Food and Chemical Toxicology 125 (2019) 182–189

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Short-term regulation of the hepatic activities of cytochrome P450 and glutathione *S*-transferase by nose-only cigarette smoke exposure in mice

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ARTICLE INFO

Keywords:
Cigarette smoke
Cytochrome P450
Glutathione *S*-Transferase
Oxidative stress
Drug metabolism

ABSTRACT

The present study aimed to determine the effects of cigarette smoke on the regulation of hepatic cytochrome P450 (CYP) and glutathione *S*-transferase (GST) enzymes in male BALB/c mice exposed to nose-only cigarette smoke for 4 days. There were no significant increases in serum liver injury markers (alanine aminotransferase and aspartate aminotransferase) or oxidative stress (total antioxidant capacity, malondialdehyde, and glutathione disulfide/reduced glutathione) following cigarette smoke exposure, but malondialdehyde was elevated in the bronchoalveolar lavage fluid of smoke-exposed mice. Additionally, the hepatic microsomal protein levels of Cyp1a and Cyp2b, and the activities of ethoxyresorufin *O*-deethylase, pentoxyresorufin *O*-depenylase, and chlorzoxazone 6-hydrxylase, were elevated in smoke-exposed mice. Interestingly, the hepatic activities of GST toward 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, and ethacrynic acid, but not cumene hydroperoxide were enhanced by cigarette smoke exposure, which was consistent with the increased expression levels of mu- and pi-class GSTs, but not alpha-class GSTs, observed in immunoblot analyses. These findings indicate that the short-term inhalation of cigarette smoke induces drug-metabolizing enzymes such as CYP1A, CYP2B, and mu/pi-class GSTs in the absence of hepatic injury and oxidative stress. Furthermore, smoking may alter hepatic drug metabolism, as well as the disposition and toxicity of xenobiotics, including some therapeutic drugs and cigarette smoke constituents.

1. Introduction

Smoking is a well-established risk factor for a variety of chronic disorders including cardiovascular diseases, respiratory disorders and certain types of cancers (Ambrose and Barua, 2004; Cataldo et al., 2010; Alexandrov et al., 2016). Cigarette smoking remains highly prevalent; there are more than 1 billion smokers worldwide (Bilano et al., 2015). In addition to the respiratory system, cigarette smoke contains a variety of toxic ingredients that have negative impacts on most bodily organs. However, the mechanisms underlying these processes remain elusive; thus, it is important to determine the pulmonary and systemic effects of cigarette smoke.

Cytochrome P450 (CYP) enzymes play a critical role in the metabolism of xenobiotics through a variety of structures. The expression levels of CYPs and other drug-metabolizing enzymes may be altered in response to xenobiotics, as well as by pathophysiological conditions such as diabetes, long-term alcohol consumption, and inflammation (Zanger and Schwab, 2013). A majority of exogenous mutagens are metabolically activated to form reactive metabolites that bind with proteins and nucleic acids. For example, CYPs can metabolize tobacco smoke constituents, including polycyclic aromatic hydrocarbons and nitrosamines, into potent alkylating agents. Drug-metabolizing enzymes

Abbreviations: AhR, aryl hydrocarbon receptor; BALF, bronchoalveolar lavage fluid; CDNB, 1-chloro-2,4 - dinitrobenzene; CHP, cumene hydroperoxide; COPD, chronic obstructive pulmonary disease; CYP, cytochrome P450; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; EROD, 7-ethoxyresorufin-*O*-deethylase; GC, gas chromatograph; GST, glutathione *S*-transferase; LC–MS/MS, liquid chromatography tandem mass spectrometry; MDA, malondialdehyde; PROD, 7-pentoxyresorufin-*O*-dependentylase; SBD-F, 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid; TOSC, total oxidant scavenging capacities; TPM, total particulate matter; XRE, xenobiotics response element

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https://doi.org/10.1016/j.fct.2018.12.035
Received 18 May 2018; Received in revised form 10 December 2018; Accepted 21 December 2018
Available online 03 January 2019
0278-6915/

in the liver and the lungs can be either induced or inhibited by cigarette smoking (Kawamoto et al., 1993; O'Malley et al., 2014) and these alterations may change the disposition and toxicity of therapeutic drugs and environmental chemicals.

The glutathione *S*-transferase (GST) family is one of most abundant and important classes of detoxification enzyme in the liver. The main function of GSTs is to conjugate electrophilic compounds with glutathione (GSH), thereby making these compounds less active and enabling their excretion (Hayes and Pulford, 1995). Thus, genetic polymorphisms and the altered expression levels of GSTs are associated with cancer susceptibility and resistance to chemotherapy (Di Pietro et al., 2010). Previous epidemiological studies have suggested a relationship between GST polymorphisms and the risk of smoking-related cancers (Reszka and Wasowicz, 2001). Moreover, GSTM1 gene deletion is associated with emphysema in lung cancer patients, and with chronic bronchitis in heavy cigarette smokers (Harrison et al., 1997; He et al., 2002). Moreover, the pi-class GST gene polymorphism is one of the determinants of susceptibility to chronic obstructive pulmonary disease (COPD; Baranova et al., 1997). Taken together, these studies indicate that GST enzymes are protective against cigarette smoke-induced lung injury.

