Letter

Impact of cigarette smoke exposure on the expression of cardiac hypertrophic genes, cytochrome P450 enzymes, and oxidative stress markers in rats

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ABSTRACT — Various experimental and clinical studies strongly support a cigarette smoke-heart disease association and suggest possible mechanisms, unfortunately, the involvement of genetic modulations remain unexplored. Thus, the main aim of the current study was to evaluate the effects of sub-chronic cigarette smoke exposure on the mRNA expression of cardiac hypertrophy genes, cytochrome P450 (CYP) enzymes, and the oxidative stress markers in heart rats. For this purpose, Wistar albino rats were exposed to increasing doses of passive cigarette smoke 2, 4, 8, and 24 cigarettes per day for 7 consecutive days. The mRNA expression of fifteen cardiac genes was determined using real-time polymerase chain reaction. Our results showed that the levels of hypertrophic genes; atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain were significantly induced, whereas the anti-hypertrophic gene α-myosin heavy chain was dramatically inhibited, in heart tissues of passive-smoke-exposed groups compared with normal-control groups. This was accompanied with a significant induction of CYP enzymes; CYP1A1, CYP2C11, CYP2E1, and CYP3A2, and the expression of oxidative stress genes, heme oxygenase 1, catalase, cyclooxygenase, and glutathione S-Transferase. The ability of cigarette smoke to induce cardiac hypertrophic genes, CYPs enzymes, and oxidative stress, collectively explore the molecular mechanism of cigarette smoke-induced cardiac diseases and brings further investigative attention to the public health issue of the injurious effects of chronic passive smoke exposure. In conclusion, sub-chronic environmental tobacco smoke exposure increases the incidence of cardiovascular diseases through modulation of cardiac genes.

Key words: Cigarette smoke, Cardiovascular disease, Cytochrome P450, Hypertrophic genes, Oxidative stress genes

INTRODUCTION

The most important determinants of human health trends are the increase in smoking related morbidity and mortality (Murray and Lopez, 1997). Tobacco related deaths are expected to increase to 10 million within the next 20-30 years, of which approximately 70% will occur in the developing countries (Murray and Lopez, 1997). Cigarette smoke has been widely investigated in terms of epidemiological and pathological studies in relation to human diseases. In this regards, it has been reported that tobacco smoking is responsible for over 11% of cardiovascular diseases (CVD) and 90% lung cancer cases, the main causes of death in the western world (Zhang *et al*., 2002; Ezzati *et al*., 2005). These detrimental effects are not only present in active smokers, but also to innocent, as passive smokers, who are exposed to cigarettes not-by-choice. In that, breathing other people's smoke has been shown to increase a person's risk of CVD by approximately 24% (Law *et al*., 1997). Recent studies have shown that approximately a million North American suffer myocardial infarction, in which prevalence studies suggest that cigarette smoking is responsible for about one third of all CVD deaths (Chen and Boreham, 2002).

Cigarette smoke contains more than 4,000 substances, several of which are known to cause cancer in humans or animals. Recent experimental and epidemiological studies in tobacco smoke particulate have detected about fourteen

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polycyclic aromatic hydrocarbons (PAHs) in each cigarette in a range of 1,000-1,600 ng/cigarette, heavy metals, and many other carcinogenic compounds (Ding *et al*., 2005). Interestingly, it has been reported that PAH compounds found in cigarette are able to differentially modulate the expression of certain genes. For example, it is well established that PAHs induce CVD, at least in part, through modulating the xenobiotic metabolizing enzymes (XMEs), particularly the cytochrome P450 (CYP). In that, cigarette smoking appeared to induce total body drug metabolism, as indicated by decreased antipyrine half-life and increased its clearance (Vahakangas *et al*., 1983). In addition, cigarette smoke exposure induces hepatic CYP enzymes, especially CYP1A2, in both rats and hamsters (Nishikawa *et al*., 2004). Although there are a substantial number of drugs acting either directly or indirectly on the heart, little is known about the metabolic capacity of heart muscle cells and tissues to express these CYPs in response to cigarette smoke (Lanca *et al*., 2004; Wang *et al*., 2003). Such studies strongly suggest a link between cigarette smoking-induced modulation of gene expression and smoking related morbidity and mortality. However, the precise molecular alterations in cardiac genes induced by smoking in CVD remain to be elucidated.

