1.15% (w/v)]. Consequently, they cut into pieces, and homogenized separately in cold sucrose solution (1 g of tissue in 5 ml of 0.25 M sucrose). After homogenizing, the tissues were separated by different ultracentrifugation. The final pellets were reconstituted in cold sucrose, and the

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-ANP</td>
<td>5'-GGG GCC TAC GAA GAT CCA GC-3'</td>
<td>5'-TCC AAT CCT GTC AAT CCT ACC C-3'</td>
</tr>
<tr>
<td>r-ANP</td>
<td>5'-GGG GCC TAC GAA GGT CAA-3'</td>
<td>5'-TAT CTT CAG TGC CAG AAG AGT-3'</td>
</tr>
<tr>
<td>m-BNP</td>
<td>5'-AGT CCT TCG TCA AGG CA-3'</td>
<td>5'-CCG ATC CGG TCT ATC TTG TGC-3'</td>
</tr>
<tr>
<td>r-BNP</td>
<td>5'-CAG AAG CTG CGT CAT GCG ATG-3'</td>
<td>5'-TGT AGG GCC TCG CCT ATC TTG-3'</td>
</tr>
<tr>
<td>m-β-actin</td>
<td>5'-TAT TGG CAA CCA GGG GGG CT-3'</td>
<td>5'-GGG ATA GAG GTC TTT ACG GAT-3'</td>
</tr>
<tr>
<td>r-β-actin</td>
<td>5'-CCA GAT CAT GTG TGA GAC CCT ACA-3'</td>
<td>5'-GTA CGA CCA GAA GCA TAC-3'</td>
</tr>
<tr>
<td>m-CT-1</td>
<td>5'-CTC TCT AAT ATC ATT CCT ACC CC-3'</td>
<td>5'-GCT CAT GGA CTT CCT CCA GAA-3'</td>
</tr>
<tr>
<td>m-COX-2</td>
<td>5'-CTG GTG CCT GGT ATG ATG-3'</td>
<td>5'-GGC AAT GGG GTT CTA ATG-3'</td>
</tr>
<tr>
<td>m-Cyp1a1</td>
<td>5'-GGT TAA CCA TGA CCA CCA GCT-3'</td>
<td>5'-GTC CCA AAT ACA AGA GAG TGA-3'</td>
</tr>
<tr>
<td>m-Cyp1b1</td>
<td>5'-AAT GAG GAG TGG GGC GGC ACA-3'</td>
<td>5'-GGC GTG TGG AAT GGT GAC-3'</td>
</tr>
<tr>
<td>m-Cyp2b10</td>
<td>5'-GGG AAC CTC TGT CAG ATG-3'</td>
<td>5'-CCC AGG TCG ACT GTG AA-3'</td>
</tr>
<tr>
<td>m-Cyp2b19</td>
<td>5'-CAC AAA GCC TCT CTC ACC GAT-3'</td>
<td>5'-ACA AGG GAG CAA CCC ACA CTC-3'</td>
</tr>
<tr>
<td>m-Cyp2b9</td>
<td>5'-GGT GCA GGT CAG CTA GTT ATG C-3'</td>
<td>5'-GCC CAT TGG CAA AAA ATA TAC C-3'</td>
</tr>
<tr>
<td>m-Cyp2c9</td>
<td>5'-TGG TCC ACC CAA CAA AAG AAA TTA-3'</td>
<td>5'-GCA GAG AGG CAA ATC CAT TCA-3'</td>
</tr>
<tr>
<td>m-Cyp2c38</td>
<td>5'-CCA TTA CTT TTA GCA ATG GAA ACA GT-3'</td>
