BJP BRITISH JOURNAL OF PHARMACOLOGY

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British Journal of Pharmacology (2009), 158, 1808–1819

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

3-Methylcholanthrene and benzo(a)pyrene modulate cardiac cytochrome P450 gene expression and arachidonic acid metabolism in male Sprague Dawley rats

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Background and purpose: There is a strong correlation between cytochrome P450 (P450)-dependent arachidonic acid metabolism and the pathogenesis of cardiac hypertrophy. Several aryl hydrocarbon receptor (AhR) ligands were found to alter P450-dependent arachidonic acid metabolism. Here, we have investigated the effect of 3-methylcholanthrene (3-MC) and benzo(a)pyrene (BaP), two AhR ligands, on the development of cardiac hypertrophy.
Experimental approach: Male Sprague Dawley rats were injected (i.p.) daily with either 3-MC (10 $mg \cdot kg^{-1}$) or BaP (20 $mg \cdot kg^{-1}$) for 7 days. Then hearts were removed, and the heart to body weight ratio and the gene expression of the hypertrophic markers and P450 genes were determined. Levels of arachidonic acid metabolites were determined by liquid chromatography-electron spray ionization-mass spectrometry.
Key results: Both 3-MC and BaP increased the heart to body weight ratio as well as the hypertrophic markers, atrial natriuretic peptide and brain natriuretic peptide. 3-MC and BaP treatment increased the gene expression of CYP1A1, CYP1B1, CYP2E1, CYP4F4, CYP4F5 and soluble epoxide hydrolase. Both 3-MC and BaP treatments increased the dihydroxyeicosatrienoic acids (DHETs) : epoxyeicosatrienoic acids (EETs) ratio and the 20-hydroxyeicosatetraenoic acid (20-HETE) : total EETs ratio. Treatment with benzo(e)pyrene, an isomer of BaP that is a poor ligand for the AhR, did not induce cardiac hypertrophy in rats, confirming the role of AhR in the development of cardiac hypertrophy. Treatment with the ω-hydroxylase inhibitor, HET0016, significantly reversed BaP-induced cardiac hypertrophy.
Conclusions and implications: 3-MC and BaP induce cardiac hypertrophy by increasing the ratio of DHETs : EETs and/or the ratio of 20-HETE : total EETs, through increasing soluble epoxide hydrolase activity.
British Journal of Pharmacology (2009) **158,** 1808–1819; doi:10.1111/j.1476-5381.2009.00461.x; published online 4 November 2009

Keywords: aryl hydrocarbon receptor; benzo(a)pyrene; cytochrome P450; epoxyeicosatrienoic acid; hydroxyeicosatetraenoic acid; 3-methylcholanthrene; soluble epoxide hydrolase

Abbreviations: 3-MC, 3-methylcholanthrene; AhR, aryl hydrocarbon receptor; ANP, atrial natriuretic peptide; BaP, benzo(a)pyrene; BeP, benzo(e)pyrene; BNP, brain natriuretic peptide; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; HO-1, haem oxygenase-1; LC-ESI-MS, liquid chromatography-electron spray ionization-mass spectrometry; P450, cytochrome P450; PCR, polymerase chain reaction; sEH, soluble epoxide hydrolase

Introduction

Heart failure affects more than five million people in North America with about half a million of new cases every year (Zordoky *et al.*, 2008). Cardiac hypertrophy and fibrosis precede heart failure and prolonged hypertrophy is a significant risk factor for heart failure (Carreno *et al.*, 2006). Epidemiological and clinical studies clearly demonstrated that exposure to aryl hydrocarbon receptor (AhR) ligands is associated with the development of heart disease (Burstyn *et al.*, 2005). Benzo(a)pyrene (BaP), which is an AhR ligand and one of the component of cigarette smoke, was found to be positively associated with mortality from ischaemic heart disease upon exposure (Burstyn *et al.*, 2005). Recent studies have shown that approximately one million North Americans suffer from a myocardial infarction, in which prevalence

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Received 2 April 2009; revised 19 June 2009; accepted 13 July 2009

studies suggest that cigarette smoking is responsible for about one-third of all heart disease deaths (Chen and Boreham, 2002).

Earlier studies linked the development of cardiovascular diseases to the activation of the AhR signalling pathways (Korashy and El-Kadi, 2006). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a potent AhR ligand, was found to mediate cardiotoxicity through enlarging right and left ventricles, decreasing responsiveness to β-adrenoceptor agonists, causing abnormalities of conduction and heart contractility, and subcutaneous and pericardial oedema, reflecting a progression to heart failure (Heid *et al.*, 2001). In chicken embryo, TCDD caused cardiac hypertrophy that was characterized by increase in heart size (Kanzawa *et al.*, 2004) and was accompanied by induction of the cardiac muscle proteins, atrial natriuretic peptide (ANP), β-myosin heavy chain and troponin (Korashy and El-Kadi, 2006).

The development of cardiovascular diseases by AhR ligands could be attributed to the cardiac expression of both AhR and its heterodimeric partner, aryl hydrocarbon receptor nuclear translocator (ARNT), which forms a complex with AhR leading to gene transcription (Korashy and El-Kadi, 2006). Both AhR mRNA and protein are strongly expressed in left ventricle of healthy, ischaemic and dilative cardiomyopathy subjects. Moreover, in ischaemic and dilative cardiomyopathy patients, the AhR protein level was twofold higher than in healthy subjects (Mehrabi *et al.*, 2002). The expression of AhR and ARNT in the developing myocardium and cardiac septa of chick embryos treated with TCDD was found to be consistent with the development of cardiac hypertrophy and septal defects (Walker *et al.*, 1997).