Previous studies investigating the effects of cigarette smoke on drug-metabolizing enzymes have focused on CYP regulation in extrahepatic tissues. Although hepatic drug metabolism plays critical roles in drug disposition and toxicity, there is little information regarding the regulation of hepatic drug-metabolizing enzymes, especially GSTs, by cigarette smoke. Thus, the purpose of this study was to investigate the alterations in hepatic activities of CYPs and GSTs in BALB/c mice following short-term inhalation of cigarette smoke, which may be indicative of changes in the disposition and toxicity of xenobiotics.

2. Materials and methods

2.1. Chemicals and reagents

GSH, glutathione disulfide (GSSG), tetraethoxypropane, β-nicotinamide adenine dinucleotide (NADH), alpha-keto-gamma-methiolbutyric acid, 2-2′-azobisamidinopropane, ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F), β-nicotinamide adenine dinucleotide phosphate (NADPH), tolbutamide, 4-hydroxytolbutamide, resorufin, ethoxyresorufin, pentoxyresorufin, 5,5-dithiobis (2-nitrobenzoic acid), 4-hydroxymidazolam, chlorzoxazone, 6-hydroxychlorzoxazone, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), cumene hydroperoxide (CHP), ethacrynic acid (EA) and cotinine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Midazolam was purchased from Bukwang Pharm. Co. (Seoul, Republic of Korea) and 1′-hydroxymidazolam was obtained from BD Gentest Co. (Woburn, MA, USA). All other chemicals and solvents were of reagent grade or better. Anti-cytochrome b_5 (Cyp b_5), Cyp reductase, Cyp1a, Cyp2c, Cyp2e1, Cyp3a, and the alpha-, mu- and pi-class GST antibodies were purchased from Detroit R & D (Detroit, MI, USA). The anti-Cyp2b antibody was purchased from Millipore (Billerica, MA) and the anti-GAPDH and G-b antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-Cyp2a was obtained from Thermo Fisher Scientific (Rockford, USA).

2.2. Animals

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Korea Institute of Toxicology (Approval No. 1602-0077). Male BALB/c mice (7 weeks of age) were purchased from Orient Bio Inc. (Seongnam, Korea), housed in the laboratory animal facility under specific pathogen-free conditions, and acclimated for 1 week prior to the experiments. The animal facility was maintained at 22 ± 3 °C, had a relative humidity of 50 ± 20%, was refreshed 10–20 times/h using air ventilation, and had a light intensity of 150–300 Lux on a 12-h light/dark cycle. Ultraviolet (UV)-irradiated food (PMI Nutrition International, Richmond, IN, USA) and filtered tap water were provided ad libitum to the experimental animals. Each group consisted of ten mice and each hepatic microsomal or cytosolic sample was isolated from two pooled livers. Based on our previous results showing regulation of hepatic drug metabolizing enzyme in diabetic animals (Park et al., 2016), the difference between two groups and coefficient of variation were 25–30% and 15% of control mean values, respectively. Thus, the sample size per each group was calculated to be four to six.

2.3. Cigarette smoke exposure

3R4F reference cigarettes (9.4 mg tar and 0.73 mg nicotine) were purchased from the University of Kentucky (Lexington, KY, USA). In accordance with ISO 3402 standards, the cigarettes were conditioned at 22 ± 1 °C and 60 ± 2% relative humidity for 48 h prior to use. Cigarette smoke was generated using a CSM 2080 30-port smoking machine (CH Technologies; Westwood, NJ, USA) in conformity with the ISO standard 3308 regimen (35 mL puff volume, 2 s puff duration, 60 s between puffs, no vent blocking). The generated cigarette smoke was diluted with filtered clean air and directed into nose-only inhalation chambers (NITC-36; HCT, Incheon, Korea). The mice were randomly divided into two groups of 10 males each; the control group was provided with filtered clean air and the smoking group was exposed to cigarette smoke at total particulate matter (TPM) concentrations of 800 μg/L for 4 consecutive days. The daily exposure was performed in the following phases: 1 h exposure, followed by 1 h smoke free, followed by 1 h exposure. The smoke concentration was measured using a gravimetric method with a 44 mm Cambridge filter pad (GE Healthcare; Little Chalfont, UK). The particle size distribution was determined using a 7-stage cascade impactor (Model 135 MiniMOUDI; MSP Corp., Shoreview, MN, USA). The mean mass aerodynamic diameters and geometric standard deviations of smoke aerosol were 0.75 μm and 1.89, respectively. Temperature and relative humidity of the exposure atmosphere in the exposure chamber were 23.4 ± 0.4 °C and 46.6 ± 1.3%, respectively. The mean carbon monoxide concentrations measured by multi-gas monitor (M40, Industrial Scientific Corporation, Oakdale, PA, USA) were 0 ± 0 and 1350 ± 143 ppm in control and cigarette smoke groups, respectively. The nicotine level analyzed by using LC-MS/MS (Agilent 6410B, Agilent Technologies, Santa Clara, CA, USA) was approximately 40.2 mg/m^3 in the cigarette smoke-exposed group.

