

## ORIGINAL ARTICLE

# In vitro analysis of factors influencing CYP1A2 expression as potential determinants of interindividual variation

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## Keywords

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## Abstract

Individual differences in drug metabolism contribute to interindividual variation that characterizes responses to drugs and risk in exposure to foreign chemicals. Large individual differences are found in expression levels of *CYP1A2*, a major drug-metabolizing enzyme. Underlying causes for this variation are not well understood. Several factors, including tobacco smoking, consumption of cruciferous vegetables, and sex, have been associated with modulating *CYP1A2* expression. To understand factors regulating expression of *CYP1A2* in establishing a causal relationship, this study examined effects of cigarette smoke condensate (CSC), indole-3-carbinol (I3C), and 17 $\beta$ -estradiol (estradiol) on *CYP1A2* expression in in vitro systems using human liver and lung cells. Treatment with CSC (2–25  $\mu$ g/mL) significantly increased levels of *CYP1A2* in six cell lines examined, in a concentration- and time-dependent manner. Fold changes in expression levels relative to controls varied among cell lines. *CYP1A2* enzymatic activity also increased with CSC exposure. Treatment of H1299 and HepB3 cells with dietary agent I3C (50 and 100  $\mu$ mol/L) increased *CYP1A2* expression. In human cell lines H1299 and H1395, treatment with estradiol (10 and 100 nmol/L) significantly reduced expression of *CYP1A2*. Using ChIP assays, effects of CSC on histone modifications were analyzed. Increases in H3K4me3 and H4K16ac were observed at several segments in the *CYP1A2* gene, whereas H3K27me3 decreased, following CSC treatment. These results suggest that *CYP1A2* expression is affected epigenetically by CSC. Additional studies will be needed to further establish regulatory mechanisms underlying effects of various environmental, dietary, and endogenous factors on *CYP1A2* expression in better predicting individual variation.

## Abbreviations

AhR, aryl hydrocarbon receptor; AzadC, 5-aza-2'-deoxycytidine; CSC, cigarette smoke condensate; DMF, 3',4'-dimethoxyflavone; Estradiol, 17 $\beta$ -estradiol; HDAC, histone deacetylase; I3C, indole-3-carbinol; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TSA, trichostatin.

## Introduction

The human cytochrome P450 1A2 (*CYP1A2*) enzyme is a major drug-metabolizing enzyme, metabolizing about 15% of clinical drugs such as clozapine, theophylline, and tacrine (Zhou et al. 2009). *CYP1A2* also plays an important role in bioactivation of various procarcinogens, including heterocyclic amines, found in foods and other

products, and tobacco-specific nitrosamines (Hecht 2006). *CYP1A2* is well represented in human liver, accounting for about 15% of total P450 content (Shimada et al. 1994), and is also in human lung tissue (Wei et al. 2002; Ding and Kaminsky 2003; Choudhary et al. 2005; Iba et al. 2010). Large individual differences are found in expression levels of *CYP1A2* (Hammons et al. 1985; Kalow and Tang 1991; Schweiki et al. 1993). Underlying

causes for this variation are not well understood. *CYP1A2* is genetically polymorphic (<http://www.cypalleles.ki.se/cyp1a2.htm>). However, the low frequency of functionally different variant alleles in populations and results of several genotype-phenotype association and other studies indicate limited function of *CYP1A2* genetic polymorphism as a cause of interindividual variation in *CYP1A2* expression (Jiang et al. 2006; Ghotbi et al. 2007; Klein et al. 2010; Spina and de Leon 2015; Vukovic et al. 2016). Other factors must play a critical role.

Several factors in human exposures have been associated with the modulation of *CYP1A2* activity, including smoking and various dietary agents. Cigarette smoking induces *CYP1A2* activity, as shown by lower plasma concentrations of phenacetin or accelerated metabolism of caffeine in smokers compared to nonsmokers (Pantuck et al. 1972; Kotake et al. 1982). In tissue samples from patients of known smoking status, *CYP1A2* content was 3.5-fold higher in smokers than in nonsmokers (Sesardic et al. 1988). Other studies have also found this relationship. A 1.66-fold increase in *CYP1A2* activity was observed for smokers consuming 11–20 cigarettes daily ( $n = 863$ ) (Tantcheva-Poor et al. 1999). Smokers had significantly higher paraxanthine:caffeine ratio (*CYP1A2* activity) than nonsmokers in both Koreans ( $n = 150$ ) and Swedes ( $n = 194$ ) (Ghotbi et al. 2007). Additionally, induction of *CYP1A2* has been demonstrated to dissipate after quitting smoking (Faber and Fuhr 2004). Clinical studies have shown that smoking can reduce plasma concentrations of many *CYP1A2* substrate drugs, which has significant implications for drug efficacy and safety (Kroon 2007).

Increased *CYP1A2* activity is associated with intake of cruciferous vegetables. Diets containing 500 g/day of broccoli were ingested, which led to a 25% increase in the elimination of *CYP1A2*-derived metabolites of caffeine in patients (Kall et al. 1996). Using a randomized, cross-over feeding trial in humans, dose effects of cruciferous vegetables were investigated (Peterson et al. 2009). Compared with basal diet, basal plus single dose of cruciferous diet increased *CYP1A2* activity and basal plus double dose of cruciferous diet resulted in further increases, with men experiencing greater dose-response than women. In a recent phase I study of indole-3-carbinol (I3C), a major indole found in cruciferous vegetables, conducted in women, *CYP1A2* levels were found to be elevated after a 4-week dose period of 800 mg daily (Reed et al. 2005).

As another source of variation, sex differences in *CYP1A2* activity have been demonstrated. Lower *CYP1A2* activity was reported in female human liver microsomes compared with male human liver microsomes (Parkinson et al. 2004). Additionally, using both urinary and plasma caffeine metabolic rates, significantly lower *CYP1A2*

activity has been reported in women compared to men in Caucasian (Relling et al. 1992; Bock et al. 1994; Carrillo and Benitez 1996) and Chinese populations (Ou-Yang et al. 2000). Other substrates have been utilized to investigate the activity of *CYP1A2* in humans. In results from a study analyzing plasma levels of thiothixene in 42 patients after oral administration, men were found to display significantly higher rate of clearance than women (Ereshefsky et al. 1991). In a study with Chinese schizophrenic nonsmoking patients, women had a significantly higher clozapine plasma concentration than men after normalizing for dose and weight (Tang et al. 2007).

Demonstration of a direct causal relationship in the effect of these factors on *CYP1A2* expression has, however, been limited. In this study, the effect of cigarette smoke condensate (CSC) on the expression of *CYP1A2* was determined in several in vitro model systems, including both human liver and lung cell lines. The lung is an important portal of entry, and environmental risk factors are important in the etiology of lung disease (Rom et al. 2000). Local levels of carcinogen-metabolizing enzymes should influence an individual's lung cancer risk by modifying cellular response to carcinogens. The effects of 17 $\beta$ -estradiol (estradiol) and dietary agent I3C were also examined. In this comprehensive study, each factor was shown to modulate *CYP1A2* expression in the cellular systems, demonstrating a causal effect. In a mechanistic assessment, findings from the study support the involvement of epigenetics in the induction by CSC. These results are consistent with the increasing evidence of the involvement of epigenetics in the regulation of *CYP1A2* expression being reported (Tang and Chen 2015).