Aryl hydrocarbon receptor ligands activate the expression of genes in both the Phase I and Phase II aryl hydrocarbon gene battery (Nebert *et al.*, 2000). This includes the xenobiotic-metabolizing cytochrome P450 (P450) enzymes from the CYP1A and CYP1B subfamilies, in addition to NAD(P)H-quinone oxidoreductase 1, an aldehyde dehydrogenase, and several phase II-conjugating enzymes, including glutathione S-transferase A1 and UDP-glucuronosyltransferase 1A1 (Nebert *et al.*, 2004). Many P450 genes have been shown to be expressed in the rat heart as well as the human heart, and their levels have been reported to be altered during cardiac hypertrophy and heart failure (Thum and Borlak, 2002; Zordoky *et al.*, 2008). With regard to the CYP1 family, the expression of CYP1A1 mRNA has been reported in the right ventricle and left atrium of patients with dilated cardiomyopathy (Thum and Borlak, 2000; 2002). We previously demonstrated the significant induction of CYP1A1 and CYP1B1 mRNA in rat hearts of isoprenaline-induced cardiac hypertrophy (Zordoky *et al.*, 2008).

It has been previously shown that there is a strong correlation between the P450-dependent arachidonic acid metabolism and pathogenesis of cardiac hypertrophy. Several reports showed the alteration of arachidonic acid metabolism by AhR ligands. AhR ligands such as β-naphthoflavone and 3-methylcholanthrene (3-MC) were found to decrease the formation of epoxyeicosatrienoic acids (EETs) (Capdevila *et al.*, 1990; Lee *et al.*, 1998) and increase levels of 19-hydroxyeicosatetraenoic acid (19-HETE) in rat liver microsomes (Lee *et al.*, 1998). In a chick embryo model, TCDD induced arachidonic acid metabolism in kidney and liver but not in heart (Nakai *et al.*, 1992; Rifkind *et al.*, 1994). In *Stenotomus chrysops* (scup fish), BaP did not alter total EETs formation in the heart (Schlezinger *et al.*, 1998). The controversy over the amounts of arachidonic acid metabolite formed demonstrates the unpredictable effects of AhR ligands on arachidonic acid epoxygenation or ω-hydroxylation among different organs or species.

In the current study we investigated the effect of two potent AhR ligands; 3-MC and BaP, on the development of cardiac hypertrophy in rats. This was investigated by studying the effect of 3-MC and BaP treatment on the induction of hypertrophic markers and expression of different P450 genes in the rat heart. In addition, we investigated whether the exposure of rats to 3-MC and BaP led to changes in P450 catalysed arachidonic acid metabolism in the heart. Furthermore, we investigated the mechanism by which AhR ligands induced cardiac hypertrophy.

Methods

Animals and treatment

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National institute of Health Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Sprague Dawley rats weighing 300–350 g were obtained from Charles River Canada (St. Constant, QC, Canada). All animals were allowed free access to food and water throughout the treatment period. Animals were injected i.p. with 10 $mg \cdot kg^{-1}$ 3-MC, 20 $mg \cdot kg^{-1}$ BaP, 20 $mg \cdot kg^{-1}$ benzo(e)pyrene (BeP) or 20 $mg \cdot kg^{-1}$ BaP with 0.1 $mg \cdot kg^{-1}$ ω-hydroxylase inhibitor, *N* - hydroxy - *N'* - (4 - butyl - 2 - methylphenyl) formamidine (HET0016) daily for 7 days (n = 6). The HET0016 dose of 0.1 $mg \cdot kg^{-1}$ was based on a report by Lv *et al.* (2008) who showed that, at this dose, HET0016 selectively inhibited 20-HETE formation in rats. Weight-matched controls received the same volume of corn oil and/or saline daily for 7 days (n = 6). 3-MC, BaP and BeP were dissolved in corn oil, whereas HET0016 was dissolved in saline. Animals were killed under isoflurane anaesthesia, 24 h following the last injection. Hearts were excised, immediately frozen in liquid nitrogen and stored at –80°C until analysis.

RNA extraction and cDNA synthesis

Total RNA from the frozen tissues was isolated using TRIzol reagent (Invitrogen) 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. Thereafter, first-strand cDNA synthesis was performed by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions.

Quantification by real time-polymerase chain reaction (PCR)

Quantitative analysis of specific mRNA expression was performed by real time-PCR, by subjecting the resulting cDNA to

Table 1 Primers sequences used for real time-PCR reactions

Gene	*Forward primer*	*Reverse primer*
CYP1A1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
CYP1B1	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA
CYP2B1	AACCCTTGATGACCGCAGTAAA	TGTGGTACTCCAATAGGGACAAGATC
CYP2B2	CCATCCCTTGATGATCGTACCA	AATTGGGGCAAGATCTGCAAA
CYP2C11	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA
CYP2C13	AGGAAAACGGATGTGTTTGG	TTGATGTCCTTTGGGTCAAC
CYP2E1	AAAGCGTGTGTGTGTTGGAGAA	AGAGACTTCAGGTTAAAATGCTGCA
CYP2J3	CATTGAGCTCACAAGTGGCTTT	CAATTCCTAGGCTGTGATGTCG
CYP4A3	CTCGCCATAGCCATGCTTATC	CCTTCAGCTCATTCATGGCAATC
CYP4F1	CCCCCAAGGCTTTTTGATG	GAGCGCAACGGCAGCT
CYP4F4	CAGGTCTGAAGCAGGTAACTAAGC	CCGTCAGGGTGGCACAGAGT
CYP4F5	AGGATGCCGTGGCTAACTG	GGCTCCAAGCAGCAGAAGA
CYP4F6	TCACTTGACCTTGATGAAGAACAAC	AAGAGAGGTGGATATCACGGAAG
sEH	CACATCCAAGCCACCAAGCC	CAGGCCTCCATCCTCCAG
ANP	GGAGCCTGCGAAGGTCAA	TATCTTCGGTACCGGAAGCTGT
BNP	CAGAAGCTGCTGGAGCTGATAAG	TGTAGGGCCTTGGTCCTTTG
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; PCR, polymerase chain reaction; sEH, soluble epoxide hydrolase.

PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems) according to the manufacturer's instructions. The primers used in the current study were chosen from previously published studies (Bleicher *et al*., 2001; Kalsotra *et al*., 2002; Hirasawa *et al*., 2005; Rollin *et al*., 2005; Baldwin *et al*., 2006; Jin *et al*., 2006) and are listed in Table 1. Melting curve (dissociation stage) was performed at the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

Real time-PCR data analysis

The real time-PCR data were analysed using the relative gene expression, that is, ($\Delta\Delta$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 (GAPDH) and relative to a calibrator. The untreated control was used as the calibrator when the change of gene expression by 3-MC and BaP is being studied.

Microsomal preparation and Western blot analysis

Microsomal protein was prepared from the heart tissue as described previously (Barakat *et al*., 2001). Heart microsomal protein concentration was determined by the Lowry method using bovine serum albumin as a standard (Lowry *et al*., 1951). Western blot analysis was performed using a previously described method (Gharavi and El-Kadi, 2005). Briefly, 20 μg of heart microsomal protein from each treatment group was separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C in blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (TBS), 5% skim milk, 2% bovine serum albumin and 0.5% Tween-20. After blocking, the blots were incubated with a primary polyclonal goat anti-rat CYP1A1 antibody, rabbit anti-rat CYP1B1, rabbit anti-rat CYP2E1 for 4 h or rabbit anti-human soluble epoxide hydrolase (sEH) for 12 h at 4°C. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody for CYP1A1 or goat anti-rabbit IgG secondary antibody for CYP1B1, CYP2E1 and sEH was carried out for 2 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ, USA). The intensity of the protein bands were quantified, relative to the signals obtained for actin, using ImageJ software (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

Microsomal incubation

Heart microsomes (1 mg protein·mL^{-1}) were incubated in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH 7.4) at 37°C in a shaking water bath (50 rpm). A pre-equilibration period of 5 min was used. The reaction was initiated by the addition of 1 mM NADPH. Arachidonic acid was added to a final concentration of 50 μM and incubated for 30 min. The reaction was terminated by the addition of 600 μL ice cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. Arachidonic acid metabolites were extracted twice by 1 mL ethyl acetate and dried using speed vacuum (Savant, Farmingdale, NY, USA). The concentrations of these eicosanoids in the samples were calculated by comparing the ratios of peak heights to their corresponding standards. Incubation conditions were optimized so that the rate of metabolism was linear with respect to incubation time and microsomal protein concentration.

Separation of different arachidonic acid metabolites by liquid chromatography-electron spray ionization-mass spectrometry (LC-ESI-MS)

Extracted arachidonic acid metabolites were analysed using LC-ESI-MS (Waters Micromass ZQ 4000 spectrometer) methods as described previously (Zordoky *et al*., 2008). The

samples (10 μL) were separated on reverse phase C18 column (Kromasil, 250 × 3.2 mm) using a linear gradient mobile phase system with water/acetonitrile with 0.005% acetic acid as mobile phase at flow rate of 0.2 mL·min^{-1}. The mobile phase system started at 60% acetonitrile, linearly increased to 80% acetonitrile in 30 min, increased to 100% acetonitrile in 5 min and held for 5 min. 4-hydroxybenzophenone was used as internal standard.

Determination of total P450 content and haem oxygenase activity

The total P450 content was performed on pooled microsomal fractions of six rat hearts and were measured spectrophotometrically using the reduced-CO spectral absorbance as described previously (Omura and Sato, 1964).

Haem oxygenase (HO) activity was assayed in pooled microsomal fractions as described previously (Sinal *et al.*, 1995).

Statistical analysis

Data are presented as mean ± standard error of the mean. Comparative gene expression and metabolite formation across groups was analysed using a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls *post hoc* comparison. A result was considered statistically significant where $P < 0.05$.

Materials

TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). High-Capacity cDNA Reverse Transcription Kit and SYBR Green SuperMix were purchased from Applied Biosystems (Foster City, CA, USA). Real time-PCR primers were synthesized by Integrated DNA Technologies Inc. (San Diego, CA, USA) according to previously published sequences. 3-MC, BaP, BeP, arachidonic acid, haemin, and 4-hydroxybenzophenone, and anti-goat IgG with horseradish peroxidase secondary antibody were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Arachidonic acid metabolite standards and HET0016 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Acrylamide, *N'N'*-bis-methylene-acrylamide, ammonium persulphate, β-mercaptoethanol, glycine, nitrocellulose membrane (0.45 μm) and TEMED were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Chemiluminescence Western blotting detection reagents were purchased from GE Healthcare Life Sciences, Piscataway, NJ, USA. CYP1A1 goat polyclonal primary antibody was purchased from Oxford Biomedical Research (Oxford, MI, USA). CYP1B1 rabbit polyclonal primary antibody was purchased from BD Gentest (Woburn, MA, USA). CYP2E1 rabbit polyclonal primary antibody was purchased from Abcam (Cambridge, UK). Goat anti-rabbit IgG with horseradish peroxidase secondary antibody, sEH rabbit polyclonal primary antibody and actin goat polyclonal primary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON, Canada).

Results

Effect of 3-MC and BaP treatment on hypertrophic markers and the heart to body weight ratio

In order to investigate whether 3-MC and BaP treatment caused cardiac hypertrophy in the treated rats, we measured the cardiac gene expression of the hypertrophic markers, ANP and brain natriuretic peptide (BNP) relative to control rats. Our results showed that 3-MC treatment caused a significant induction of both hypertrophic markers, ANP and BNP by threefold and twofold respectively (Figure 1A). Similarly, BaP treatment caused a significant induction of both hypertrophic markers, ANP and BNP by 3.5- and 1.8-fold respectively (Figure 1A). In addition, 3-MC and BaP treatment caused a significant increase in the heart to body weight ratio by about 16.5% and 8.7% respectively (Figure 1B).