In this study we have designed experiments to address the effect of sub-chronic exposure to cigarette smoke *in vivo* on the expression of cardiac target genes involved in CVD, hypertrophic genes (atrial natriuretic peptide ANP, brain natriuretic peptide BNP, and β- and α-myosin heavy chain β-MHC and α-MHC), metabolizing enzymes (CYP1A1, CYP2C11, CYP2E1, and CYP3A2), and oxidative stress markers (heme oxygenase-1 HO-1, catalase, and glutathione S Transferase GSTA1). In the current study we showed that sub-chronic environmental tobacco smoke exposure increases the incidence of cardiovascular diseases through modulation of cardiac genes.

MATRIALS AND METHODS

Materials

Cigarettes (manufactured by Philip Moris) purchased from local markets, TRIzol reagent was purchased from Invitrogen Co. (Grand Island, NY, USA). The High-Capacity cDNA reverse transcription kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA, USA). Rat forward and reverse primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA). Diethyl pyrocarbonate (DEPC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON, Canada).

Animals

Adult male Wistar Albino (WA) rats approximately 3 months age ranging in weight from 200-230 g, obtained from the Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia, were housed in metabolic cages under controlled environmental conditions (25ºC and a 12 hr light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water unless otherwise indicated. The protocol of this study has been approved by Research Ethics Committee of College of Pharmacy, King Saud University (Riyadh, Saudi Arabia) and the animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals by National Institute of Health.

Cigarette smoke exposure

The passive smoke exposure was implemented in a closed plastic chamber (50 × 40 × 35 cm) with 12 ventilation holes (1 cm in diameter). In dose-response experiments, six WA rats were placed in each chamber of a total three chambers and exposed to a commercial filter-tipped cigarette (manufactured by Philip Moris with a tar content of 7.0 mg, nicotine content of 0.75 mg) smoke generated from 2, 4, 8, or 24 cigarettes/day over a 8-hr exposure period for 7 consecutive days. The cigarette smoke was introduced into the chamber with a three-way smoking device through the ventilation holes. The puff volume was 50 ml, and the puff interval was 30 sec by the use of timed draw-back mimicking normal smoking inhalation volume and cigarette burn rate. For control group, WA rats were placed in similar chamber, to avoid distressing the animals, but did not receive cigarette smoke (sham-exposed). On the eighth day, animals were euthanized under ether anesthesia, thorax was opened and heart tissues were rapidly dissected, lavaged with phosphate-buffered saline (PBS), and then kept in -20°C freezer until use (Kim *et al*., 2004).

RNA extraction and cDNA synthesis

After homogenization of heart tissue samples with a glass tissue homogenizer in 10 volumes of ice chilled PBS, total tissue RNA was isolated using TRIzol reagent (Invitrogen®) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio (> 1.8). Thereafter, first strand cDNA synthesis was performed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems®), according to the manufacturer's instructions and as described previously (Korashy and El-Kadi, 2012). Briefly, 1.5 µg of total RNA from each sample was added to a mixture of 2.0 µl of

10x reverse transcriptase buffer, 0.8 µl of 25x dNTP mix (100 mM), 2.0 µl of 10x reverse transcriptase random primers, 1.0 µl of MultiScribe reverse transcriptase, and 3.2 µl of nuclease-free water. The final reaction mixture was kept at 25°C for 10 min, heated to 37°C for 120 min, heated for 85°C for 5 sec, and finally cooled to 4°C.

Quantification of mRNA expression by real-time polymerase chain reaction (RT-PCR)

Quantitative analysis of specific mRNA expression was performed by RT-PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems®). The 25-µl reaction mixture contained 0.1 µl of 10 µM forward primer and 0.1 µl of 10 µM reverse primer (40 nM final concentration of each primer), 12.5 µl of SYBR Green Universal Mastermix, 11.05 µl of nuclease-free water, and 1.25 µl of cDNA sample. The fold change in the level of target genes (Table 1) between treated and untreated cells were corrected by the levels of β-ACTIN. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. The RT-PCR data were analyzed using the relative gene expression (i.e.,ΔΔ CT) method, as described in Applied Biosystems User Bulletin No. 2 and explained further by Livak and Schmittgen (2001). Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene β-ACTIN and relative to a calibrator. The fold change in the level target genes between treated and untreated cells, corrected by the level of β-actin, was determined using the following equation: fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