## Materials and Methods

### Cell lines and treatment conditions

The human liver and lung cell lines, SNU-387 (hepatocellular carcinoma), HepB3 (hepatocellular carcinoma), A549 (lung carcinoma), H1395 (lung adenocarcinoma), H1792 (lung adenocarcinoma), and H1299 (non-small cell lung carcinoma), were obtained from the American Type Culture Collection (Manassa, VA). The cells were cultured in growth medium as recommended by the supplier and routinely maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cigarette smoke condensate (CSC) was purchased from Murty Pharmaceuticals (Lexington, KY) and was prepared using a smoking machine designed for Federal Trade Commission testing. The particulate matter from Kentucky standard cigarettes (1R3F; University of Kentucky, Lexington, KY) was collected on Cambridge glass fiber filters and the amount of CSC obtained was determined by weight increase on the filter. CSC was

prepared by dissolving the collected smoke particulates in dimethyl sulfoxide (DMSO) to yield a 4% solution (w/v). The CSC was diluted into DMSO and aliquots were stored at  $-80^{\circ}\text{C}$ . In treatment experiments, cells (400,000 cells per plate) were cultured in 100 mm dishes in appropriate media with DMSO alone or with treatment agent. 3',4'-Dimethoxyflavone (DMF), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), estradiol, I3C, 5-aza-2'-deoxycytidine (AzadC), and trichostatin (TSA) were purchased from Sigma-Aldrich (St. Louis, MO). At appropriate times, cells were harvested, and processed for further analysis. Cell proliferation was assessed by MTT techniques using the Cell Titer 96 Non-Radioactive Cell Proliferation Assay (Promega; Madison, WI).

### RNA isolation and Quantitative Real-time PCR (QRT-PCR) analysis

Total cellular RNA was isolated from the cells using the Qiagen RNeasy isolation kit (Qiagen; Valencia, CA) according to the manufacturer's instructions. The concentration of RNA was determined by NanoDrop<sup>®</sup> Spectrophotometer (ThermoScientific; Wilmington, DE). The OD260/OD280 nm ratios of all RNA samples were determined to be between 1.7 and 2.0 to ensure that all RNA samples are highly pure. RNA integrity was verified by Experion<sup>™</sup> (Bio-Rad; Hercules, CA). Real-time PCR reactions were then performed using a SyberGreen PCR master mix on iQ<sup>™</sup>5 Multicolor Real-time PCR Detection System (Bio-Rad; Hercules, CA). The primers for *CYP1A2* and *GADPH* ( $\beta$ -actin) were synthesized by Sigma-Aldrich Oligo Division (Woodlands, TX). Primers were as follows: *CYP1A2* – forward 5'-CCACACCAGCCATTACAA CCCTGCC-3' and reverse 5'-TGCGCTGGGTCATCCTTG ACAGTGC-3'; *GADPH* – forward 5'-GAAGGTGAAGG TCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGA TTTC-3'. Reactions were carried out in triplicates in a 96-well plate in a total volume of 15  $\mu\text{L}$ . Each reaction mixture contained 7.5  $\mu\text{L}$  of SyberGreen PCR master mix, 3.8  $\mu\text{L}$  of sterile nuclease-free water, 0.6  $\mu\text{L}$  of forward primer (20  $\mu\text{mol/L}$ ), 0.6  $\mu\text{L}$  of reverse primer (20  $\mu\text{mol/L}$ ). PCR conditions were as follows:  $50^{\circ}\text{C}$  for 2 min,  $95^{\circ}\text{C}$  for 10 min,  $95^{\circ}\text{C}$  for 15 sec,  $60^{\circ}\text{C}$  for 1 min (40 cycles). Quantification of relative mRNA levels was carried out by determining the threshold cycle ( $C_T$ ), which is defined as the cycle at which the reporter fluorescence exceeds by 10 times the standard deviation of the mean baseline emission for cycles 3–10. *GADPH* was used as an internal control. The mRNA levels of *CYP1A2* were normalized to those of *GADPH* according to the following formula:  $C_T$  (target) –  $C_T$  (*GADPH*) =  $\Delta C_T$ . Thereafter, the relative mRNA levels of *CYP1A2* after treatment were calculated using the  $\Delta\Delta C_T$  method:  $\Delta\Delta C_T$

(treatment) –  $\Delta C_T$  (vehicle) =  $\Delta\Delta C_T$  (treatment). The fold changes of mRNA levels were expressed as  $2^{-\Delta\Delta C_T}$ . All PCR reactions were performed in triplicate in three independent experiments.

### Cytochrome (CYP) 1A2 activity assay

The P450-Glo<sup>™</sup> *CYP1A2* (Luciferin-1A2) assay (Promega; Madison, WI) was used to determine *CYP1A2* enzymatic activity in H1395, H1299, SNU-397, and HepB3 cells treated with CSC. This assay provides a luminescent method to measure *CYP1A2* activity via the conversion of the inactive luciferin-1A2 into the active form by *CYP1A2* enzyme to produce luminescence. The amount of luminescence produced is directly proportional to *CYP1A2* activity. Cells were treated with 2–25  $\mu\text{g/mL}$  CSC for 16 h. Control cells were treated with vehicle (DMSO) only. Assaying was conducted according to manufacturer's instructions. The luminescences were measured in a Chameleon plate reader (Hidex; Turku, Finland) at 700 nm.

### Chromatin immunoprecipitation assay

SNU-387 cells were treated with CSC (25  $\mu\text{g/mL}$ ) or vehicle (DMSO) only for 24 h. ChIP assays were performed using a Millipore Magna ChIP<sup>™</sup> A assay kit (Millipore; Temecula, CA) following the manufacturer's protocol. Antibodies used in the immunoprecipitations were purchased from Millipore and recognized trimethylated histone H3 lysine 4 (H3K4me3), acetyl histone H4 lysine 16 (H4K16ac), trimethylated histone H3 lysine 27 (H3K27me3). The immunoprecipitated DNA was eluted in a total volume of 30  $\mu\text{L}$ . DNA (3  $\mu\text{L}$ ) was analyzed by real-time PCR using the following conditions:  $95^{\circ}\text{C}$  for 10 min,  $95^{\circ}\text{C}$  for 20 sec,  $60^{\circ}\text{C}$  for 1 min (40 cycles). Eight subregions were analyzed in the 5'-flanking region of the *CYP1A2* gene (GenBank, NCBI Reference Sequence: NG\_008431.1): Reg-1, 8456-8549; Reg-2, 28319-28405; Reg-3, 28521-28560; Reg-4, 28791-28814; Reg-5, 29081-29154; Reg-6, 30378-30402; Reg-7, 31261-31271; Reg-8, 31431-31510. The sequence of primer sets used in the analysis were as followed: Reg-1, forward GCTACAGCCTACCAGGACTC and reverse CGCCATCC ATTCGGATCCTT; Reg-2, forward ATCTTGGCTCACC GCAACCT and reverse AAATTAGCTGGGCGTGATGG; Reg-3, forward CTCGGCCTCCCAAAGTGCTAGAA and reverse TGCACAGCCCACACCGAAGGAGT; Reg-4, forward GGAAAGGTGGGAAAGGAGTAAC and reverse AG GTGCC ATTCTCGTCACAT; Reg-5, forward ACTGTC ACAGCCAAGAGGAATC and reverse GGTGGAGGTT AGGCTCGGTTT; Reg-6, forward CACCTCCCAAGTTC AAGCCATT and reverse AGCACTTTGAGAGGCCGAG