2.4. Preparation of hepatic microsomes and cytosol

Each liver homogenate sample was obtained from two mice and the pooled livers were homogenized in threefold volumes of ice-cold buffer consisting of 0.154 M KCl, 50 mM Tris–HCl, and 1 mM EDTA (pH 7.4). The homogenate was centrifuged (10,000 g, 20 min) and then the supernatant fraction was collected and centrifuged (104,000 g, 65 min) again; the cytosolic fraction was used for the GST assays. Microsomal pellets were suspended and recentrifuged (104,000 g, 65 min) and then the microsomes were diluted to an equivalent of 1.0 g of tissue/mL of buffer. Protein content was determined using the Lowry method with bovine serum albumin (BSA) as the standard.

2.5. Determination of GSH and lipid peroxidation levels

The total GSH (GSH and GSSG) concentration was measured with a high-performance liquid chromatography (HPLC; SCL-10A; Shimadzu, Kyoto, Japan) system equipped with a fluorescence detector (RF-10AXL; Shimadzu), with excitation at 385 nm and emission at 515 nm, using an SBD-F derivatization method that has been described elsewhere (Ryu et al., 2013). The GSSG concentration was measured with a 5,5-dithiobis (2-nitrobenzoic acid)-GSSG reductase recycling assay (Oh

et al., 2010), and the extent of lipid peroxidation was estimated by measuring malondialdehyde (MDA) levels in the liver and bronchoalveolar lavage fluid (BALF) using an HPLC system equipped with a fluorescence detector (Oh et al., 2012). The MDA–TBA complex was monitored using fluorescence detection with excitation at 515 nm and emission at 553 nm, and the MDA concentration was calculated using a calibration curve prepared with tetraethoxypropane.

2.6. Total oxy-radical scavenging capacity assay

Total oxy-radical scavenging capacity (TOSC) values were obtained with a gas chromatograph (GC) flame ionization detector (FID), as described elsewhere (Oh et al., 2012). Peroxyl radicals were generated by thermal homolysis of 60 mM 2-2′-azobisamidinopropane at 35 °C in 100 mM potassium phosphate buffer (pH 7.4), and reactions with 0.3 mM alpha-keto-gamma-methiolbutyric acid were carried out in 10-ml rubber septa-sealed vials at a final reaction volume of 1 ml. Ethylene production was measured with a gas chromatographic analysis of 200 µl aliquots taken from the headspace of the vials, and total ethylene formation was quantified from the area under the kinetic curve; samples were monitored in sequence at 20-min intervals within a 60-min period. Analyses were performed with a Shimadzu-2010 (Shimadzu Corp.; Tokyo, Japan) GC equipped with a DB-05 capillary column (30 m × 0.32 mm i.d.). The oven, injection, and FID temperatures were 60, 180, and 180 °C, respectively, and helium was used as the carrier gas at a flow rate of 30 mL/min. TOSC values were calculated using the following equation: TOSC = 100 − (SA/CA × 100), where SA and CA are the integrated areas from the curves defining the sample and blank reactions, respectively. The specific TOSC was calculated by dividing experimental TOSC by liver weight.

2.7. Immunoblotting analysis

For immunoblot analyses of the CYP and GST isoforms, microsome and cytosol samples were diluted to 1 mg/mL with a loading buffer that contained a reducing agent. Aliquots of the lysates (10 µg) were resolved using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes (Bio-Rad Laboratories; Hercules, CA, USA), and then blocked using 5% skim milk powder in 0.1% Tween 20 in phosphate-buffered saline (PBST). The membranes were incubated with anti-CYP reductase (1:1000), anti-cytochrome b_5 (1:1000), anti-Cyp1a (1:100,000), anti-Cyp2a5 (1:4000), anti-Cyp2b (1:5000), anti-Cyp2c (1:5000), anti-Cyp2e1 (1:10,000), anti-Cyp3a (1:3000), and anti-GST alpha (1:100,000), -GST mu (1:10,000), or -GST pi (1:2000) antibodies in PBST containing 2–5% skim milk powder overnight at 4 °C. Proteins were detected by enhanced chemiluminescence on a Fusion SL2 (Vilber Lourmat; Collégien, France) and quantified using the FusionCapt Advance Solo 4 software (Vilber Lourmat).

2.8. Enzyme assay

Microsomal 7-ethoxyresorufin-*O*-deethylase (EROD) and 7-pentoxyresorufin-*O*-dependentylase (PROD) activities were determined using a modified version of the method of Park et al. (2016). Chlorzoxazone 6-hydroxylase activity was determined by measuring the formation of 6-hydroxy chlorzoxazone with a liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI/MS/MS) system consisting of a Shimadzu 20AD-XR HPLC system (Shimadzu) and a 3200 QTRAP LC–MS/MS system equipped with a Turbo V Ion Spray™ source (Applied Biosystems; Foster City, CA, USA) operated in the negative ion mode (Park et al., 2016). Coumarin 7-hydroxylase activity was determined by measuring the formation of 7-hydroxy coumarin using the LC–ESI/MS/MS system operated in the positive ion mode. Briefly, 1 mg/mL microsomes were preincubated at 37 °C in the presence of 1 mM NADPH and 0.1 M potassium phosphate buffer (pH 7.4). Reactions were initiated by addition of the coumarin (100 µM) and were then quenched by adding two-fold volume of ice-cold acetonitrile (Lee and Kim, 2013). Midazolam and tolbutamide hydroxylase activities in microsomes were determined by measuring the formation of 1′- or 4-hydroxymidazolam and 4-hydroyxy tolbutamide, respectively, using a modified method of Lee et al. (2015) with a LC–ESI/MS/MS system consisting of a Shimadzu 20AD-XR HPLC system (Shimadzu) and a 3200 QTRAP LC–MS/MS system equipped with a Turbo V Ion Spray™ source (Applied Biosystems) operated in the positive ion mode.