<td>5'-CCA CAA GAC ACT GTG CTT CCT CTC-3'</td>
</tr>
<tr>
<td>m-Cyp2c40</td>
<td>5'-TCC GGT TTT TGA CAA GGT TTT CTC TAC-3'</td>
<td>5'-TGG CCA AGT TCC TCA AGG TAT TCC-3'</td>
</tr>
<tr>
<td>m-Cyp2c44</td>
<td>5'-CTT TAC AAG GAG CGA TGA TCC CC-3'</td>
<td>5'-TGT TCC TCC TCG ATC TTG C-3'</td>
</tr>
<tr>
<td>m-Cyp2e1</td>
<td>5'-CCC AAG TCT TTA ACC AAG TTG GC-3'</td>
<td>5'-CTT CCA TGT GGG TCC ATT GA-3'</td>
</tr>
<tr>
<td>m-Cyp2j1</td>
<td>5'-GTG TCA TGG ACA TCA GTC ACC GGG A-3'</td>
<td>5'-GGT CCA CAG CAG TGA AGA TGA-3'</td>
</tr>
<tr>
<td>m-Cyp2j3</td>
<td>5'-GGG AAG AGG AGG AGC GTC CT-3'</td>
<td>5'-GCA GCC CCT GCT CAA ACT GAC T-3'</td>
</tr>
<tr>
<td>m-Cyp2j5</td>
<td>5'-TGT GAA TCG CTG TAT GAC ACC G-3'</td>
<td>5'-TG AAG TGG TGC CTT TGA ATG-3'</td>
</tr>
<tr>
<td>m-Cyp2j9</td>
<td>5'-GGG AAT GGT CTA AGC CTG CAT TT-3'</td>
<td>5'-GAG TGA CTG GCC GAC TTA TAA A-3'</td>
</tr>
<tr>
<td>m-Cyp4a10</td>
<td>5'-GTG CTG AGG TGG ACA CAT TCA T-3'</td>
<td>5'-TGC GGC CAG AGC ATA GAA GAT C-3'</td>
</tr>
<tr>
<td>m-Cyp4a12</td>
<td>5'-TGA CCC CAG CTT ATG ACT-3'</td>
<td>5'-TGT TGC CTA ACC TAC GCC-3'</td>
</tr>
<tr>
<td>m-Cyp4a14</td>
<td>5'-TGT CCG TCA GGA GCA ATA TAC G-3'</td>
<td>5'-GCC AAT CCA GAG AGG AAG CAA-3'</td>
</tr>
<tr>
<td>m-Cyp4f13</td>
<td>5'-CCC TAA ACC GAG CAG GTT CTT C-3'</td>
<td>5'-GAT TCG CAG GAT TGG CAC-3'</td>
</tr>
<tr>
<td>m-Cyp4f15</td>
<td>5'-CCT GTG CTT GTG TCC TTA GGA-3'</td>
<td>5'-GAC GGG AAA TGA CCG TGA CT-3'</td>
</tr>
<tr>
<td>m-Cyp4f16</td>
<td>5'-CCG CCT CAG TGT TTT CCC TC-3'</td>
<td>5'-TGG CCA AGT GAC CTG AAA ACC-3'</td>
</tr>
<tr>
<td>m-Cyp4f18</td>
<td>5'-CTG CAT GTG CCT CTC ATC G-3'</td>
<td>5'-GGT TGG TGA CTA GCT CCG AAA-3'</td>
</tr>
<tr>
<td>m-EPHX2</td>
<td>5'-GAA AGG ATT CAC AAG ATG CAT TG-3'</td>
<td>5'-GGC CAG GCT GTG CTT CTC TGC-3'</td>
</tr>
<tr>
<td>r-EPHX2</td>
<td>5'-CAC ATC CAA GCC ACC AAG CC-3'</td>
<td>5'-CAG GCC TCC ATC CTC CAG-3'</td>
</tr>
</tbody>
</table>