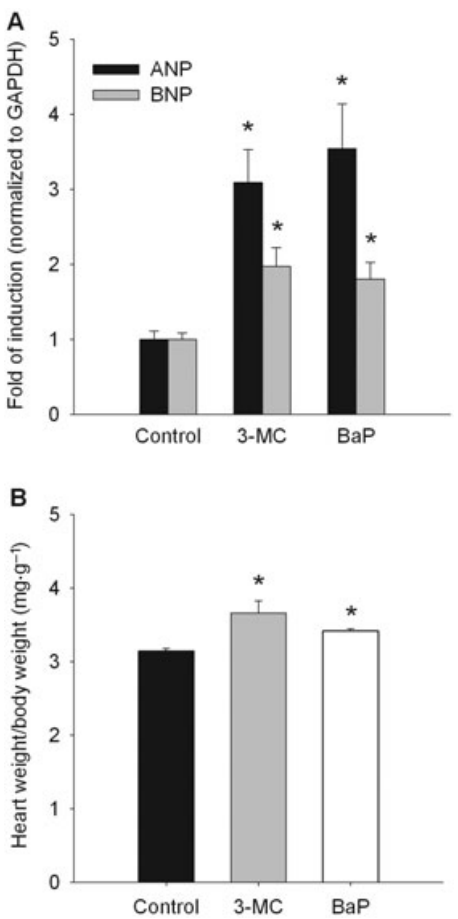

Figure 1 Effect of 3-methylcholanthrene (3-MC) and benzo(a)pyrene (BaP) treatments on the hypertrophic markers. Sprague Dawley rats received daily injections of 3-MC (10 mg·kg^{-1}) and BaP (20 mg·kg^{-1}) for 7 days while weight-matched controls received the same volume of corn oil. (A) Gene expression of the hypertrophic markers, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) was determined in the heart. (B) Heart to body weight ratio (mg·g^{-1}) was determined for each animal after seven daily IP injections of 3-MC, BaP or corn oil. Results are presented as mean ± SEM ($n = 6$). *$P < 0.05$ compared with control.

Effect of 3-MC and BaP treatment on P450 gene expression
To examine the effect of 3-MC and BaP on the expression of several *P450* genes in the heart, total RNA was extracted from the heart of control, 3-MC- and BaP-treated rats. Thereafter, the expression of different genes was measured using reverse transcription followed by real time-PCR, as described in the methods.

Figure 2A shows the effect of 3-MC or BaP treatment on CYP1 family gene expression. Our results demonstrate that both 3-MC and BaP treatments caused significant induction of *CYP1A1* gene expression in the heart by about 160- and 150-fold respectively (Figure 2A). In addition, 3-MC or BaP treatment caused significant induction of CYP1B1 (Figure 2A).

With regard to the CYP2B subfamily, neither 3-MC nor BaP caused any significant change in the expression of CYP2B1 or CYP2B2 in the heart of treated rats (Figure 2B). Similarly, there was no change in *CYP2C11* and *CYP2C13* gene expression in the hearts of rats treated with either 3-MC or BaP (Figure 2C). On the other hand, both 3-MC and BaP treatments caused significant induction of CYP2E1 in the heart (Figure 2D), whereas CYP2J3 expression was not significantly altered by 3-MC or BaP.

3-MC caused a significant induction of CYP4A3 gene expression in the heart by fourfold. Nevertheless, CYP4A3 gene expression did not change significantly in the hearts of BaP-treated rats (Figure 3A). With regard to CYP4F1, there was no significant change in the gene expression in the hearts of both 3-MC- and BaP-treated rats compared with control. However, unlike CYP4F1, both 3-MC and BaP treatments caused induction of CYP4F4 in the heart (Figure 3A). Figure 3B shows the relative gene expression of CYP4F5 and CYP4F6. CYP4F5 was significantly induced in both 3-MC- and BaP-treated rats. Nevertheless, the same treatments did not cause any significant change in the relative gene expression of CYP4F6.

Effect of 3-MC and BaP treatment on sEH gene expression
The enzyme sEH is a major determinant of EET level; therefore, we determined the effect of 3-MC and BaP treatments on expression of the sEH gene. Total RNA was extracted from the