AT; Reg-7, forward CAAGCCAAG AGTTGATCCTTC and reverse AGCTTCTGGCTCTAGGTGAGT; Reg-8, forward CTCCACACCAGCCATTACAA and reverse ATCGGAAACAGAAGT CAAGAG. Results are expressed as fold changes relative to control for percent of input.

### Statistical analysis

Prism IV software (GraphPAD Software; San Diego, CA) was used for graphical analyses. Data were analyzed for statistical significance using one-way ANOVA followed by Dunnett's test. Differences with  $P < 0.05$  were considered statistically significant.

### Results

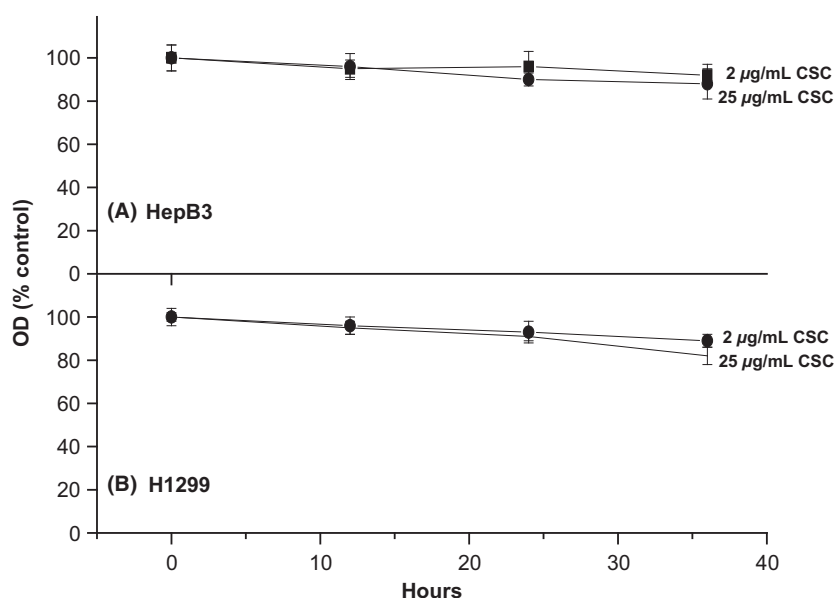
Increased levels of *CYP1A2* have been found to be associated with cigarette smoking in population studies (Pantuck et al. 1972; Kotake et al. 1982; Sesardic et al. 1988; Tantcheva-Poor et al. 1999; Faber and Fuhr 2004; Ghotbi et al. 2007; Kroon 2007). Using an in vitro approach, this study investigated the effect of CSC on *CYP1A2* expression in human liver and lung cell lines. Two liver cell lines and four lung cell lines were included. Under the conditions of the CSC concentrations employed in these experiments and the duration of exposure, inhibition of cell growth did not exceed 15%. Figure 1 shows a representation of these results.

Treatment with CSC significantly increased the level of *CYP1A2* in each of the six cell lines examined (Fig. 2).

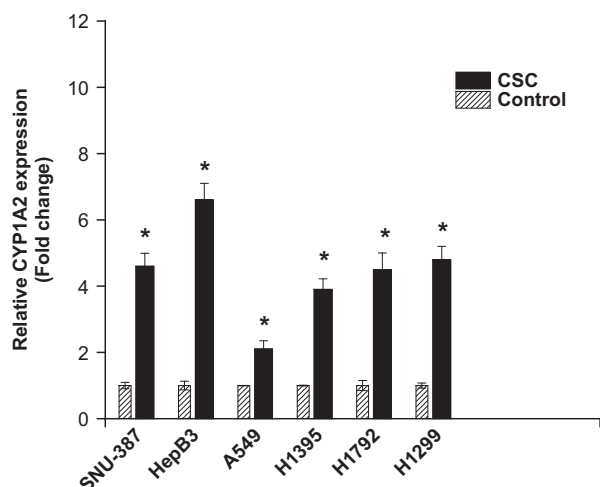
Fold changes in the expression levels relative to controls varied among the cell lines. Changes in *CYP1A2* expression levels varied with time of exposure (4–36 h), as shown in Figure 3. Increases in levels of *CYP1A2* expression were also concentration-dependent in the range 2–25  $\mu\text{g}/\text{mL}$  CSC (Fig. 4). Using the P450-Glo™ *CYP1A2* assay system, *CYP1A2* enzymatic activity was examined. In each of the cell lines, *CYP1A2* activity increased with CSC exposure (Fig. 5). These increases in *CYP1A2* activity were concentration-dependent.

The effects of estradiol and the dietary agent, I3C, on *CYP1A2* expression were also examined. In the human cell lines H1299 and H1395, treatment with estradiol (10 and 100 nmol/L) significantly reduced the expression levels of *CYP1A2* (Fig. 6). Interestingly, *CYP1A2* expression was not significantly changed with either concentration of estradiol in A549 cells. Treatment of H1299 and HepB3 cells with I3C (50 and 100  $\mu\text{mol}/\text{L}$ ) increased the level of *CYP1A2* expression, as shown in Figure 7. At 100  $\mu\text{mol}/\text{L}$  I3C, the increase was significant in both cell lines.

To explore the potential underlying pathway involved in the induction of *CYP1A2* expression by CSC, the effect of DMF on CSC-inducible expression of *CYP1A2* was determined. DMF is a competitive antagonist of the aryl hydrocarbon receptor (AhR) that inhibits AhR-mediated induction of cytochrome P450 expression (Lee and Safe 2000). Treating cells with CSC and DMF resulted in only a slight reduction in the *CYP1A2* expression level resulting from treatment with CSC alone (Fig. 8). In contrast,



**Figure 1.** Effect of CSC on human liver and lung cells proliferation. HepB3 (A) and H1299 (B) cells were treated with 2 or 25  $\mu\text{g}/\text{mL}$  CSC for 12, 24, or 36 h and assayed by MTT techniques. Data are presented as mean  $\pm$  SD of at least three determinations.