© 2012 Informa UK, Ltd.
supernatant, cytosols, were all stored at –80°C. Thereafter, microsomal and cytosolic protein concentrations were determined by the Lowry method using bovine serum albumin as a standard (Lowry et al. 1951).

Western blot analysis
Western blot analysis was performed using a previously described method (Anwar-mohamed et al. 2010). Briefly, 10 µg of heart cytosol for sEH, 20 µg of heart microsomes for Cyp4a and Cyp4f, or 50 µg of H9c2 total cell lysate for sEH were 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 the following primers: primary polyclonal mouse anti-rat Cyp4a, primary polyclonal rabbit anti-human Cyp4f2, primary polyclonal goat anti-mouse/rat sEH, primary polyclonal rabbit anti-mouse actin, or primary polyclonal goat anti-mouse Gapdh overnight at 4°C. Incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody for Cyp4f2 and actin or goat anti-mouse IgG secondary antibody for Cyp4a, or rabbit anti-goat IgG secondary antibody for sEH and Gapdh was carried out for another 2 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
Heart 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. 14,15-EET and its metabolites 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 (Zordoky et al., 2010).

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 at a density of 1–1.5 × 10⁶ cells per well in a 6-well tissue culture plate. On 60 to 80% confluence (2–3 days), appropriate stock solutions of As(III) were directly added to the culture media for 6 h, thereafter total RNA was isolated.

Transfecting H9c2 cells with EPHX2 siRNA
H9c2 cells were plated onto 12-well cell culture plates. Each well of cells was transfected with EPHX2 siRNA, or Silencer® Select Negative Control #2 siRNA at the concentration of 100 nM using INTERFERin reagent according to manufacturer’s instructions (Polyplus). EPHX2 siRNA sequences were sense: GAU CGA UCC CAG UUU UCA Att, and antisense: UUG AAA ACU GGG AUC GAU. Transfection efficiency was determined using real-time PCR to detect EPHX2 mRNA post-transfection at 24 h. Thereafter, cells were treated for additional 6 h post-transfection with 5 µM As(III) to determine EPHX2, ANP, and BNP mRNA levels.
Statistical analysis
Data are presented as mean ± standard error of the mean (SEM). Control and treatment measurements at 6 or 24 h were compared using Student’s t test or 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 As(III) treatment on hypertrophic markers ANP, BNP, and CT-1
To examine the effect of As(III) treatment on cardiac hypertrophy markers, we measured the cardiac gene expression of the hypertrophic markers atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and cardiотrophin-1 (CT-1) relative to control mice. Our results showed that As(III) treatment significantly induced the hypertrophic markers ANP, BNP, and CT-1 by 3600%, 250%, and 1800%, respectively compared to control (Figure 1A).

Effect of As(III) treatment on \( \text{P}4\text{G}5\text{O} \) gene expression
To examine the effect of As(III) treatment on the cardiac expression of several \( \text{P}4\text{G}5\text{O} \) genes, total RNA was extracted from the heart of both control and As(III)-treated mice. Thereafter, the expression of different genes was measured using reverse transcription followed by real-time PCR as described under materials and methods.

As(III) significantly induced Cyp1b1 mRNA levels by 150% compared to control (Figure 1B). With regard to Cyp2 family, As(III) treatment significantly induced Cyp2b9, Cyp2b10, and Cyp2b19 mRNA levels by 800%, 70%, and 150%, respectively compared to control (Figure 1B). Furthermore, As(III) treatment also significantly induced Cyp2c38 and Cyp2c40 by 160% and 383%, respectively, while it did not affect Cyp2c29 or Cyp2c44 (Figure 2A). Regarding the effect of As(III) on Cyp2e1 mRNA levels, As(III) significantly inhibited heart Cyp2e1 mRNA levels by 75%, compared to control (Figure 2A). For the Cyp2j subfamily, As(III) significantly inhibited heart Cyp2j5, Cyp2j11, Cyp2j13 mRNA levels by 90%, 64%, and 52%, respectively compared to control (Figure 2B). Interestingly, As(III) significantly induced heart Cyp2j9 mRNA levels by 100%, compared to control (Figure 2B).

With regard to the major \( \text{CYP} \) \( \omega \)-hydroxylases, the cardiac gene expression of Cyp4a and Cyp4f was assessed. Figure 3 shows the effect of As(III)-induced toxicity on cardiac Cyp4a10, Cyp4a12, and Cyp4a14 mRNA levels at 6 h after As(III) administration. Of interest, As(III) administration failed to significantly affect heart Cyp4a10 and Cyp4a12 mRNA levels, while significantly inhibited Cyp4a14 mRNA levels by 66%, compared to control (Figure 3A). In a different pattern to that of Cyp4a, acute As(III) toxicity caused a significant induction of heart Cyp4f15 and Cyp4f18 mRNA levels by 120% and 140%, respectively compared to control while it did not significantly affect Cyp4f13 and Cyp4f16 mRNA levels (Figure 3B).

Effect of As(III) treatment on EETs and DHETs metabolites formation
To investigate the effect of As(III) treatment on \( \text{CYP} \) gene expression was further translated to the protein level, microsomal protein was prepared from hearts of control and animals treated with As(III) for 6 h. Gene expressions were determined by real-time PCR. Results are presented as mean ± SE \((n = 6)\). \( *p < 0.05 \) compared to control.