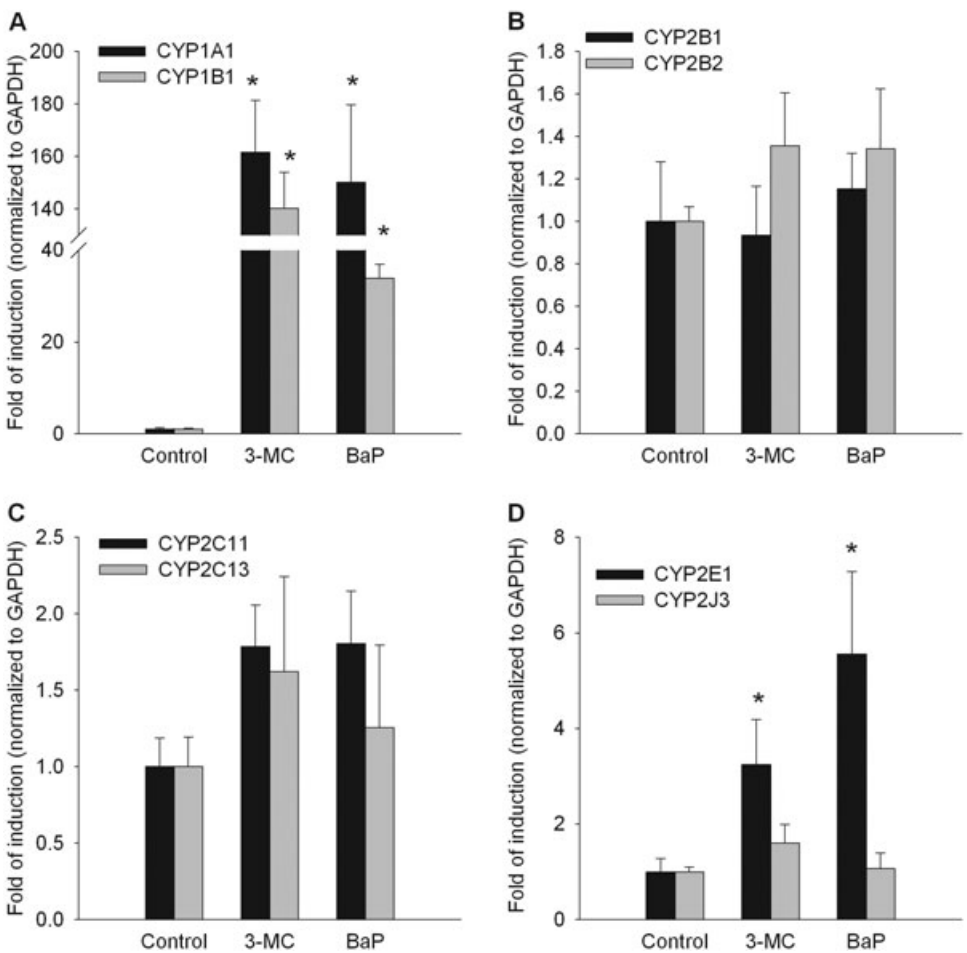

Figure 2 Effect of 3-methylcholanthrene (3-MC) and benzo(a)pyrene (BaP) treatment on gene expression of the CYP1 and CYP2 families. Sprague Dawley rats received daily injections of 3-MC (10 mg·kg^{-1}) and BaP (20 mg·kg^{-1}) for 7 days while weight-matched controls received the same volume of corn oil. Total RNA was isolated from the heart of control, 3-MC- and BaP-treated rats, and the relative gene expression of (A) CYP1A1 and CYP1B1, (B) CYP2B1 and CYP2B2, (C) CYP2C11 and CYP2C13 and (D) CYP2E1 and CYP2J3 was determined by real time-PCR. Results are presented as mean ± SEM ($n = 6$). *$P < 0.05$ compared with control.

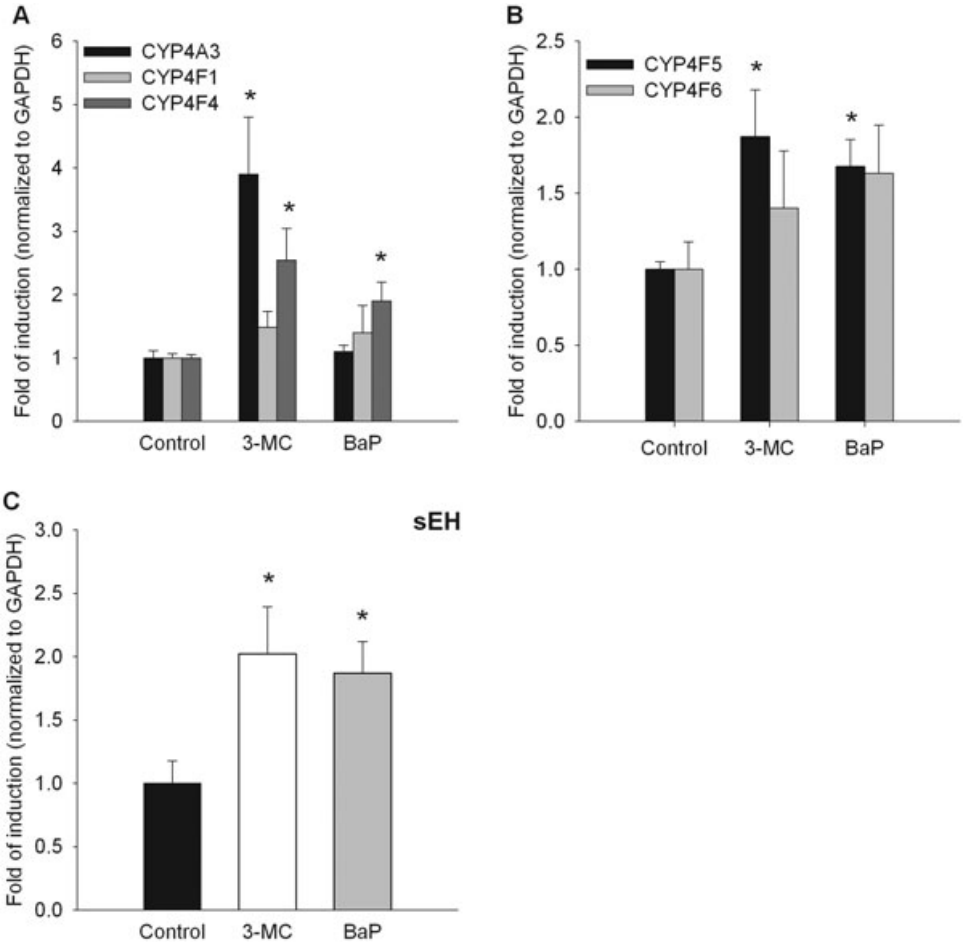

Figure 3 Effect of 3-methylcholanthrene (3-MC) and benzo(a)pyrene (BaP) treatment on gene expression of the CYP4 family and soluble epoxide hydrolase (sEH). Sprague Dawley rats received daily injections of 3-MC (10 mg·kg^{-1}) and BaP (20 mg·kg^{-1}) for 7 days while weight-matched controls received the same volume of corn oil. Total RNA was isolated from the heart of control, 3-MC- and BaP-treated rats, and the relative gene expression of (A) CYP4A3, CYP4F1 and CYP4F4, (B) CYP24F5 and CYP4F6 and (C) sEH was determined by real time-PCR. Results are presented as mean ± SEM (n = 6). *P < 0.05 compared with control.

heart of control, 3-MC- and BaP-treated rats. Thereafter, the expression of sEH gene was measured using reverse transcription followed by real time-PCR as described under *Methods*. 3-MC and BaP treatments caused a significant induction of sEH gene expression in the heart (Figure 3C).