**Figure 2.** Effect of CSC on *CYP1A2* expression in human liver and lung cells. Cells were treated with 25  $\mu\text{g}/\text{mL}$  CSC for 24 h. Controls were treated with vehicle (DMSO) only. Expression levels were quantified by real-time PCR. Fold changes in expression levels in CSC-treated cells are in comparison to controls. Data are presented as mean  $\pm$  SD of at least three determinations. \*Denotes a significant difference ( $P < 0.05$ ) compared to controls. CSC cigarette smoke condensate; DMSO, dimethyl sulfoxide.

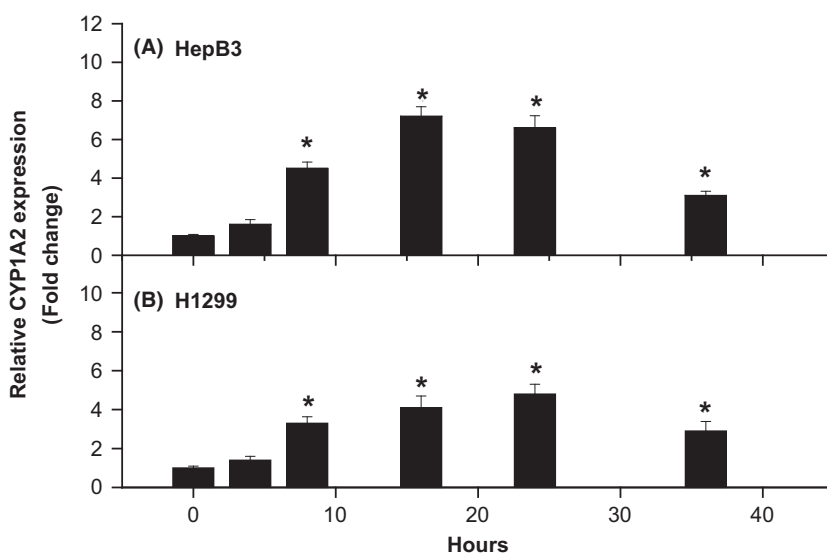
DMF strongly inhibited TCDD-induced *CYP1A2* expression.

There is increasing evidence of the involvement of epigenetics in the regulation of *CYP1A2* expression (Tang and Chen 2015), suggesting another pathway that may be

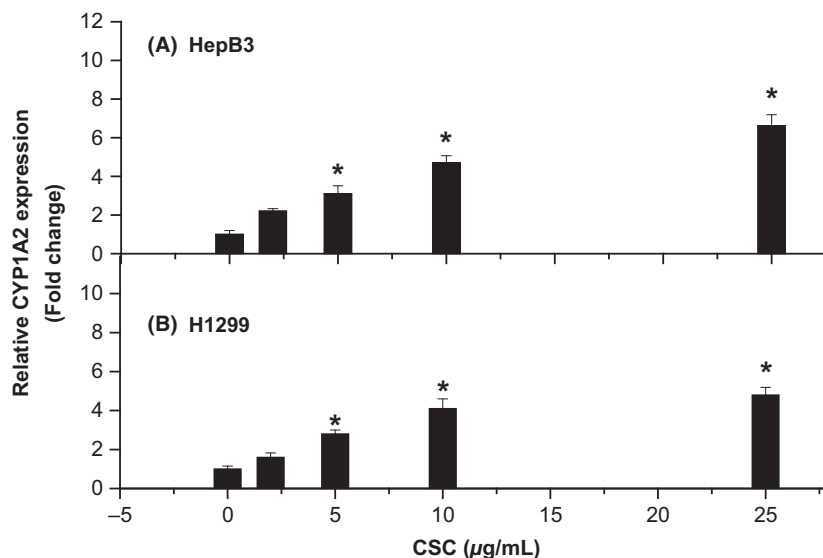
involved in CSC induction of *CYP1A2*. Figure 9 shows the effect of AzadC, a DNA methylation inhibitor (Hackanson and Daskalakis 2014), and TSA, a histone deacetylase (HDAC) inhibitor (Monneret 2005), on *CYP1A2* expression levels in SNU-387 and H1299 cell lines. Both agents increased *CYP1A2* expression in a concentration-dependent manner. Although either agent increased CSC-inducible *CYP1A2* expression slightly, the effect was not significant (Fig. 10). Given these results indicating the potential of epigenetics in the regulation of *CYP1A2* expression, the effect of CSC on histone modifications in *CYP1A2* was explored in cells treated with CSC. Three histone modifications, H3K4me3, H4K16ac, and H3K27me3, were analyzed by ChIP assay. Eight specific segments in the 5'-flanking region in the *CYP1A2* gene were included. Increases in H3K4me3 and H4K16ac were detected in several segments of the gene (Reg-3, Reg-5, and Reg-6 for H3K4me3; Reg-2, Reg-5, and Reg-8 for H4K16ac); a decrease in H3K27me3 was detected in Reg-4 (Fig. 11). Comparison of several these histone modifications (H3K4me3 at Reg-3 and H4K16ac at Reg-5) in two other cell lines demonstrated similar results (Fig. 12).

## Discussion

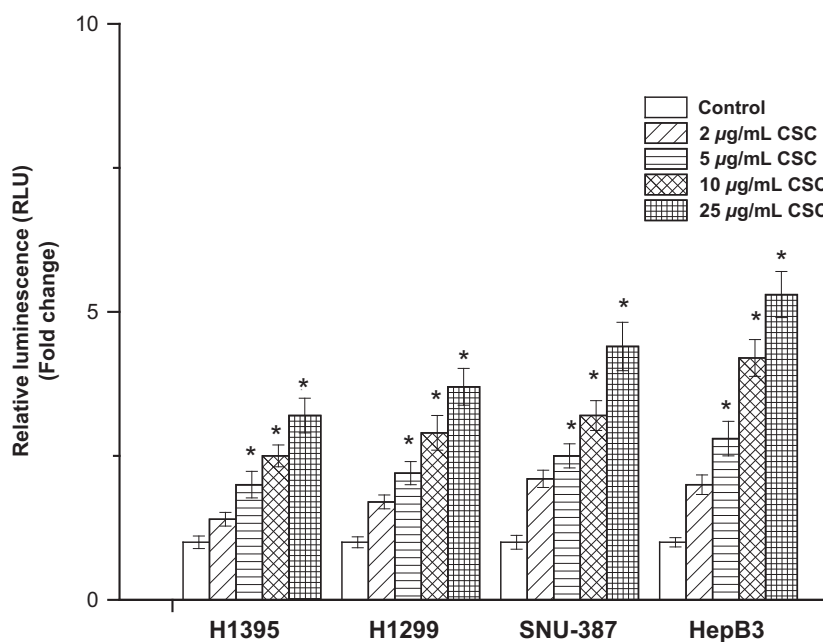
Individual variation in the expression of major drug-metabolizing enzymes is associated with substantial individual differences in bioavailabilities and clearance of drugs and other xenobiotics and presents a major challenge in evaluating drug safety and efficacy and in



**Figure 3.** Effect of length of CSC exposure on *CYP1A2* expression. HepB3 (A) and H1299 (B) cells were treated with 25  $\mu\text{g}/\text{mL}$  CSC for 4–36 h. Controls were treated with vehicle (DMSO) only. Expression levels were quantified by real-time PCR. Fold changes in expression levels in CSC-treated cells are in comparison to controls. Data are presented as mean  $\pm$  SD of at least three determinations. \*Denotes a significant difference ( $P < 0.05$ ) compared to controls. CSC, cigarette smoke condensate; DMSO, dimethyl sulfoxide.