Cytosolic GST activity was determined using either CDNB, DCNB, CHP, or EA as a substrate. GST activities toward CDNB, DCNB, and EA were measured using the method of Habig et al. (1974) and GST activity toward CHP was determined using the method of Reddy et al. (1984). Briefly, the samples were incubated at 30 °C in the presence of 1 mM CDNB, 5 mM DCNB, 0.2 mM EA, and 1 mM CHP for each substrate separately, and 1 mM GSH in 0.1 M potassium-phosphate buffer (pH 6.5). The rate of formation of the colored product was monitored for 4 min using a GENios microplate reader (Tecan; Männedorf, Switzerland).

2.9. Determination of cotinine

Hepatic concentration of cotinine was determined using a modified method of Irene and Ping (2013). Samples were diluted with two-fold volume of ice cold acetonitrile and centrifuged at 1000 × *g* for 10 min at 4 °C. Supernatants were subjected to LC-ESI/MS/MS. The sample injection volume was 10 µL, and separation was performed on an Eclipse XDB-C8 column (4.6 × 150 mm i.d., 5.0 µm; Agilent, Santa Clara, CA, USA) with a SecurityGuard C_{18} guard column (2.0 × 4.0 mm i.d.; Phenomenex, Torrance, CA, USA) maintained at 30 °C. HPLC mobile phases consisted of A (5 mM ammonium formate containing 0.1% (v/v) formic acid) and B (acetonitrile containing 0.1% (v/v) formic acid). A linear gradient of the two solvents was used (0–0.5 min, 95% A; 3.5 min, 60% A; 6.0 min, 60% A; 6.5 min, 10% A; 7.0 min, 10% A; 7.5 min, 95% A).

2.10. Data analysis

All data were analyzed with unpaired Student's *t*-tests for two-group comparisons, and P values < 0.05 were considered to indicate statistical significance unless indicated otherwise. The data are presented as means ± standard deviation (SD).

3. Results

There were no significant differences between the control and smoking groups in of body weight, liver weight, relative liver-to-body weight, or protein concentrations in the cytosol or microsomes (Table 1). Additionally, cigarette smoke did not induce liver injury, as assessed by the serum levels of alanine aminotransferase (36.0 ± 3.7 IU/L in control mice; 39.1 ± 6.9 IU/L in smoking mice) and aspartate aminotransferase (43.3 ± 9.6 IU/L in control mice; 47.9 ± 10.6 IU/L in smoking mice). In contrast, activity of lactate dehydrogenase, a marker for cytotoxicity, was increased in the BALF from smoking group

Table 1
Body weight, liver weight, relative liver weight, and microsomal/cytosolic protein levels in control and smoking mice.

	Control	Smoking
Body weight (g)	21.6 ± 1.14	21.6 ± 0.7
Liver weight (g)	1.14 ± 0.08	1.08 ± 0.07
Liver/body weight (%)	5.27 ± 0.20	5.01 ± 0.31
Microsomal protein levels (mg/g liver)	28.2 ± 2.3	27.8 ± 3.0
Cytosolic protein levels (mg/g liver)	107.7 ± 4.7	102.9 ± 4.0

Values represent means ± SD of 10 mice or five pooled liver samples.

Table 2
Comparison of oxidative stress markers between control and smoking mice.

	Control	Smoking
Hepatic TOSC value (TOSC/g liver)	43210 ± 4210	39270 ± 3750
Hepatic MDA (nmol/g liver)	11.3 ± 2.8	12.1 ± 2.4
Hepatic total GSH (nmole/g liver)	8320 ± 1449	7232 ± 1341
Hepatic GSSG (nmole/g liver)	222.6 ± 25.4	303.4 ± 116.7
Hepatic GSSG/GSH (%)	2.98 ± 1.02	4.53 ± 1.15

Values represent means ± SD of five pooled liver samples or ten plasma samples.
TOSC, total oxy-radical scavenging capacity; MDA, malondialdehyde; GSH, glutathione; GSSG, glutathione disulfide.

(130.6 ± 55.6 U/L in control mice; 309.4 ± 60.9 U/L in smoking mice, $P < 0.001$). And hepatic concentration of cotinine, a biomarker for smoke exposure, was 2.4 ± 0.7 pmoL/mg protein in smoking group, showing systemic exposure to cigarette smoke.

3.1. Comparisons of oxidative stress between control and smoking mice

To determine whether cigarette smoke induced oxidative stress in the liver, TOSC, MDA, and GSH levels were measured (Table 2). TOSC and MDA assays were performed to evaluate total antioxidant capacity and lipid peroxidation, respectively. The TOSC and MDA levels of the control and smoking groups did not significantly differ and, similarly, the hepatic levels of total GSH and GSSG, and the ratio of GSSG to reduced GSH, were not changed by cigarette smoke. In contrast, MDA levels in the BALF were elevated by cigarette smoke (0.58 ± 0.29 μM in control mice; 1.18 ± 0.40 μM in smoking mice, $P < 0.01$).