To investigate whether the effect of As(III) treatment on \( \text{CYP} \) gene expression was further translated to the protein level, microsomal protein was prepared from hearts of control and mice treated with As(III) for 24 h. Thereafter, Cyp4a, and Cyp4f protein expression levels were determined using Western blot analysis relative to actin as an endogenous control. Our results show that As(III) treatment failed to significantly affect cardiac Cyp4a and Cyp4f protein expression compared to control (Figure 3C).

Effect of As(III) treatment on EETs and DHETs metabolites formation
To investigate the effect of As(III) treatment on the formation of \( \text{P}4\text{G}5\text{O} \)-derived arachidonic acid metabolites, heart microsomes of either control or 24 h As(III)-treated mice were incubated with 50 \( \mu \)M arachidonic acid for 30 min. Thereafter, arachidonic acid metabolites were measured using LC-ESI-MS. In comparison to control animals, in heart microsomes of As(III)-treated mice, the formation of 14,15- and 11,12-EETs were significantly lower than control values by 83% and 82%, respectively.
We also measured the levels of enzymatic hydration products of EETs, the DHETs. As shown in Figure 4B, in the heart microsomes of As(III)-treated mice, the formation of 14,15-, 11,12-, 8,9-DHETs were significantly higher than control values by 300%, 331%, and 419%, respectively (Figure 4B).

In order to investigate the effect of As(III) treatment on the total epoxygenase activity, we calculated the sum of all the products of epoxygenase enzymes, namely the total EETs and DHETs in control and mice treated with As(III) for 24 h. The total epoxygenase activity was not significantly altered in the heart microsomes of As(III)-treated mice as compared to the control (Figure 5A). On the other hand, to determine the effect of As(III) treatment on P450 ω-1- and ω-hydroxylases activity, we determined the formation of 19- and 20-HETE, respectively, in heart microsomes from control and As(III)-treated mice. As(III) treatment did not significantly alter 19- or 20-HETE formation in heart microsomes of As(III)-treated mice (Figure 5B and 5C). Moreover, to determine the relative formation of 20-HETE compared with total EETs in As(III) treatment, we calculated the 20-HETE: total EET ratio. As(III) treatment significantly induced the ratio of 20-HETE: EET by 75% compared to control (Figure 5D).
Effect of arsenic on CYP-mediated arachidonic acid metabolism

To investigate the mechanism responsible for lower EETs levels in the heart microsomes of As(III)-treated mice, the expression of EPHX2 gene was determined. Our results show that As(III) treatment significantly induced EPHX2 gene expression in the heart by 450%, compared to control (Figure 6A). To confirm whether or not the induction of EPHX2 gene is translated to functional protein, sEH protein expression and catalytic activity were induced by 400% and 384%, respectively, in the heart cytosols of mice treated with As(III) for 24 h (Figure 6B and 6C).

Effect of As(III) treatment on COX-2 expression

The fact that As(III) was not able to increase 20-HETE formation in heart microsomal incubations despite the significant induction of Cyp4f15 and Cyp4f18 mRNA levels prompted us to examine its effect on COX-2 which is known to metabolize 20-HETE to 20-hydroxy-PGE$_2$ (Imig 2004). Our results showed that As(III) significantly induces COX-2 expression by 125%, compared to control (Figure 7).

Effect of As(III) treatment on hypertrophic markers and sEH mRNA and protein expression levels in H9c2 cells

To examine whether the induction of hypertrophic markers and sEH gene expression is due to the direct effect of As(III) on the cardiomyocytes, the cardiac derived H9c2 cells were treated with increasing concentrations of As(III) (1–10 µM). Thereafter, the expression of ANP, BNP, and EPHX2 was measured using real-time PCR as described under materials and methods.

Our results demonstrated that As(III) significantly induced ANP and BNP in a dose-dependent manner. ANP mRNA level was induced by 44%, 330%, and 1200% compared to control with As(III) concentrations of 1, 5, and 10 µM, respectively (Figure 7A). On the other hand, BNP mRNA level was induced by 10%, 150%, and 318% compared to control with As(III) concentrations of 1, 5, and 10 µM, respectively (Figure 7A). In accordance with our in vivo results, the induction of sEH was 90% and 140% higher than control with As(III) concentrations of 5 and 10 µM, respectively (Figure 7B). To investigate whether the changes of EPHX2 gene expression is further translated to the protein expression levels, we measured the sEH protein expression levels in H9c2 cells treated with As(III) (5 µM) for 24 h. Our results demonstrated that As(III) significantly increased sEH protein expression levels in these cells by 50% compared to control (Figure 7C).