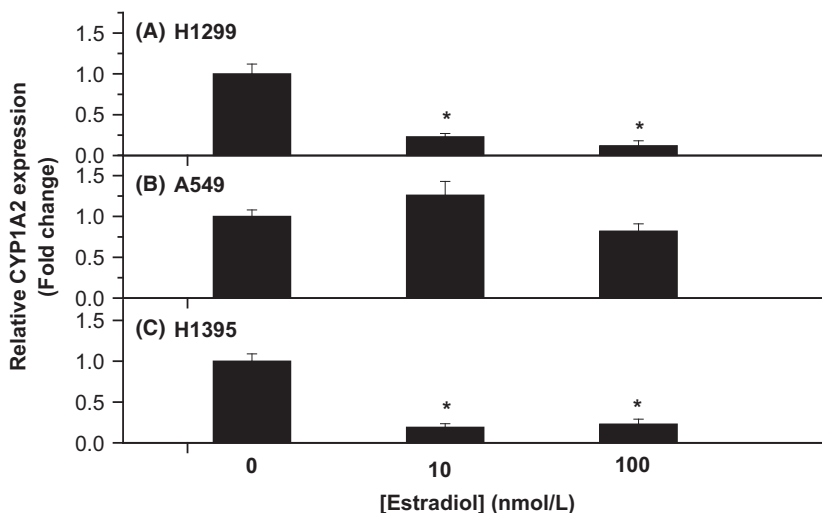
**Figure 4.** Dependence of CYP1A2 expression on concentration of CSC. HepB3 (A) and H1299 (B) cells were treated with 2–25 µg/mL CSC for 24 h. Controls were treated with vehicle (DMSO) only. Expression levels were quantified by real-time PCR. Fold changes in expression levels in CSC-treated cells are in comparison to controls. Data are presented as mean ± SD of at least three determinations. \*Denotes a significant difference ( $P < 0.05$ ) compared to controls. CSC, cigarette smoke condensate; DMSO, dimethyl sulfoxide.



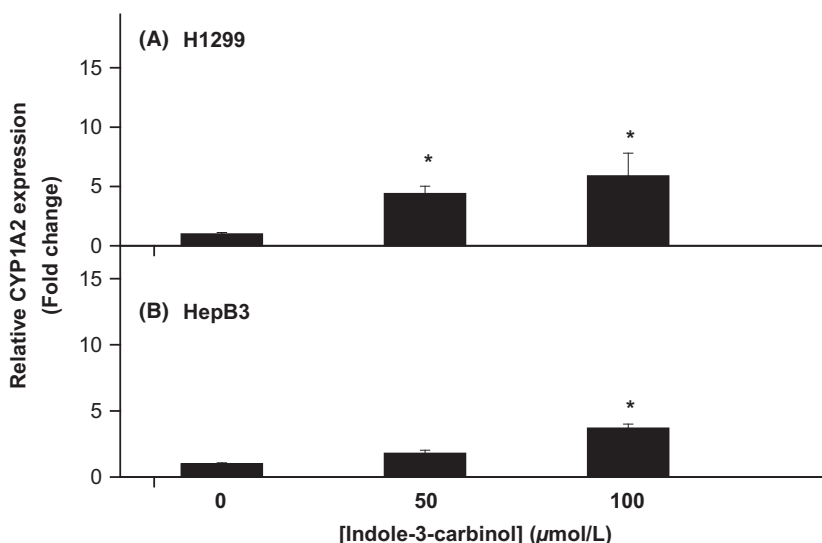
**Figure 5.** Effect of CSC on CYP1A2 activity in human liver and lung cells. Cells were treated with 2–25 µg/mL CSC and CYP1A2 enzymatic activity was measured using the P450-Glo™ CYP1A2 assay. Fold changes in activity levels in CSC-treated cells are in comparison to controls. Data are presented as mean ± SD of at least three determinations. \*Denotes a significant difference ( $P < 0.05$ ) compared to controls. CSC, cigarette smoke condensate.

assessing individual risk in exposure to foreign chemicals. Large individual differences in expression levels of CYP1A2 have been established (Hammons et al. 1985;

Kalow and Tang 1991; Schweiki et al. 1993). Although smoking has been recognized as a critical factor associated with levels of CYP1A2 expression (Pantuck et al. 1972;



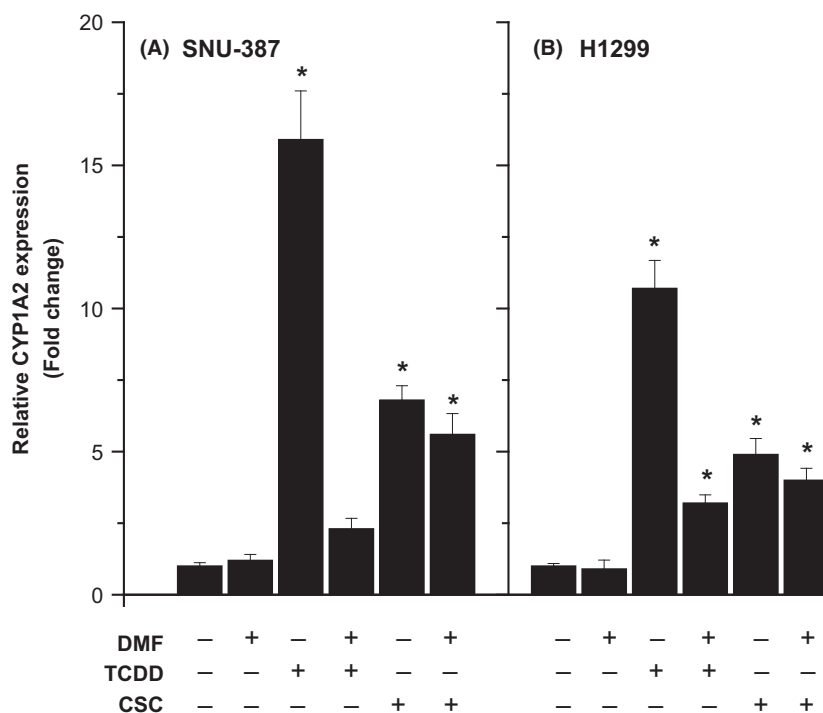
**Figure 6.** Effect of estradiol on *CYP1A2* expression in human lung cells. H1299 (A), A549 (B) and H1395 (C) cells were treated with 10 or 100 nm estradiol. Controls were treated with vehicle (DMSO) only. Expression levels were quantified by real-time PCR. Fold changes in expression levels in treated cells are in comparison to controls. Data are presented as mean  $\pm$  SD of at least three determinations. \*Denotes a significant difference ( $P < 0.05$ ) compared to controls. DMSO, dimethyl sulfoxide.