Statistical analysis

The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat® for Windows (Systat Software, Inc, CA, USA). One-way analysis of variance (ANOVA) followed by Dunnett's test was carried out to assess which treatment groups showed a significant difference from the control group. The differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Cigarette smoking is a major health risk contributing to cardiovascular morbidity and mortality. Recent experimental and epidemiological studies in tobacco smoke particulate have reported the ability of cigarette smoke to differentially modulate the expression of certain genes (Ding *et al*., 2005). Although evidence supporting the correlation between cigarette smoking and the increased incidence of CVD has increased in the last decade, no studies have been conducted to examine the impact of cigarette smoke on the genetic modulation of cardiac genes.

Several previous studies have examined the impact of the exposure level of cigarette on the actual *in vivo* toxicities under similar exposure condition. In that, it has been

Table 1. Primers sequences used for Real-Time PCR reactions

Gene	5'→3' Forward primer	5'→3' Reverse primer
ANP	GGAGCCTGCGAAGGTCAA	TATCTTCGGTACCGGAAGCTGT
BNP	CAGAAGCTGCTGGAGCTGATAAG	TGTAGGGCCTTGGTCCTTTG
β-MHC	ATCAAGGGAAAGCAGGAAGC	CCTTGTCTACAGGTGCATCA
α-MHC	TCCTTTATCGGTATGGAGTCTG	TGATCTTGATCTTCATGGTGCT
CYP1A1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
CYP1A2	AACAAGGGACACAACGCTGAAT	GGAAGAGAAACAAGGGCTGAG
CYP2C11	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA
CYP3A2	CTGCTTTCAGCTCTCACACT	CAGTCGGTGCTTATGCTT
CYP2E1	AAAGCGTGTGTGTGTTGGAGAA	AGAGACTTCAGGTTAAAATGCTGCA
HO-1	TGCTCGCATGAACACTCTGGAGAT	ATGGCATAAATTCCCACTGCCACG
CATALASE	CCCGAGTCCAGGCTCTTCT	CGGCCTGTACGTAGGTGTGA
GSTA1	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA
COX2	TGTATGCTACCATCTGGCTTCGG	GTTTGGAACAGTCGCTCGTCATC
β-ACTIN	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA

reported that chronic cigarette smoke exposure (four pieces cigarette daily 30 min each, for a 2-hr exposure total, for 24 weeks) to rats significantly increased the lipid peroxidation levels in tissues of passive-smoke-exposed compared with normal-bred control groups (Kim *et al*., 2004). Furthermore, sub-chronic exposure of mice to cigarette smoke generated from 3, 6, or 9 cigarettes/day for 4 days, caused upregulation of chemokines and inflammatory mediators (Vlahos *et al*., 2006). In addition, Izzotti *et al*. (1992) showed that exposure of the whole-body of male Sprague-Dawley rats to mainstream cigarette smoke (once daily for up to 40 consecutive days) resulted in increased DNA adducts formation in the heart more than those detected in the lung of the same animals, whereas no adduct was revealed in liver DNA.

To our knowledge, this is the first *in vivo* study addresses the influences of cigarette smoke on cardiac hypertrophic genes, metabolizing enzymes, and oxidative stress markers. Initially, no significant differences were found between the smoke-exposed and control groups in baseline rat body weight (218 ± 17 vs. 230 ± 12 g), or survival rate (100%, 18/18 rats vs. 6/6), data not shown. Our current study demonstrates for first time that sub-chronic exposure of rats to cigarette smoke dramatically increased the expression of most, if not all, cardiac target genes that are involved in the CVD pathogenesis. This conclusion is supported by several pieces of evidence. The findings that cigarette smoke increased the expression of several hypertrophic gene markers, ANP, BNP and β-MHC by approximately 14-, 4-, and 8-fold, respectively (Fig. 1A-C), while inhibited the mRNA expression of α-MHC, anti-hypertrophic gene marker by 70% after exposure to 24 cig./day (Fig. 1D) resulting in hypertrophied heart muscle which subsequently leads to heart failure. In this context, it has been shown that some cardiotoxic drugs, such as doxorubicin, induced cardiotoxicity in rats through the modulation of hyertrophic genes, particularly β-MHC and α-MHC (Zordoky *et al*., 2010).