The effect of knockdown of EPHX2 on As(III)-mediated induction of ANP, and BNP mRNA levels

We took a genetic approach to confirm whether or not EPHX2 is involved in the As(III)-mediated induction of the hypertrophic markers ANP and BNP. For this purpose, H9c2 cells were transfected with rat EPHX2 siRNA or Silencer negative control siRNA for 24 h, and then the cells were treated with 5 µM As(III). Our results showed that EPHX2 siRNA significantly decreased EPHX2 mRNA by 45% as compared to control (Figure 9A). On the other hand, As(III) was able to increase EPHX2 mRNA levels by 75% compared to control. When the cells were transfected with EPHX2
siRNA, and then treated with As(III) there was a statistically significant decrease in EPHX2 mRNA levels by 62% compared to As(III) alone. Furthermore, the silencer select negative control siRNA did not affect the constitutive level of EPHX2 mRNA, eliminating the possibility that the inhibitory effects of EPHX2 siRNA might have been due to any toxicity.

Looking at ANP and BNP mRNA levels, As(III) alone significantly induced ANP and BNP mRNA levels by 70% and 90%, respectively, compared to control (Figure 9B). EPHX2 siRNA alone did not significantly affect ANP or BNP mRNA levels. Interestingly, when H9c2 cells were transfected with EPHX2 siRNA and then treated with As(III), the As(III)-mediated induction of ANP and BNP was significantly decreased by 87% and 47%, respectively, compared to As(III) alone (Figure 9B).

**Discussion**

Results from our lab and others have previously shown that As(III) significantly alters P450 enzymes in the liver, lung, and kidney (Medina-Diaz et al., 2009; Maier et al., 2000; Anwar-Mohamed et al., 2009; Wu et al., 2009; Seubert et al., 2002). However, there are no studies about the effect of As(III) on P450 enzymes in the heart. P450 enzymes are considered one of the major metabolic pathways for arachidonic acid in addition to the cyclooxygenase and the lipoxygenase pathways. P450 epoxygenases metabolize arachidonic acid to several regioisomers of EETs, while P450 \( \omega-1 \)- and \( \omega \)-hydroxylases metabolize arachidonic acid to 19- and 20-HETE (Roman, 2002). In the present study, acute As(III) toxicity has been induced by a single IP injection of 12.5 mg/kg of sodium arsenite in C57Bl/6 mice as this dose has been shown previously to induce cardiotoxicity in mice (Yanez et al. 1991; Liu et al. 2002).

In the current study we have demonstrated for the first time that As(III) induces the hypertrophic markers ANP, BNP, and CT-1 to different extents. In an attempt to examine the effects of As(III) on P450, we examined the effect of As(III) on the gene expression of different P450s. Our results demonstrated that As(III) induced Cyp1b1 mRNA levels. However, the induction of Cyp1b1 by As(III) cannot be simply attributed to the AhR-dependent pathway which governs the Cyp1b1 gene expression in response to AhR-ligands (Bhattacharyya et al. 1995). As(III) is not known to be an AhR ligand, yet it has been shown to induce inflammation as it increases serum tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)) (Das et al. 2009). Similarly, it has been reported that TNF-\( \alpha \) is a potent stimulator of Cyp1b1 gene expression in some liver cells (Piscaglia et al. 1999; Umannova et al. 2007). Thus, it is plausible that As(III) might have
Effect of arsenic on CYP-mediated arachidonic acid metabolism

The effects of arsenic on the metabolism of arachidonic acid were studied. Arsenic induced the expression of the Cyp1b1 gene, which metabolizes arachidonic acid to both mid-chain HETEs and EETs (Choudhary et al., 2004). Looking at the Cyp2 family, which includes important P450 epoxygenases, arsenic differentially affected their mRNA levels. Heart Cyp2b9, Cyp2b10, and Cyp2b19 mRNA levels were all induced in response to arsenic treatment. Furthermore, heart Cyp2c38 and Cyp2c40 were also induced while Cyp2c29 and Cyp2c44 mRNA levels were not altered. Although, Cyp2b subfamily members belong to the class of epoxygenases, the major P450 epoxygenases are members of the Cyp2c and Cyp2j subfamilies (Kroetz and Zeldin, 2002).