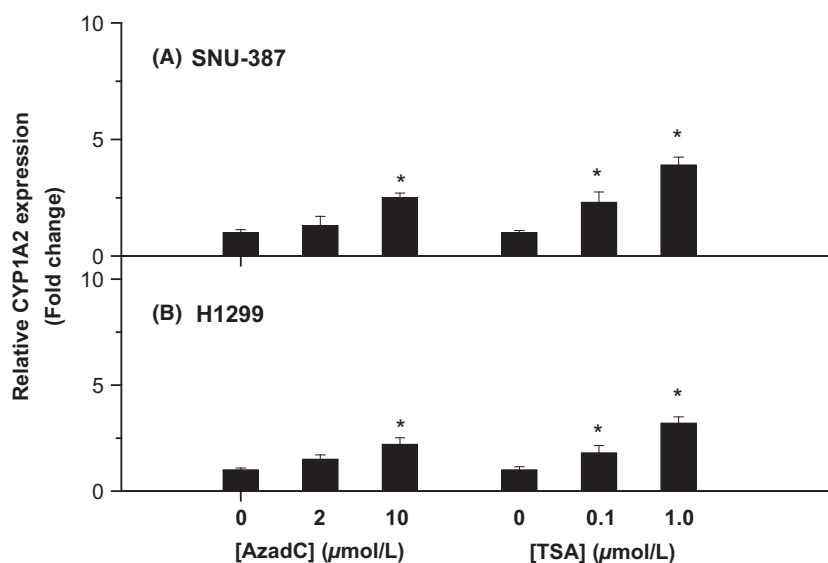
**Figure 7.** Effect of indole-3-carbinol on *CYP1A2* expression in human liver and lung cells. H1299 (A) and HepB3 (B) cells were treated with 50 or 100  $\mu$ mol/L indole-3-carbinol (I3C). Controls were treated with vehicle (DMSO) only. Expression levels were quantified by real-time PCR. Fold changes in expression levels in treated cells are in comparison to controls. Data are presented as mean  $\pm$  SD of at least three determinations. \*Denotes a significant difference ( $P < 0.05$ ) compared to controls. DMSO, dimethyl sulfoxide.

Kotake et al. 1982; Sesardic et al. 1988; Tantcheva-Poor et al. 1999; Faber and Fuhr 2004; Ghotbi et al. 2007; Kroon 2007), only limited evidence of a causal relationship has been reported. Using CSC exposure in an in vitro approach, results from the study provide the first comprehensive determination of the causal effect of CSC on *CYP1A2* expression, showing that *CYP1A2* expression

levels were increased in cells treated with CSC. This was demonstrated in human liver and lung cells. The effect was in a concentration- and time-dependent manner. These results support observations of *CYP1A2* expression levels being higher in smokers (Pantuck et al. 1972; Kotake et al. 1982; Sesardic et al. 1988; Tantcheva-Poor et al. 1999; Faber and Fuhr 2004; Kroon 2007) in

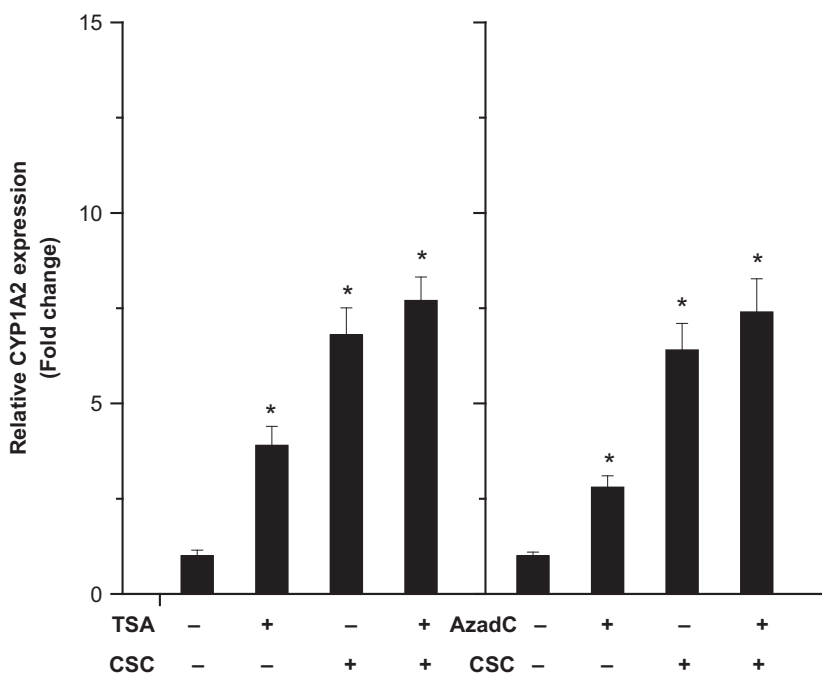


**Figure 8.** Effect of DMF on TCDD or CSC-induced expression of CYP1A2 in human liver and lung cells. SNU-387 (A) and H1299 (B) cells were treated with 10  $\mu\text{mol/L}$  DMF, 10 nm TCDD, or 25  $\mu\text{g/mL}$  CSC alone, or DMF plus TCDD or DMF plus CSC. Controls were treated with vehicle (DMSO) only. Expression levels were quantified by real-time PCR. Fold changes in expression levels in treated cells are in comparison to controls. Data are presented as mean  $\pm$  SD of at least three determinations. \*Denotes a significant difference ( $P < 0.05$ ) compared to controls. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CSC cigarette smoke condensate; DMSO, dimethyl sulfoxide.