CYP enzymes are known to play important role in the pathogenesis of CVD (Korashy and El-Kadi 2006a; Elbekai and El-Kadi, 2006). Therefore, we examined the effect of exposure to cigarette smoke on the expression of various CYP genes (CYP1A1, CYP1A2, CYP2C11, CYP2E1, and CYP3A2) in both control (sham-exposed) and cigarette-exposed rats using RT-PCR. Fig. 2A shows that exposure to cigarette smoke for 7 days significantly increased CYP1A1, but not CYP1A2 (data not shown), mRNA expression level in a dose-dependent manner. The maximum induction (13-fold) was observed at the highest dose tested (24 Cig/day). The ability of cigarette smoke to induce the expression of CYP1A1, a well-known can-

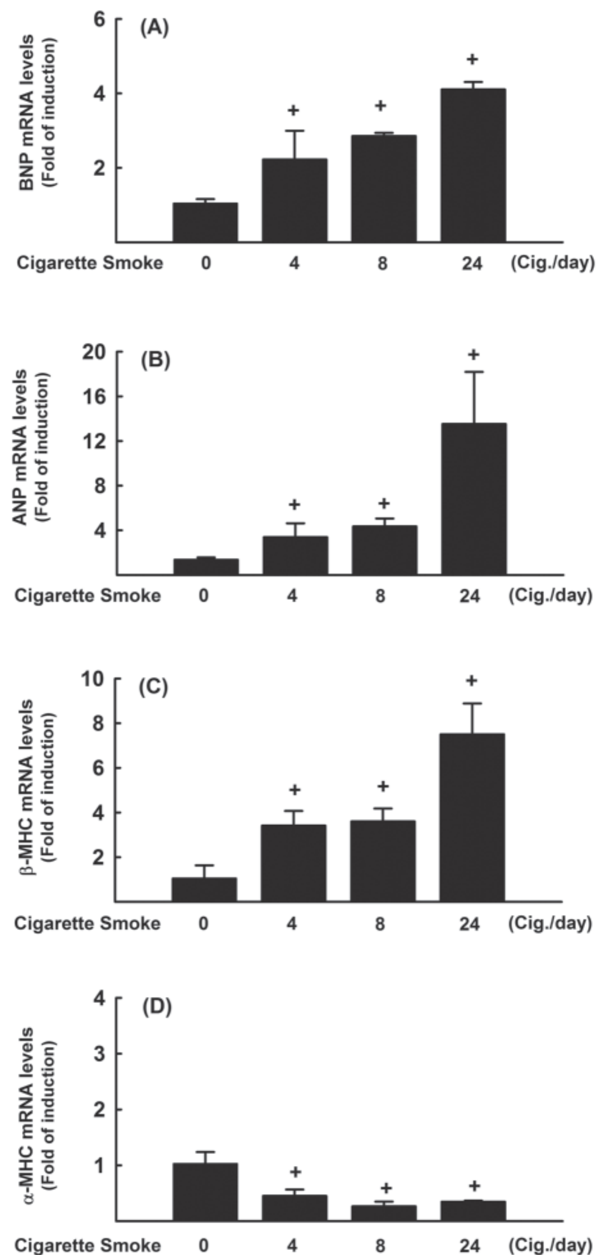

Fig. 1. Effect of exposure to cigarette smoke on BNP (A), ANP (B), β-MHC (C), and α-MHC (D) mRNA levels. Rats were exposed to cigarette smoke with increasing doses of cigarette as described under Materials and methods section. Thereafter, total RNA was isolated using TRIzol reagent and BNP, ANP, β-MHC, and α-MHC mRNAs were quantified using RT-PCR normalized to β-ACTIN housekeeping gene. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. Values are presented as means ± S.E.M. (n = 6). +$P < 0.05$, compared to control (0 Cig./day).

cer-activating gene, is of a major interest because of its role in bioactivating procarcinogens and environmental pollutants such as PAHs into carcinogenic and mutagenic intermediates (Guengerich, 2004). In this context, it is well documented that CYP1A1 bioactivates PAHs to epoxide and diol-epoxide intermediates that subsequently lead to DNA and protein adducts formation (Shimada and Fujii-Kuriyama, 2004). The current knowledge of the mechanism of CYP1A1 induction by PAHs suggests a transcriptional regulation, in which the binding of PAH to cytosolic transcription factor, the aryl hydrocarbon receptor (AhR), is the first step in a series of events leading to carcinogenicity and mutagenicity (Whitlock, 1999) through binding to xenobiotic responsive elements (XRE) located in the promoter region of *CYP1A1* resulting in the initiation of the mRNA transcription process (Whitlock, 1999; Pollenz, 2002).