Effect of 3-MC and BaP treatment on P450 and sEH protein expression

To investigate whether the induction in P450 and sEH gene expression by 3-MC and BaP is further translated to functional protein, microsomal protein was prepared from hearts of control, 3-MC- and BaP-treated rats. Thereafter, CYP1A1, CYP1B1, CYP2E1 and sEH protein levels were determined using Western blot analysis. The protein level of CYP1A1 was increased by treatment with 3-MC or BaP (Figure 4). Similarly, 3-MC or BaP treatment caused a significant induction of the protein level of CYP1B1 and CYP2E1. Moreover, sEH gene expression was found to be further translated to protein (Figure 4) as, 3-MC or BaP treatment increased sEH protein level. The level of CYP4A3 was below the detection level and, the antibodies for rat CYP4F were not commercially available.

Effect of 3-MC and BaP treatment on arachidonic acid metabolism

To investigate the effect of 3-MC and BaP treatment on the formation of arachidonic acid metabolites, heart microsomes from control, 3-MC- or BaP-treated rats were incubated with 50 μM arachidonic acid for 30 min. Thereafter, arachidonic acid metabolites were determined using LC-ESI-MS. In microsomes from hearts of 3-MC-treated rats, the formation of 5,6-, 8,9-, 11,12- and 14,15-EET were significantly lower than control (Figure 5A). In a similar manner, in BaP-treated rats, the levels of 5,6-, 8,9-, 11,12- and 14,15-EET were significantly lower than in control rats (Figure 5A).

We also measured levels of enzymatic hydroxylation of EETs products, dihydroxyeicosatrienoic acids (DHETs). As shown in Figure 5B, the formation of 5,6-DHET, 11,12-DHET and 14,15-DHET was inhibited in 3-MC-treated rats, compared with control. Similarly, in BaP-treated rats, the formation of these compounds was significantly lower than in control rats. On the other hand, levels of 8,9-DHET were not significantly altered in either 3-MC- or BaP-treated rats (Figure 5B).

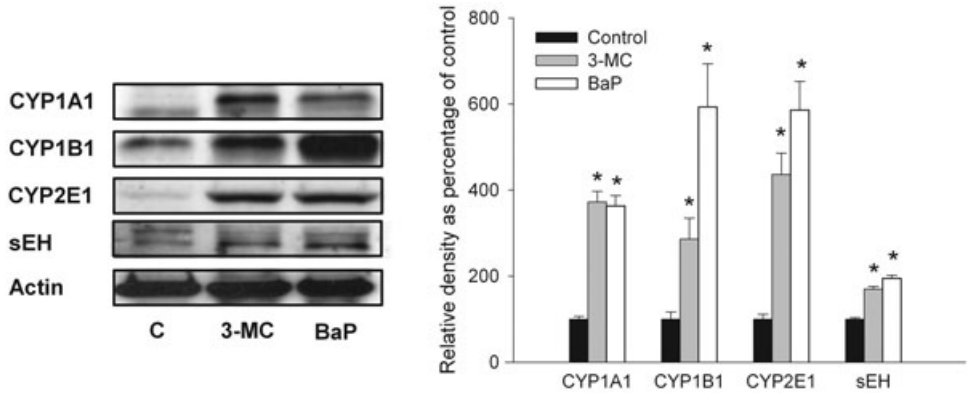

Figure 4 Effect of 3-methylcholanthrene (3-MC) and benzo(a)pyrene (BaP) treatment on the protein level of cytochrome P450 (P450) and soluble epoxide hydrolase (sEH). Sprague Dawley rats received daily injections of 3-MC (10 mg·kg^{-1}) and BaP (20 mg·kg^{-1}) for 7 days while weight-matched controls received the same volume of corn oil. Heart microsomal protein was isolated from the hearts of control, 3-MC- and BaP-treated rats; 20 μg of microsomal protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). CYP1A1, CYP1B1, CYP2E1 and sEH proteins were detected using the enhanced chemiluminescence method. This experiment was repeated three times; one representative result is shown. The graph represents the relative normalized amount of P450 and sEH protein (mean ± SE, $n = 3$), which was calculated by dividing the levels of P450 and sEH by the level of actin in the same sample, and the results are expressed as percentage of the control values taken as 100%. *$P < 0.05$ compared with control.