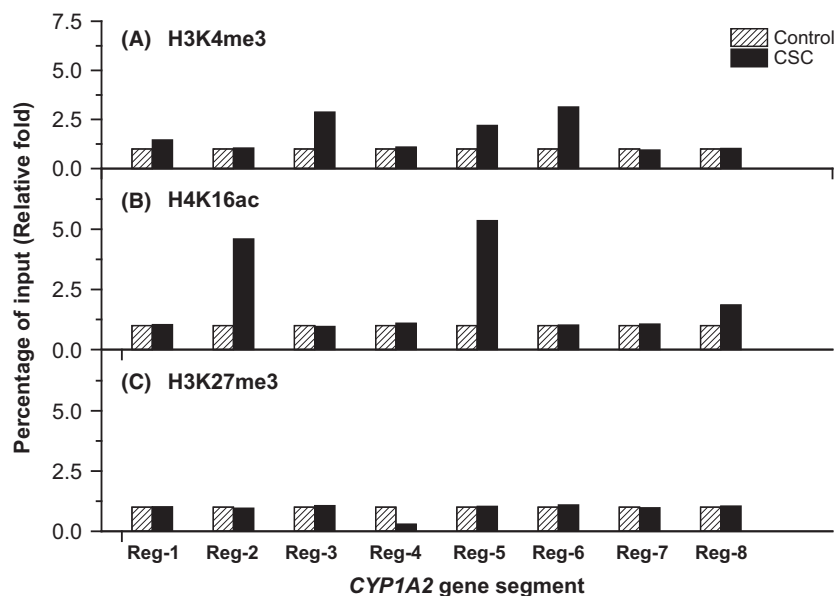


**Figure 9.** Effect of TSA or AzadC on CYP1A2 expression in human liver and lung cells. SNU-387 (A) and H1299 (B) cells were treated with 0.1 or 1.0  $\mu\text{mol/L}$  TSA for 24 h, or 2 or 10  $\mu\text{mol/L}$  AzadC for 72 h. Controls were treated with vehicle (DMSO) only. Expression levels were quantified by real-time PCR. Fold changes in expression levels in treated cells are in comparison to controls. Data are presented as mean  $\pm$  SD of at least three determinations. \*Denotes a significant difference ( $P < 0.05$ ) compared to controls. TSA, trichostatin; DMSO, dimethyl sulfoxide.

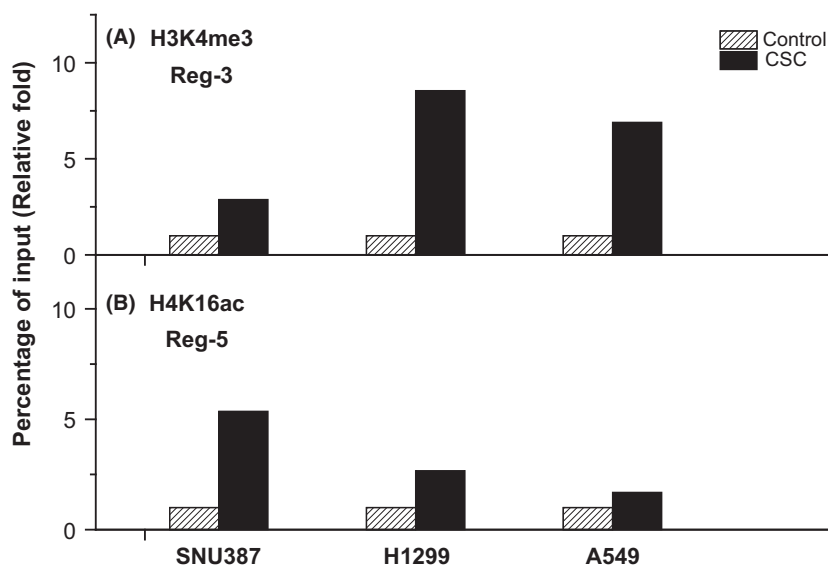




**Figure 10.** Effect of TSA or AzadC and CSC on *CYP1A2* expression. SNU-387 were treated with 1  $\mu\text{mol/L}$  TSA, 10  $\mu\text{mol/L}$  AzadC or 25  $\mu\text{g/mL}$  CSC alone, or TSA plus CSC or AzadC plus CSC. Controls were treated with vehicle (DMSO) only. Expression levels were quantified by real-time PCR. Fold changes in expression levels in treated cells are in comparison to controls. Data are presented as mean  $\pm$  SD of at least three determinations. \*Denotes a significant difference ( $P < 0.05$ ) compared to controls. CSC, cigarette smoke condensate; DMSO, dimethyl sulfoxide.



**Figure 11.** Effect of CSC on histone modifications on the *CYP1A2* gene. SNU-387 cells were treated with vehicle (DMSO) or 25  $\mu\text{g/mL}$  CSC for 24 h and subjected to ChIP analysis with antibodies targeted to the indicated histone modifications. The DNA was amplified by real-time PCR using primers specific for eight segments of the *CYP1A2* gene (Reg-1, 8456-8549; Reg-2, 28319-28405; Reg-3, 28521-28560; Reg-4, 28791-28814; Reg-5, 29081-29154; Reg-6, 30378-30402; Reg-7, 31261-31271; Reg-8, 31431-31510). Data are expressed as fold changes relative to control for percent of input. CSC, cigarette smoke condensate; DMSO, dimethyl sulfoxide.



**Figure 12.** Comparison of the effect of CSC on histone modifications in the *CYP1A2* gene in SNU-387, H1299, and A549 cells. Cells were treated with vehicle (DMSO) or 25  $\mu\text{g}/\text{mL}$  CSC for 24 h and subjected to ChIP analysis with antibodies targeted to H3K4me3 (A) or H4K16ac (B). The DNA was amplified by real-time PCR using primers specific for Reg-3 (H3K4me3) or Reg-5 (H4K16ac) in the *CYP1A2* gene. Data are expressed as fold changes relative to control for percent of input. CSC cigarette smoke condensate; DMSO, dimethyl sulfoxide.

demonstrating directly a causal relationship. Only two earlier in vitro studies were found. CSC was shown to upregulate the expression of *CYP1A2* in HepG2 cells, although *CYP1A2* expression was not the focus of the study (Washio et al. 2011). Using cDNA array analysis, exposure of human vascular endothelial cells to CSC (30  $\mu\text{g}/\text{mL}$ ; 24 hrs) increased *CYP1A2* expression (Nordskog et al. 2003). In an animal model study, *CYP1A2* expression was induced in Sprague–Dawley rats exposed to passive smoking (He et al. 2015).

Experiments were conducted to explore possible mechanisms involved in induction of *CYP1A2* expression by CSC. There is evidence that induced expression can be mediated by AhR as demonstrated by studies using AhR null mice (Fernandez-Salguero et al. 1995; Schmidt et al. 1996), suggesting the presence of polycyclic aromatic hydrocarbons in cigarette smoke may contribute to the inductive effect of cigarette smoke (Hoffmann et al. 1997). In our study, treating cells with CSC and DMF resulted in only a partial reduction in *CYP1A2* expression levels induced by treatment with CSC alone. DMF is a competitive antagonist of AhR that inhibits AhR-mediated induction of cytochrome P450 expression (Lee and Safe 2000). These results indicated that alternative pathways are involved.

Expression of *CYP1A2* has been shown to be affected epigenetically. Results from our laboratory showed that human hepatic *CYP1A2* expression is linked to the methylation status of the CCGG site adjacent to the AP-1 site in human *CYP1A2* gene's promoter region

(Hammons et al. 2001). Other studies examining *CYP1A2* expression and epigenetic changes have been reported. CpG sites of the *Cyp1a2* promoter were undermethylated in the mouse liver when compared to sites in the lung and kidney, suggesting that DNA methylation regulates the tissue-specific expression of the *Cyp1a2* gene (Jin et al. 2004). Treatment with a combination of TSA and AzadC enhanced expression of *CYP1A2* in HeLa cells, suggesting that cooperation between DNA methylation and histone acetylation is important in *CYP1A2* expression (Nakajima et al. 2003). The methylation of two separate core CpG sites was found to be strongly associated with the *CYP1A2* mRNA levels in human liver samples (Ghotbi et al. 2009). Additionally, *CYP1A2* expression in human hepatoma B16A2 cells was strongly induced by treatment with AzadC (Ghotbi et al. 2009). Regulation of *CYP1A2* by DNA methylation and histone modification was demonstrated in the differential expression of *CYP1A2* in human primary hepatocytes compared to human embryonic stem cell-derived hepatocytes (Park et al. 2015). Inhibition of DNA methyltransferases (DNMT) increased expression levels of *CYP1A2*. Enrichment of active histone modification H3K4me3 modulated expression of *CYP1A2*; the presence of repressive histone modification H3K27me3 found in the cells was associated with down-regulation of *CYP1A2* transcription.