Mechanistically, the presence of XRE DNA sequences in the promoter region of several hypertrophic genes, particularly ANP, BNP, and β-MHC (Thackaberry *et al*., 2005; Turhan *et al*., 2005; Borlak and Thum 2002; Kvasnicka *et al*., 2001; Savouret *et al*., 2003), may suggest a mechanism of induction of hypertrophic genes by cigarette smoke exposure. This is supported by the observations that AhR activation by PAHs, the main constituent of cigarette and the most potent CYP1A1 inducer (Mimura and Fujii-Kuriyama, 2003), caused an induction of hypertrophic genes in rat cardiomyocye H9c2 cells which was associated with induction of several CYP1A1 gene (Zordoky and El-Kadi, 2010). Another supporting evidence for the role of CYP1A1 induction is the findings both AhR mRNA and protein are strongly expressed in ischemic and dilative cardiomyocyte (Korashy and El-Kadi, 2006a). Furthermore, it has been shown that 17β-estradiol, a cardioprotective mediator, is metabolized in the cardiac fibroblasts by the CYP1A1 to its toxic hydroxyestradiol metabolite (Dubey *et al*., 2003; Pelzer *et al*., 2000; Zordoky *et al*., 2008), suggesting that induction of CYP1A1 by cigarette smoke decrease the cardiac levels of cardioprotective mediators. Taken together, these studies strongly suggest a direct role for CYP1A1 induction in cardiotoxicity induced by cigarette smoke exposure.

With regard to the CYP2 family, CYP2C11 and CYP2E1 mRNA levels were induced by approximately 3.2- and 2.5-fold, respectively, at the highest dose tested, 24 cig/day (Fig. 2B and C). The induction of CYP2C11 and CYP2E1 by cigarette exposure may suggest another mechanism of cardiotoxicity, in which it has been reported that CYP2E1 expression in the microsomal function of the myocardium was found to be much higher in dilat-