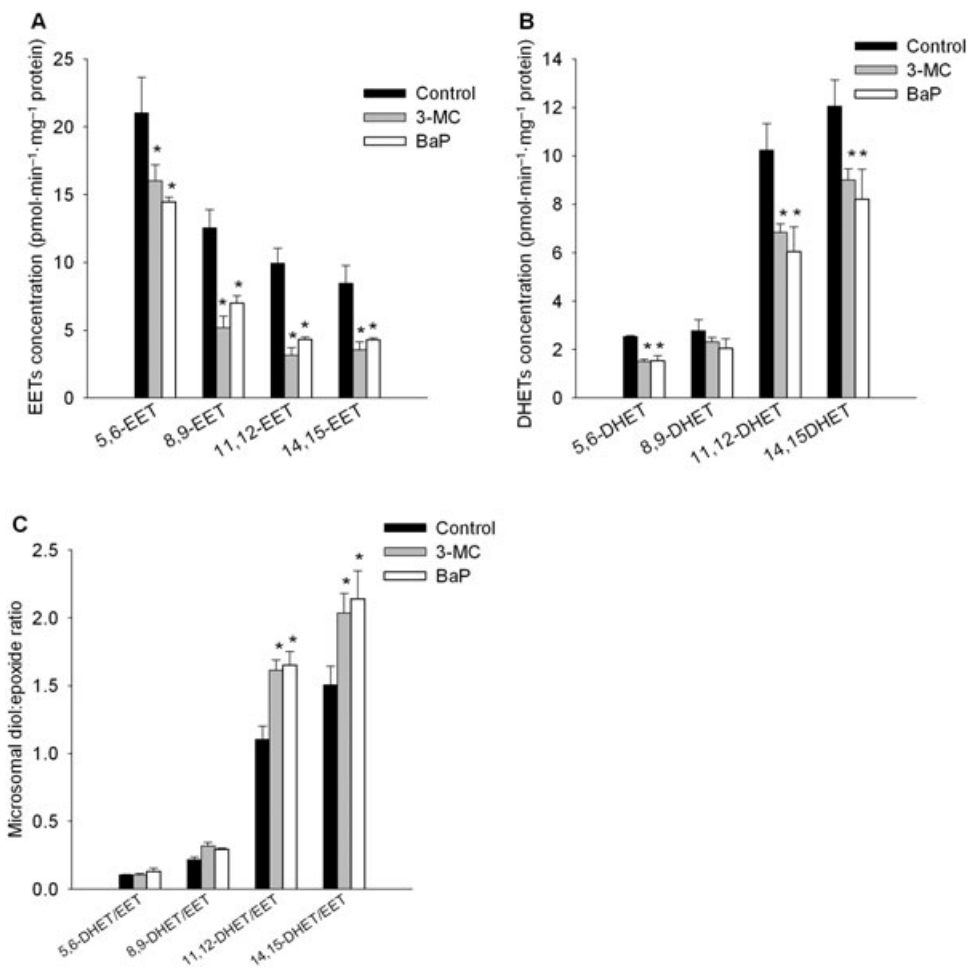

Figure 5 Effect of 3-methylcholanthrene (3-MC) and benzo(a)pyrene (BaP) treatment on (A) epoxyeicosatrienoic acids (EETs), (B) dihydroxyeicosatrienoic acids (DHETs) and (C) DHET : EET ratio. Heart microsomes from control, 3-MC- or BaP-treated rats were incubated with 50 μM arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice cold acetonitrile. EETs and DHETs were extracted twice by 1 mL ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into liquid chromatography-electron spray ionization-mass spectrometry for metabolite determination. Results are presented as mean ± SEM ($n = 5$). *$P < 0.05$ compared with control.

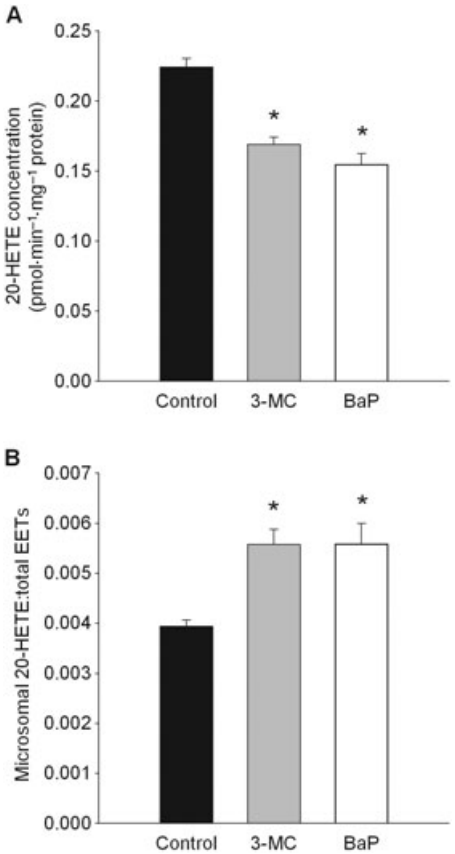

Figure 6 Effect of 3-methylcholanthrene (3-MC) and benzo(a)pyrene (BaP) treatment on (A) 20-hydroxyeicosatetraenoic acid (20-HETE) formation and (B) total 20-HETE : epoxyeicosatrienoic acid (EET) ratio. Heart microsomes of control, 3-MC- or BaP-treated rats were incubated with 50 μM arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice cold acetonitrile. EETs, DHETs and 20-HETE were extracted twice by 1 mL ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into liquid chromatography-electron spray ionization-mass spectrometry for metabolite determination. Results are presented as mean ± SEM ($n = 5$). *$P < 0.05$ compared with control.

To determine the epoxide hydrolase activity, the diol : epoxide ratio was calculated for all treatments and is represented in Figure 5C. In 3-MC-treated rats, there was a significant increase in the ratio of 8,9-, 11,12-, 14,15-DHET : EET. After treatment with BaP, these ratios were similarly increased. However the ratio of 5,6-DHET : EET was unchanged by treatment with either 3-MC or BaP (Figure 5C).

To determine the effect of 3-MC and BaP treatment on P450 ω-hydroxylase activity, we determined the formation of 20-HETE in microsomes from control, 3-MC- and BaP-treated rats. Both 3-MC and BaP treatments significantly decreased the 20-HETE formation, in comparison with the control group (Figure 6A).

Moreover, to determine the relative formation of 20-HETE compared with total EETs in both 3-MC and BaP treatments, we calculated the 20-HETE : total EET ratio. Both 3-MC and BaP treatments demonstrated significantly higher ratios of 20-HETE : EET, compared with control values (Figure 6B).

Effect of 3-MC and BaP treatment on total P450 content and HO-1 gene expression and activity

In order to examine whether the inhibition of arachidonic acid metabolite formation by P450 enzymes was due to inhibition of the P450s responsible for their formation, we determined the total P450 content in pooled microsomal fractions of rat hearts treated with either BaP or 3-MC. Our results showed that there was about a 20% decrease in the total P450 content in 3-MC- or BaP-treated rats (Figure 7A).

In an attempt to explain the decrease in the total P450 content, we determined the gene expression of HO-1 in both 3-MC- and BaP-treated rats. HO-1 was significantly induced in 3-MC- or BaP-treated rats (Figure 7B). These results are consistent with the measurement of HO activity that was similarly increased in 3-MC- and BaP-treated rats, compared with the control levels (Figure 7C).

Effect of BeP treatment on hypertrophic markers and heart to body weight ratio

In order to investigate whether the AhR activation is involved in the development of cardiac hypertrophy, we examined whether BeP, which is a poor ligand for the AhR, could induce cardiac hypertrophy. Our results showed that BeP did not alter the hypertrophic genes, ANP and BNP (Figure 8A), or the heart to body weight ratio (Figure 8B). In addition, BeP caused only a fourfold increase in *CYP1A1* gene expression (data not shown).