3.2. Effects of cigarette smoke on the hepatic microsomal CYP system

Hepatic levels of CYP reductase, Cyp b_5, and the CYP isoforms, including Cyp1a, 2a, 2b, 2c, 2e1, and 3a, were measured in the control and smoking groups (Fig. 1); these are the main isoforms involved in the bioactivation of procarcinogens and the clearance of therapeutic drugs (Zanger and Schwab, 2013). Hepatic microsomal CYP reductase and Cyp b_5, which are CYP electron transfer partner proteins, were not changed by smoking (Fig. 1A and B, respectively) whereas Cyp1a and Cyp2b protein levels were significantly increased by smoking (Fig. 1C and D, respectively). The control and smoking groups exhibited comparable levels of Cyp2a, 2c, Cyp2e1, and Cyp3a (Fig. 1E, F and G, respectively). G-beta protein, which was used as a loading control, did not exhibit significant changes (Fig. 1H).

CYP activities were determined in hepatic microsomes using ethoxyresorufin, coumarin, pentoxyresorufin, tolbutamide, chlorzoxazone, and midazolam as selective substrates for CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, and CYP3A, respectively (Lee et al., 2015, Table 3). EROD, PROD, and chlorzoxazone 6-hydroxylation levels significantly increased in the smoking group but there were no significant differences in the activities of coumarin 7-hydroxylation, tolbutamide 4-hydroxylation and midazolam 1′- or 4 hydroxylation between the control and smoking groups.

3.3. Effects of cigarette smoke on hepatic cytosolic GST enzymes

To evaluate the effects of cigarette smoke on hepatic GST enzymes, cytosolic protein levels of various GST enzymes, including alpha-, mu- and pi-class GSTs, were assessed (Fig. 2). The hepatic levels of mu- and pi-class GSTs significantly increased in the smoking group, while those of the alpha-class GSTs did not change (Fig. 2). The GAPDH protein used as a loading control and did not significantly change (Fig. 2D).

GST activities were determined using CDNB, DCNB, EA, and CHP as substrates for total GST, mu-class GST, pi-class GST, and alpha-class GST, respectively (Fujimoto et al., 2006; Guthenberg and Mannervik, 1981, Table 4) The activities of total GST, mu-class GST, and pi-class GST exhibited significant elevations following cigarette smoke exposure, whereas that of alpha-class GST did not change, which is consistent with the results of the immunoblot analyses.

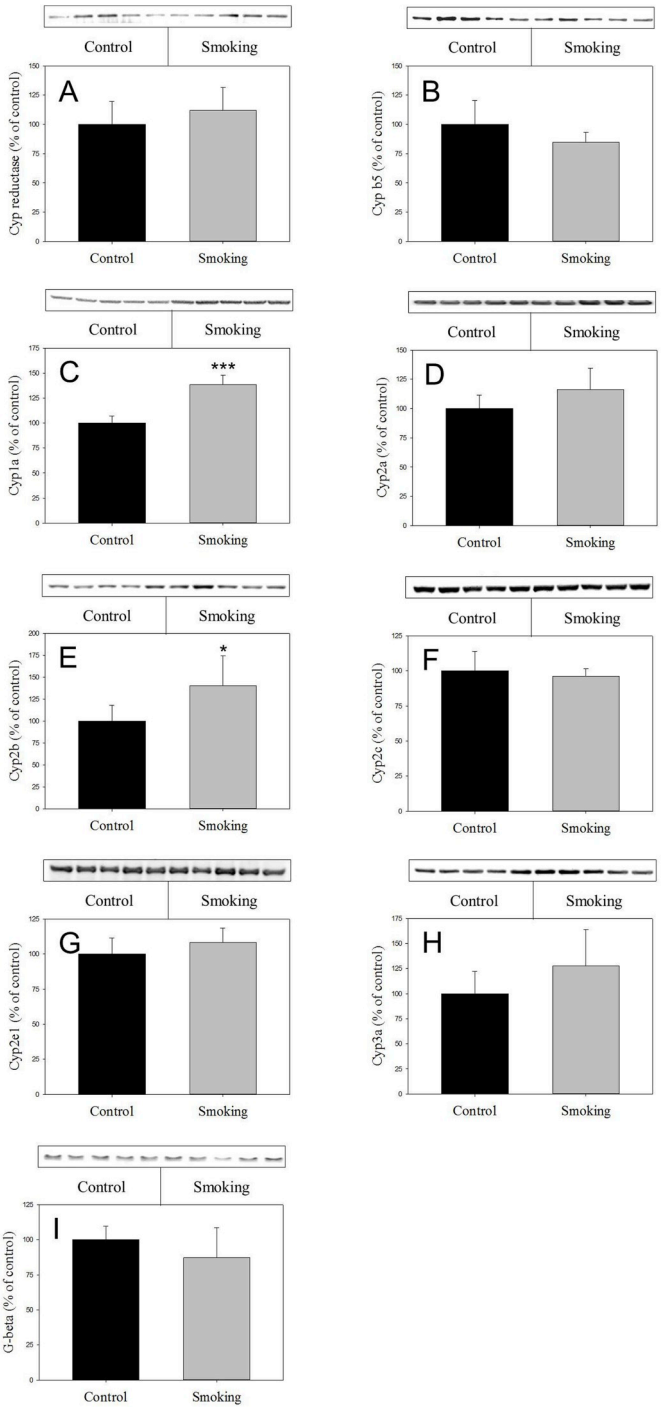

Fig. 1. Hepatic microsomal protein levels of Cyp reductase (A), Cyp b_5 (B), Cyp1a (C), Cyp2a (D), Cyp2b (E), Cyp2c (F), Cyp2e1 (G), Cyp3a (H), and G-beta (I). BABL/c mice were exposed to nose-only cigarette smoke for 4 consecutive days. Values are means ± SD for five pooled liver samples. *,*** Significantly different from control mice at $P < 0.05$ and $P < 0.001$ (Student's *t*-test), respectively.