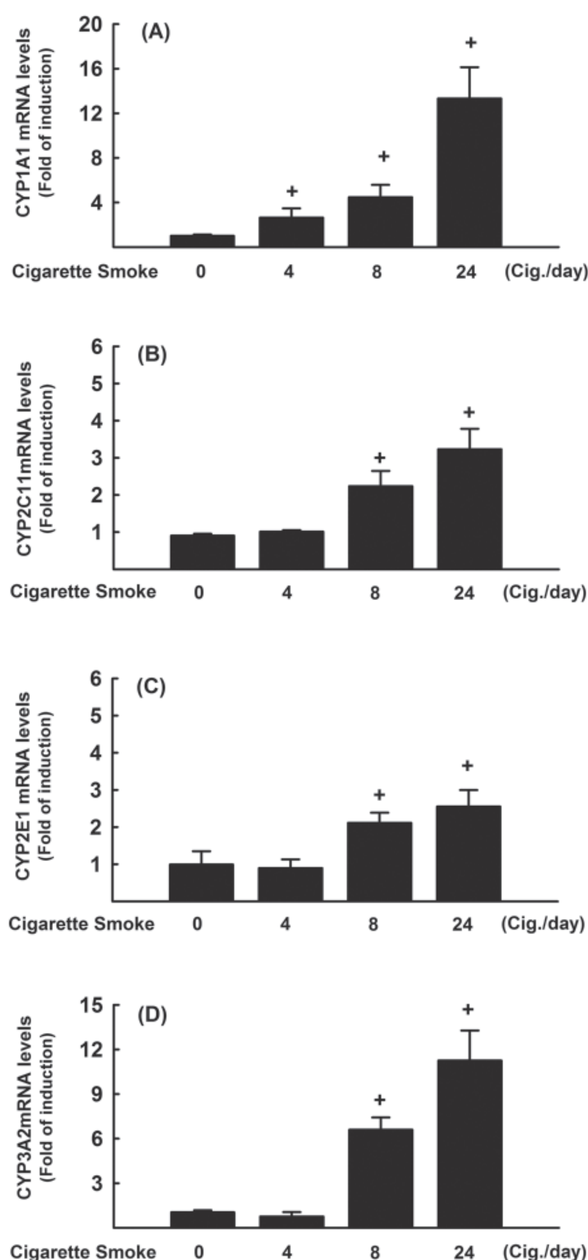

Fig. 2. Effect of exposure to cigarette smoke on CYP1A1 (A), CYP2C11 (B), CYP2E1 (C), and CYP3A2 (D) mRNA levels. Rats were exposed to cigarette smoke with increasing doses of cigarette as described under Materials and methods section. Thereafter, total RNA was isolated using TRIzol reagent and CYP1A1, CYP2C11, CYP2E1, and CYP3A2 mRNAs were quantified using RT-PCR normalized to β-ACTIN housekeeping gene. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. Values are presented as means ± S.E.M. (n = 6). +$P < 0.05$, compared to control (0 Cig./day).

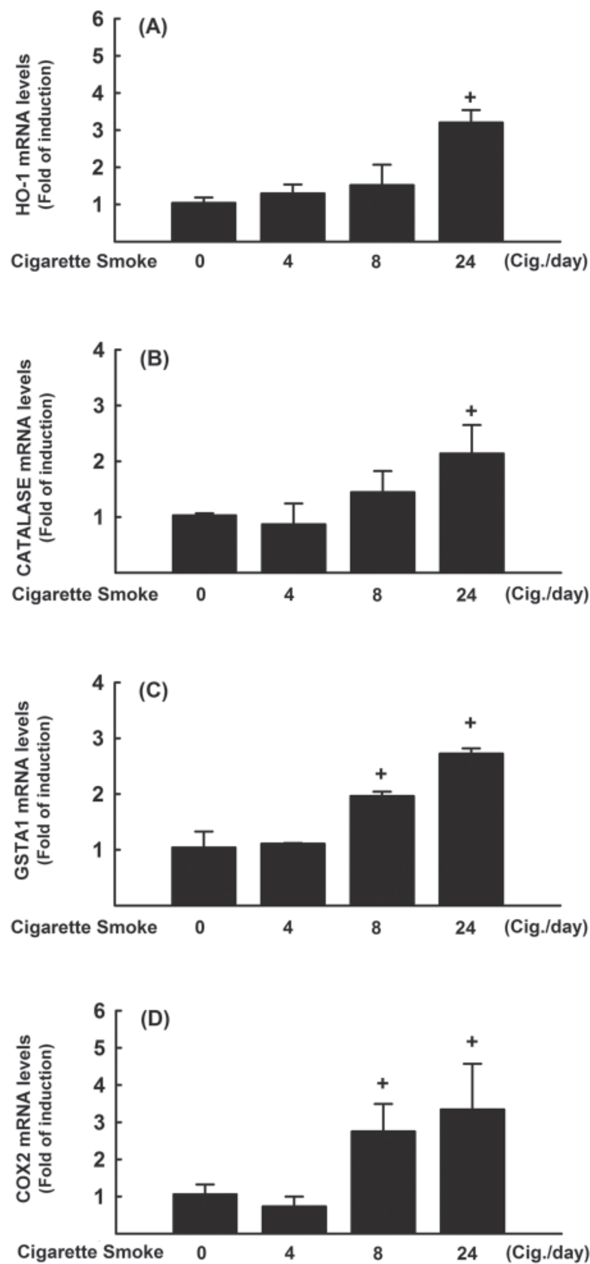

Fig. 3. Effect of exposure to cigarette smoke on HO-1 (A), CATALASE (B), GSTA1 (C), and COX2 (D) mRNA levels. Rats were exposed to cigarette smoke with increasing doses of cigarette as described under Materials and methods section. Thereafter, total RNA was isolated using TRIzol reagent and HO-1, Catalase, GSTA1, and COX2 mRNAs were quantified using RT-PCR normalized to β-ACTIN housekeeping gene. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. Values are presented as means ± S.E.M. (n = 6). $+P < 0.05$, compared to control (0 Cig./day).

ed cardiomyopathy than that in healthy human hearts (Sidorik *et al*., 2005). The physiological importance of these enzyme emerge from their ability to metabolize arachidonic acid to various cardiotoxic metabolites, particularly hydroxyeicosatetraenoic acid (HETEs) (Elbekai and El-Kadi, 2006). In addition, benzo[a]pyrene, a major constituent of cigarette smoke and strong CYP1A1 inducer, induces cardiac hypertrophy through the induction of CYP-hydroxylases, enzymes responsible for the formation of (HETE) and known cardiotoxic arachidonic acid metabolite, such as CYP1A1, CYP2C11, and CYP2E1 enzyme in the hearts of male Sprague-Dawley rats (Aboutabl *et al*., 2009; Annas *et al*., 1998).