Investigating the effect of arsenic on the P450 hydroxylases mRNA levels, we found an inhibition of Cyp2e1 and Cyp4a14 mRNA levels in the heart. On the other hand, Cyp2j9, Cyp4f15, and Cyp4f18 mRNA were induced in the heart. Furthermore, Cyp4a10 and Cyp4a12 mRNA levels were not altered in the heart. Previous results have shown that P450ω-1-hydroxylases are mainly Cyp2e1, Cyp2j9, and Cyp4a10 (Roman, 2002). Cyp4a10 is also considered a ω-1-hydroxylase as it has the capacity to produce 20-HETE in addition to 19-HETE (Roman, 2002).

To correlate the mRNA results to those of the protein expression and activity level, we found that arsenic did not alter Cyp4a or Cyp4f protein expression levels. In addition, arsenic did not alter ω-1- or ω-2-hydroxylases activity despite the increase in Cyp4f15 and Cyp4f18 mRNA levels, which is in line with the protein expression results. In the current study, we have shown that arsenic increases COX-2 expression which is in agreement with previously published studies (Chai et al., 2007; Bunderson et al., 2004). This induction of COX-2 might have contributed to the observed tendency towards the decreased formation of 20-HETE. In this regard, recent studies have shown 20-HETE to be a substrate for COX-2 (Imig, 2004).

20-HETE is known to be involved in many cardiovascular diseases and its formation has been studied extensively. It has been suggested that 20-HETE may play a role in the regulation of inflammatory responses and the pathology associated with pro-inflammatory conditions.

![Figure 6](image.png)

Figure 6. Effect of As(III) toxicity on cardiac EPHX2 gene expression (A), sEH protein expression (B), and sEH activity (C). (A) Total RNA was isolated from hearts of control and animals treated with As(III) for 6 h. Gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 6). *p < 0.05 compared to control. (B) Heart cytosolic protein was isolated from control and animals treated with As(III) for 24 h. Cytosolic protein (10 μg) was separated on a 10% SDS-PAGE. sEH, and Gapdh 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. (C) Heart cytosols of control or animals treated with As(III) for 24 h were incubated with 50 μM 14,15-EET. 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. 14,15-DHET and 14,15-DHET were extracted twice with 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.

![Figure 7](image.png)

Figure 7. Effect of As(III) toxicity on cardiac COX-2 gene expression. Total RNA was isolated from hearts of control and animals treated with As(III) for 6 h. Gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 6). *p < 0.05 compared to control.
reported to be higher in the hearts of rats with streptozotocin-induced diabetes and isoproterenol-induced cardiac hypertrophy (Yousif et al., 2009; Zordoky et al., 2008). Interestingly, inhibiting 20-HETE formation caused improvement of the cardiac function following ischemia-reperfusion injury in diabetic rats (Yousif et al., 2009) and reduced cardiomyocyte apoptosis in another ischemia-reperfusion injury model (Lv et al., 2008). In the current study, the 20-HETE: total EETs ratio was significantly higher in As(III)-treated mice, due to the lowered formation of EETs. Thus, although As(III) did not increase 20-HETE formation, its ability to increase the ratio of 20-HETE: total EETs formation is another predisposing factor that might contribute to the progression of cardiotoxicity.
Effect of arsenic on CYP-mediated arachidonic acid metabolism

To investigate the effect of P450 alteration on arachidonic acid metabolism, we performed *in vitro* incubation of heart microsomes with arachidonic acid. In the current study we have demonstrated for the first time that As(III) decreases heart 14,15- and 11,12-EET formation. This decrease in EETs formation was accompanied by a significant increase in the formation of their corresponding DHETs. In order to estimate the epoxygenase activity, the sum of the total EETs and DHETs was calculated (Zhao et al., 2006). Interestingly, the total epoxygenase activity was not altered in the heart microsomes of As(III)-treated mice. Although some heart epoxygenases were induced and others were inhibited, the end result of these differential alterations were in favor of decreased cardiac EETs formation. In order to see if this decrease was due to an increase in *EPHX2* gene expression and subsequently sEH protein expression and activity we measured *EPHX2* gene expression and sEH protein expression and activity in heart cytosols. Our results demonstrated that As(III) increased heart *EPHX2* gene expression, protein expression, and activity. Thus, the decrease in cardiac EETs, could be attributed to the increased sEH activity as total cardiac epoxygenase activity was not altered.