4. Discussion

The impacts of short-term exposure to cigarette smoke on the

Table 3
Effects of cigarette smoke on hepatic CYP activities in mice.

CYP enzyme assay	Control	Smoking
Ethoxyresorufin-*O*-deethylase (pmol/min/mg protein)	89.34 ± 3.16	99.51 ± 8.85 *
Coumarin 7-hydroxylase (pmol/min/mg protein)	6.61 ± 1.27	7.45 ± 2.27
Pentoxyresorufin-*O*-depentylase (pmol/min/mg protein)	18.43 ± 1.32	23.35 ± 2.73 **
Tolbutamide hydroxylation (nmol/min/mg protein)	0.62 ± 0.08	0.67 ± 0.06
Chlorzoxazone hydroxylation (nmol/min/mg protein)	5.07 ± 0.31	5.63 ± 0.38*
Midazolam 1′-hydroxylation (pmol/min/mg protein)	0.859 ± 0.068	0.789 ± 0.033
Midazolam 4-hydroxylation (pmol/min/mg protein)	0.104 ± 0.011	0.096 ± 0.006

Values represent the mean ± SD of five pooled liver samples. *,**Significantly different from control mice at $P < 0.05$ and $P < 0.01$ (Student's *t*-test), respectively.
CYP, cytochrome P450.

protein levels and activities of hepatic CYP and GST isoforms were determined using microsomes and cytosol, respectively, which were isolated from mice exposed to nose-only cigarette smoke. The hepatic protein levels and activities of Cyp1a, Cyp2b, and mu- and pi-class GSTs were elevated by cigarette smoke exposure in the absence of hepatic injury. Oxidative stress markers in the liver, including TOSC and MDA, were not affected by cigarette smoke but MDA levels were elevated in BALF. Additionally, the control and smoking groups exhibited comparable levels of hepatic total GSH, GSSG, and the ratio of GSSG to GSH. Taken together, the present results suggest that oxidative stress primarily occurs in the respiratory tract of subjects exposed directly to cigarette smoke.

In the present study, EROD, PROD, and chlorzoxazone hydroxylation activities were elevated in smoking mice. The increase in EROD

Table 4
Effects of cigarette smoke on hepatic GST activities in mice.

GST substrate	Control	Smoking
CDNB (nmol/min/mg protein)	1276.8 ± 97.8	1610.1 ± 121.9 **
DCNB (nmol/min/mg protein)	32.5 ± 2.4	39.0 ± 1.1 ***
EA (nmol/min/mg protein)	58.4 ± 4.8	64.6 ± 1.3 *
CHP (nmol/min/mg protein)	655.6 ± 27.6	685.6 ± 39.5

Values represent means ± SD of five pooled liver samples. *,**,***Significantly different from control mice at $P < 0.05$, $P < 0.01$, and $P < 0.001$ (Student's *t*-test), respectively.
GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; CHP, cumene hydroperoxide.