On the other hand, CYP3A2 mRNA levels were dramatically increased by exposure to cigarette smoke to approximately 6.5 and 11.2 fold at the higher doses as compared to sham-exposed rats (Fig. 2D). Although there are a substantial number of drugs acting either directly or indirectly on the heart, little is known about the metabolic capacity of heart muscle cells and tissues to express and metabolize xenobiotics and drugs. In this context, we have shown here that exposure to cigarette smoke significantly induced the CYP3A2 gene expression, an enzyme that is mainly involved in the metabolism of several drugs. In fact many currently used therapeutic modalities in CVD owe their therapeutic efficacy to their effect on CYP metabolites. Thus, interruption of the expression of these CYP by exposure to cigarette smoke may significantly contribute to CVD and treatment failure (Elbekai and El-Kadi, 2006).

Cigarette smoke comprises several oxidants and free radicals compounds, which are capable of initiating or promoting oxidative damage, that exert harmful effect in CVD (Kim *et al*., 2004). Therefore, to determine the capacity of cigarette smoke to promote oxidative stress in the heart, we measured the mRNA expression of several oxidative stress-mediated genes, such as HO-1, Catalase, and GSTA1 using RT-PCR. Our results show that exposure to cigarette smoke daily induced the mRNA expression of HO-1 and Catalase genes by 3.2- and 2.2-fold, respectively, only at the highest doses (Fig. 3A and B). On the other hand, the mRNA expression of GSTA1, an antioxidant, gene was significantly induced dose-dependently by approximately 1.96- and 2.72-fold at the higher doses (8 and 24 cig/day) (Fig. 3C). Thus, imbalance in the expression of oxidant and antioxidants genes could determine the effect of smoking exposure on oxidative damage.

The ability of cigarette smoke exposure to induce the expression of oxidative stress genes, such as HO-1, may indicate the ability of cigarette smoke to initiate or pro-

mote oxidative damage that lead to various degenerative CVDs (Dertinger *et al*., 2001). In agreement with our result, it has been previously demonstrated the ability of PAHs to produce ROS and DNA adduct proteins (Izzotti *et al*., 1994; Elbekai *et al*., 2004). Furthermore, exposure to cigarette smoke caused significant induction of COX2 mRNA level, by approximately 2.8- and 3.5-fold, respectively (Fig. 3D), that plays a role in converting arachedonic acid to prostaglandins, a and hence contribute to the pathogenesis of CVDs. Knowing that COX2 gene contains XRE DNA sequences on its upstream regulatory region, we postulate here that cigarette smoke induce the expression of COX2 gene through the activation of AhR/XRE pathway (Sherratt *et al*., 2003). However, the ability of exposure to cigarette smoke to significantly induce the gene expression of antioxidant genes, such as GSTA1, a phase II XME that plays an important role in detoxification of many toxic xenobiotics, and catalase enzyme may maintain the cellular levels of biological antioxidants, such as vitamin E and ubiquinol and therefore can protect against cardiotoxicity (Cao and Li, 2004). Therefore, imbalance of the expression of cardioprotective and cardiotoxic XMEs is the main determinant of cigarette smoke-mediated cardiotoxicity.

We conclude that the exposure to cigarette smoke caused significant induction of the hypertrophic markers, oxidative stress as well as CYP genes at mRNA level. We showed that AhR activation by exposure to cigarette smoke caused a significant induction of hypertrophic markers directly through activation XRE DNA sequences which are located in the promoter region of several hypertrophic genes or by CYP induction which would result in alteration of arachidonic acid metabolism which has been associated with cardiac hypertrophy and heart failure (Zordoky *et al*., 2008); therefore, this may be one of the mechanism by which the exposure to cigarette smoke induces cardiotoxicity.

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