In this study, *CYP1A2* expression was demonstrated to be inducible by TSA and AzadC in the cellular systems. The effect of CSC treatment on three histone modifications, H3K4me3, H4K16ac, and H3K27me3, in specific

segments in the 5'-flanking region of *CYP1A2* was determined. DNA-binding sequences of several putative regulatory factors have been characterized in the *CYP1A2* gene, including xenobiotic response element (Quattrochi et al. 1994), AP-1 element (Quattrochi et al. 1988), E-box motifs (Pickwell et al. 2003), and Ets element (Aklillu et al. 2003). Additional putative binding sites for HNF-1 and HNF-3 have also been identified farther upstream (Corchero et al. 2000). A GC box proximal to the TATA box is also a critical element for the *CYP1A2* promoter (Miyajima et al. 2009). Segments were selected for these initial experiments to include the binding sequences. Specific histone modifications were explored given their reported involvement in the expression of *CYP1A2* and several other CYPs (Okino et al. 2006; Li et al. 2009; Ovesen et al. 2011; Park et al. 2015). Increases in H3K4me3 and H4K16ac were observed at several segments, whereas H3K27me3 was shown to decrease, following CSC treatment. H3K4me3 and H4K16ac are associated with active transcription; H3K27me3 is repressive (Handy et al. 2011). This is the first report identifying specific histone modifications involved in CSC-induced expression of *CYP1A2*. These results indicate involvement of epigenetics in induction of *CYP1A2* by CSC, potentially at DNA-binding sites. Cigarette smoking has been shown in previous studies to be associated with epigenetic changes (Anttila et al. 2003; Divine et al. 2005; Szulakowski et al. 2006; Hammons and Lyn-Cook 2011; Tekpli et al. 2012). Cigarette smoke-induced chromatin remodeling by acetylating lysine residues on histone proteins to facilitate gene expression was found (Marwick et al. 2004). In addition, the activity of HDACs, which remove acetyl groups to repress transcription, was reduced in bronchial biopsies from smokers compared to nonsmokers (Ito et al. 2001).

Given the sex differences associated with *CYP1A2* expression, a hormonal effect on expression levels was investigated using estradiol. Treatment with estradiol was found to reduce *CYP1A2* expression in two of the cell systems examined. Estradiol has been shown to modulate expression of several other CYPs. Real-time RT-PCR analysis has revealed that treatment with estradiol induced *CYP1B1* mRNA expression in ER-positive MCF-7 cells (Tsuchiya et al. 2004). Luciferase reporter assays using MCF-7 cells showed a significant transactivation by estradiol with a reporter plasmid containing a region from -151 to +25 of the human *CYP1B1* gene. Specific binding of the estrogen receptor (ER) to the putative estrogen responsive element (ERE) was demonstrated by chromatin immunoprecipitation assays and gel shift analyses. Estradiol has also been shown to be involved in regulation of *CYP1A1* and *CYP1B1* in human lung cells (Kuo et al. 2013). The possible role of estrogen in regulating expression of the human *CYP3A* subfamily was examined

(Williams et al. 2004), since *CYP3A4* mRNA expression in liver was lower in women than in men (Wolbold et al. 2003). It was shown that *CYP3A4* and *CYP3A43* are downregulated by estrogen, whereas *CYP3A5* is expressed at higher levels during the secretory phase in endometrium. Estradiol derivatives also downregulated *CYP2C19* expression in Huh-7 cells via estrogen receptor ER $\alpha$ , which interacts with the newly identified ER-binding half site at position -151/-147 in *CYP2C19* promoter (Mwinyi et al. 2010). The mechanism of action of estradiol on expression of *CYP1A2* and whether the involvement of estradiol in regulation of *CYP1A2* expression underlies the higher expression of CYP1A2 in men compared to women remain, however, to be established. Computer-assisted homology search of *CYP1A2* does identify the presence of the putative half ERE at several sites in the gene.

Increased CYP1A2 activity is also associated with intake of cruciferous vegetables (Kall et al. 1996; Peterson et al. 2005). Results from this study show that I3C, an important indole in cruciferous vegetables, induces expression of *CYP1A2* in several cellular systems, suggesting that this dietary component contributes to the higher CYP1A2 activity associated with the consumption of cruciferous vegetables. In the only other report found, treatment with I3C (10 – 50  $\mu$ M) was shown to increase *CYP1A2* expression in three human breast cell lines, MCF-7, MDA-MB-231, and MCF10A (Szafer et al. 2012). Although the mechanism underlying the modulation of *CYP1A2* expression by I3C is not yet known, dietary agents can effect epigenetic changes. I3C is a hypomethylating agent, as demonstrated in several studies including results from our laboratory (Lyn-Cook et al. 2008, 2009). I3C has also been reported to decrease histone deacetylase-I (HDAC-I) expression in SEB-activated T cells (Busbee et al. 2014).

In conclusion, several factors known to be associated with *CYP1A2* expression in population studies were shown to directly modulate the expression of *CYP1A2* in this comprehensive in vitro study. Given the crucial role of drug-metabolizing enzymes in regulating the pharmacological and biological activity of drugs as well as being a critical determinant in risk to foreign chemicals, it is important to understand the regulatory features that lead to individual differences in the expression of drug-metabolizing enzymes. Results from this study clearly establish a causal relationship between tobacco smoke and *CYP1A2* expression. The effect was demonstrated in both human liver and lung cells. Further evidence is provided supporting the involvement of epigenetic mechanisms. In examining other factors, evidence is presented that estradiol may be a contributing factor in the sex difference in the expression of *CYP1A2* and that the dietary component I3C may have an important role as well in *CYP1A2*

expression. Studies to further clarify the regulatory mechanisms involved in *CYP1A2* expression in response to environmental, dietary, and endogenous factors will be needed.

## Disclaimer

The views and opinions expressed in this article are those of the authors only and do not necessarily represent the views, official policy, or position of the U.S. Food and Drug Administration or any of its affiliated institutions or agencies.

## Author Contributions

Hammons, Lyn-Cook, Xie, Pogribna, Word, Lyn-Cook, Jr participated in research design. Xie, Pogribna, Word, Lyn-Cook, Jr conducted the experiments. Xie, Pogribna, and Hammons performed data analysis. Hammons and Lyn-Cook wrote or contributed to the writing of the manuscript.

## Disclosure

None declared.

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