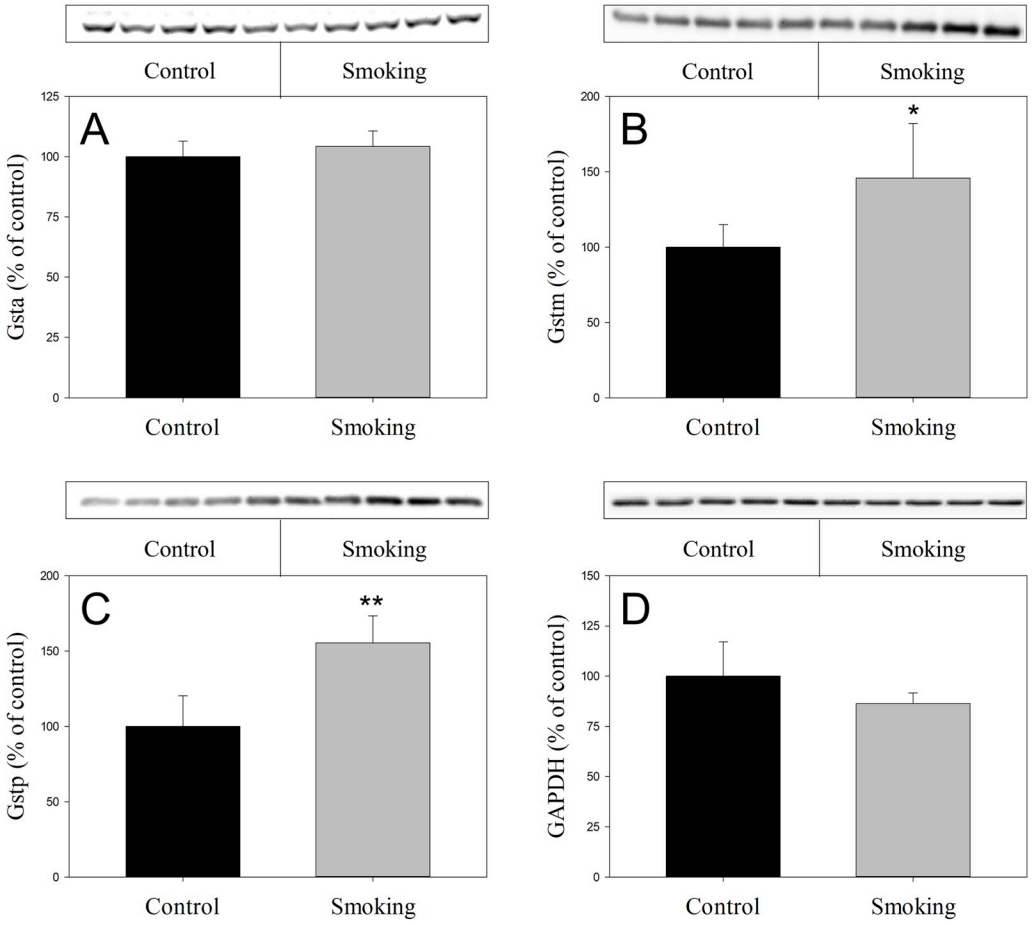

Fig. 2. Hepatic cytosolic protein levels of Gsta (A), Gstm (B), Gstp (C), and GAPDH (D). BABL/c mice were exposed to nose-only cigarette smoke for 4 consecutive days. Values represent means ± SD for five pooled liver samples. *,** Significantly different from control mice at $P < 0.05$ and $P < 0.01$ (Student's *t*-test), respectively.

activity observed in the present study is consistent with previously reported increases in Cyp1a protein levels (Pastrakuljic et al., 1997). Although pentoxyresorufin is commonly used as a probe substrate for the CYP2B subfamily (Burke et al., 1994), it is also metabolized by CYP1A2 (Kobayashi et al., 2002). Therefore, the increased levels of Cyp2b and Cyp1a proteins in the present study may have been responsible for the observed increase in PROD activity. Chlorzoxazone 6-hydroxylation is mainly mediated by CYP2E1, but it can also be metabolized by the CYP1A (Carriere et al., 1993) and CYP2C subfamilies (Draper et al., 1998; Minn et al., 2005). Thus, it is possible that the induction of Cyp1a may have been involved in the increased chlorzoxazone 6-hydroxylation activities. The 7-hydroxylation of coumarin is mainly catalyzed by CYP2A isoforms, the major enzymes involved in metabolism of nicotine to cotinine (Benowitz et al., 2016; Lake, 1999). The metabolism of tolbutamide to 4-hydroxy tolbutamide is mediated by CYP2C9 and CYP2C19 in humans (Lasker et al., 1998) and, thus, tolbutamide was used as a Cyp2c selective substrate in the present study. Midazolam is metabolized to 1′-hydroxy midazolam and 4-hydoxy midazolam by CYP3A (Turpeinen et al., 2007). In the present study, the hydroxylation activities of coumarin, tolbutamide and midazolam did not change in the smoking group, which is consistent with the immunoblot results showing no significant changes in Cyp2a, Cyp2c or Cyp3a protein levels.

Hepatic protein levels of the CYP1A and CYP2B subfamilies, but not CYP2A, CYP2C, CYP2E1, or CYP3A, were elevated in smoke-exposed mice, which is consistent with previous studies of rats (Kawamoto et al., 1993; Tatematsu et al., 2011; Ueta et al., 2003) and mice (Raza et al., 2013) exposed to cigarette smoke. In the present study, BALB/c mice that inhaled smoke for 4 days exhibited increased hepatic protein levels and activities of CYP1a1 and CYP1a2 in the absence of oxidative stress, as defined by reactive oxygen species (ROS), lipid peroxidation, and GSH levels (Raza et al., 2013). The upregulation of CYP1A expression is thought to be primarily mediated by a specific receptor, the Aryl hydrocarbon receptor (AhR; Androutsopoulos et al., 2009). Polycyclic aromatic hydrocarbons in cigarettes may induce activation of the CYP1A subfamily via AhR (O'Malley et al., 2014), but the induction of CYP1A2 by smoking does not appear to be related to the nicotine component of tobacco (Hukkanen et al., 2011). The CYP1A and CYP2B subfamilies play important roles in carcinogenesis in cigarette-related carcinogen-induced lung cancers and metabolize several therapeutic drugs, including anticancer agents (O'Malley et al., 2014; Bellec et al., 1996). CYP induction may be involved in drug-drug interaction; thus, the high inducibility of the CYP1A and CYP2B subfamilies may impose additional risks for lung cancer patients with histories of cigarette smoking (Ma and Lu, 2007; Schaffer et al., 2009). Moreover, endogenous signaling molecules, including arachidonic acid, prostaglandins, and estrogens, are metabolized by CYP1A enzymes (Nebert and Dalton, 2006), suggesting the role of CYP1A in endocrine dysregulation related to cigarette smoke.