To examine whether As(III) directly induces *EPHX2* gene expression at the cardiomyocyte level, we used H9c2 cells which is a commercially available myogenic cell line derived from embryonic rat heart ventricles (Kimes and Brandt 1976). In agreement with the *in vivo* results, As(III) treatment caused a significant induction of *EPHX2* mRNA and protein expression levels *in vitro*. Thus, the induction of *EPHX2* and subsequently sEH is a direct effect of As(III).

The inverse correlation between sEH expression and activity on one side and cardiac hypertrophy and heart failure on the other side has been previously reported (Monti et al. 2008; Xu et al. 2006; Ai et al. 2009). With regard to hypertrophy, it has been previously reported that the administration of sEH inhibitors protect against angiotensin II-induced hypertrophy in male Wistar rats and reversed chronic pressure overload-induced cardiac hypertrophy through thoracic aortic constriction in C57Bl/6 mice, as demonstrated by the decreased left-ventricular hypertrophy, reduced cardiomyocyte size hypertrophic markers expression, including atrial natriuretic factor and β-myosin heavy chain (Ai et al. 2009; Xu et al. 2006). Our results demonstrated that knocking down *EPHX2*, the gene encoding sEH, prevented against As(III)-induced hypertrophy, implying that As(III)-induced hypertrophy is in part due to inducing sEH.

Despite the importance of sEH enzyme, little is known about its transcriptional regulation in different pathophysiological and experimental conditions. In this context, previous reports have demonstrated that *EPHX2* promoter contains three activator protein-1 (AP-1) and one nuclear factor κB (NF-κB) regulatory elements (Ai et al. 2007). With the fact that As(III) is a potent activator of both AP-1 and NF-κB (Felix et al. 2005), it is thus plausible that As(III) might have induced *EPHX2* through activating one or both transcription factors. The role of EETs in the heart has been previously reported (Roman 2002). In addition to their blood pressure lowering properties, EETs are also reported to have cardioprotective effects through several mechanisms most notably by inhibiting the activation of NF-κB (Hirotani et al. 2002; Xu et al. 2006). Interestingly, As(III) has been shown to induce myocardial apoptosis through activation of NF-κB (Ghosh et al. 2009). Therefore, As(III)-induced NF-κB activation and subsequently increased sEH activity may be involved in lowering cardiac EETs formation and the subsequent myocardial apoptosis and cardiotoxicity. In addition, increased oxidative stress has been suggested as one of the main mechanisms of As(III)-induced cardiotoxicity (Liu et al. 2011). Taking into account the antioxidant properties of EETs, we can postulate that lower cardiac EETs formation potentiates As(III)-induced oxidative stress and further contributes to its cardiotoxicity.

In conclusion, acute As(III) toxicity induces several cardiac P450 and sEH enzymes. There are several mechanisms that can explain the effect of As(III) toxicity on these enzymes. Although the exact mechanism is not fully elucidated yet, As(III)-induced inflammation and/or oxidative stress contribute, at least in part, in the alteration of the P450 enzymes and sEH. The changes in P450 and sEH expression result in altered arachidonic acid metabolism as EETs formation was decreased in the heart with a subsequent increase in DHETs formation. Taking into account the physiological functions of these metabolites, the decrease in EETs due to the increase in sEH activity can be considered as a maladaptive response to As(III) cardiotoxicity. Along with the results of Liu (Liu et al. 2002), the inhibition of sEH might confer cardioprotection in patients receiving As(III) for the treatment of acute promleocytic leukaemia.

**Declaration of interest**

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant RGPIN 250139 to A.O.S. A.A-M. is the recipient of Alberta Innovates Technology Futures Scholarship, and Izaak Walton Killam memorial graduate scholarship. A.A.E. is the recipient of Egyptian Government Scholarship. S.H.K is the recipient of Alberta Innovates Health Solutions summer studentship. H.N.A is the recipient of Salman Bin Abdulaziz University scholarship, Saudi Arabia. B.N.M.Z. is the recipient of Alberta Innovates Health Solutions Scholarship.
References


Liu J, Liu Y, Powell DA, Waalkes MP, Klaassen CD. (2002). Multidrug-resistance mdr1a/1b double knockout mice are more sensitive than wild type mice to acute arsenic toxicity, with higher arsenic accumulation in tissues. Toxicology 170:55–62.