CYP2A is involved in metabolic inactivation of nicotine to cotinine and it also activates tobacco-derived pro-carcinogens including nitrosamines (Camus et al., 1993). In previous studies, the administration of nicotine decreased its own metabolism through down-regulation of hepatic CYP2A expression in male African green monkeys treated with nicotine for three weeks (Schoedel et al., 2003) and mice for three days (Stalhandske and Slanina, 1970). In contrast, upregulation of hepatic CYP2A5 was observed in mice treated with AhR ligands, although CYP2A5 was less sensitive to the AhR ligand than CYP1A1 (Arpiainen et al., 2005). Thus, the comparable activity and protein level of CYP2A between the control and smoking groups may be attributed to the opposite effects of nicotine and polycyclic aromatic hydrocarbons on expression of hepatic CYP2A.

The protein levels of mu- and pi-class, but not alpha-class, GSTs were elevated in the mice exposed to cigarette smoke in the present study. Moreover, the GST activities determined by CDNB, DCNB, and EA, but not CHP, as substrates were elevated. A previous study using a whole-body exposure chamber for 3 days reported that hepatic GST activity toward EA is increased, GST activity toward 1,2-epoxy-3-(*p*-nitrophenoxy)-propane is decreased, and hepatic GST activities for CDNB and DCNB do not significantly change in mice exposed to cigarette smoke (Eke et al., 1996). However, in that study, serum markers of hepatic injury and protein levels of GST enzymes were not reported. In rats exposed to smoke for 90 days, there was a decrease in hepatic GST activity determined using CDNB (Ramesh et al., 2010). In that study, the rats that inhaled smoke for 90 days exhibited hepatic oxidative stress, elevations in serum liver injury markers, and histopathological changes. Thus, although the exposure period and method of cigarette smoke exposure may contribute to discrepancies among studies, little is known about the regulation of hepatic GST expression by cigarette smoke. In lung tissues from patients, alpha- and pi-class GST protein levels were higher in those with mild/moderate COPD than in those with severe/very severe COPD, and were comparable between smoking and non-smoking groups (Harju et al., 2008). In the bronchial epithelium of smokers, the mRNA levels of GSTA2 and GSTP1, but not GSTM1, were elevated compared to non-smokers (Thum et al., 2006). Additionally, previous microarray analyses have demonstrated the upregulation of GSTA2 expression due to smoking in the airway epithelium of smokers (Hackett et al., 2003). However, an extensive literature review identified no studies that have investigated the regulation of hepatic GST by cigarette smoke in humans.

Although the precise mechanisms underlying the induction of hepatic mu- and pi-class GSTs by cigarette smoke remain to be elucidated, it is possible that the activation of transcription factors, including AhR, is responsible for the induction of GSTs (Higgins and Hayes, 2011). Hepatic GST activities are increased by polycyclic aromatic hydrocarbons, such as 3-methylcholanthrene, 3,4-benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Baars et al., 1978; Clifton and Kaplowitz, 1978), and the 3R4F reference cigarettes used in the present study included 14 major polycyclic aromatic hydrocarbons, including naphthalene, fluorene, phenanthrene, acenaphthene, benz[a]anthracene, and benzo[a]pyrene, at levels of 833 ± 45 ng/cigarette (Vu et al., 2015). The activation of AhR by these polycyclic aromatic hydrocarbons can increase the transcription of genes containing xenobiotic response element (XRE) sequences, which appear to have been identified in the regulatory regions of GST genes (Higgins and Hayes, 2011). Moreover, the hepatic protein levels of Gsta1/2, Gstm1, and Gstp1/2 but not Gsta3, which is a constitutive alpha-class GST isoform, exhibit a greater increase following exposure to beta-naphthoflavone, a prototype AhR ligand, in C57BL/6 mice than in DBA/2 mice, which lack functional AhRs (McLellan et al., 1991). Additionally, hepatic GST activities toward CDNB, DCNB, and EA, but not CHP, are elevated by beta-naphthoflavone (McLellan et al., 1991). Taken together, these data suggest that the activation of AhR by cigarette smoke may be responsible for the induction of mu- and pi-class GSTs. Considering that carcinogenic metabolites of tobacco smoke constituents can be detoxified by GSH conjugation reactions (Hayes and Pulford, 1995; Reszka and Wasowicz, 2001; Di Pietro et al., 2010), the increases in GST enzymes may be adaptive response to cigarette smoke. These results warrant further studies to elucidate the effects of long-term exposure to cigarette smoke on the regulation of hepatic GST expression.

In conclusion, the present study demonstrated that the hepatic protein levels and activities of Cyp1a and Cyp2b in BALB/c mice increased following the short-term exposure to nose-only cigarette smoke in the absence of hepatic injury and oxidative stress. Additionally, the activities of GST toward CDNB, DCNB, and EA but not CHP were elevated, which was consistent with the present immunoblot results showing the induction of mu- and pi-class GSTs but not alpha-class GSTs. Taken together, these results suggest the that disposition and toxicity of xenobiotics metabolized by CYP1A, CYP2B, or mu/pi-class GSTs may be altered by cigarette smoke and could be associated with interactions of cigarette smoke with various therapeutic drugs, as well as the susceptibility of smokers to smoking-related diseases.

Conflicts of interest

The authors have declared no conflicts of interest.

Acknowledgements

This research was supported by a grant (14182MFDS977) from Ministry of Food and Drug Safety in 2017 and a National Research Foundation of Korea Grant funded by the Korean Government (2016R1A2B4008382 and 2018M3A9H1021640).

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.035.

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