Effect of the ω-hydroxylase inhibitor, HET0016 on hypertrophic markers and the heart to body weight ratio

To determine the mechanism by which AhR ligands caused cardiac hypertrophy, we examined whether the inhibition of 20-HETE formation would confer cardioprotection. Our results clearly demonstrated that the ω-hydroxylase inhibitor, HET0016 prevented the BaP-mediated induction of ANP and BNP. In addition, HET0016 completely prevented the increase in heart to body weight ratio induced by BaP (Figure 8B).

Discussion

In the present study, we have demonstrated that the AhR ligands, 3-MC and BaP, caused cardiac hypertrophy in rats, manifested as significant induction of the hypertrophic markers, ANP and BNP as well as the increase of the heart to body weight ratio; both these variables have been linked to the development of cardiac hypertrophy (Shimoike *et al.*, 1997; Zordoky *et al.*, 2008). Our results are compatible with the earlier findings that AhR ligands such as TCDD induced cardiac hypertrophy and increase systemic blood pressure in adult male C57BL/6 mice through increasing the heart weight with concentric left ventricular hypertrophy (Kopf *et al.*, 2008).

In an attempt to better understand the mechanism by which AhR ligands cause cardiac hypertrophy, we assessed possible alterations in the gene expression of different *P450* genes including CYP1, CYP2 and CYP4 families. In the current study, we demonstrated that treatment with 3-MC or

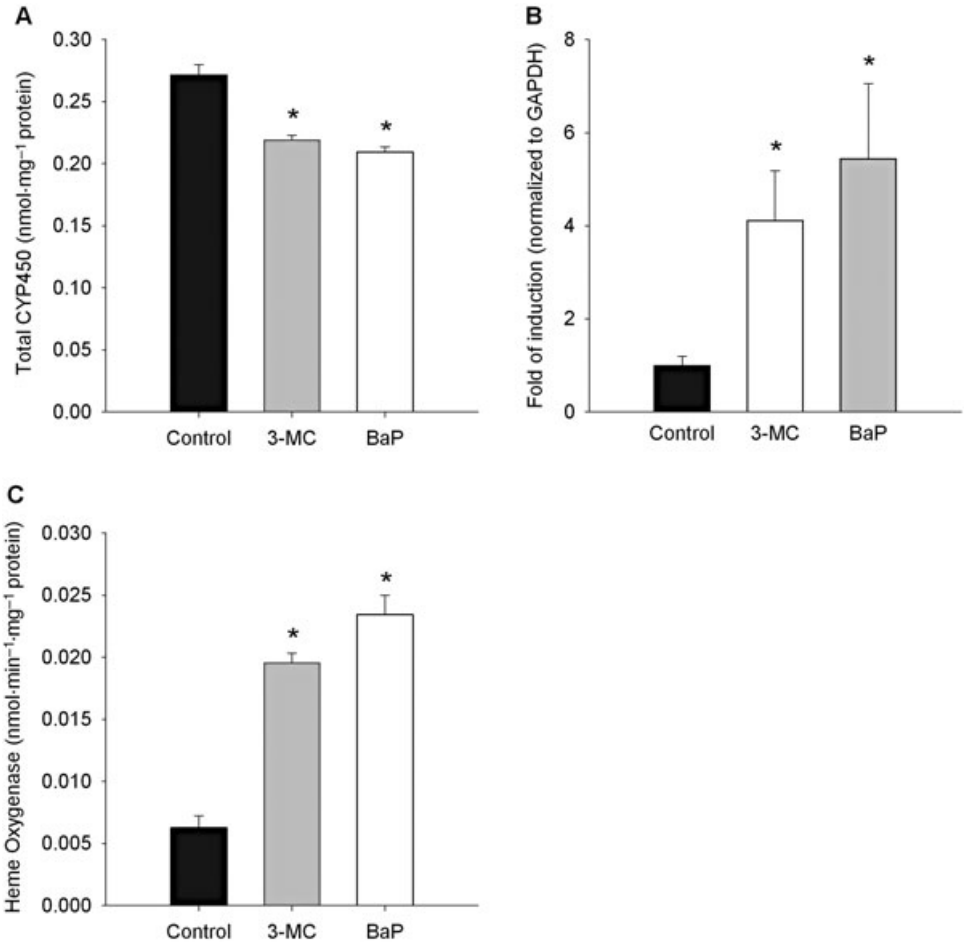

Figure 7 Effect of 3-methylcholanthrene (3-MC) and benzo(a)pyrene (BaP) treatment on (A) total cytochrome P450 (P450) content, (B) haem oxygenase-1 (HO-1) gene expression and (C) total HO activity in the heart. Sprague Dawley rats received daily injections of 3-MC (10 mg·kg^{-1}) and BaP (20 mg·kg^{-1}) for 7 days while weight-matched controls received the same volume of corn oil. (A) The total P450 content was performed on pooled microsomal fractions of six rat hearts and were measured spectrophotometrically using the reduced-CO spectral absorbance. Results are presented as mean ± SEM ($n = 4$). (B) Total RNA was isolated from the heart of control, 3-MC- and BaP-treated rats, and the relative gene expression of HO-1 was determined by real time-PCR. Results are presented as mean ± SEM ($n = 6$). (C) Total HO activity was assayed in pooled microsomal fractions of six rat hearts and was measured spectrophotometrically by determining the amount of bilirubin. Results are presented as mean ± SEM ($n = 4$). *$P < 0.05$ compared with control.

BaP significantly increased the gene expression of CYP1A1 and CYP1B1. The fact that 3-MC and BaP caused induction of the gene expression of CYP1A1 and CYP1B1 is consistent with previous reports demonstrating induction of CYP1A1 and CYP1B1 mRNA in mouse heart following BaP treatment (Shimada *et al.*, 2003). CYP1A1 has been shown to be involved in ω-terminal HETEs synthesis, whereas CYP1B1 can metabolize arachidonic acid to both mid chain HETEs and EETs (Choudhary *et al.*, 2004). These results confirm our proposal on the role of 3-MC and BaP in the development of cardiac hypertrophy through the induction of the P450-dependent arachidonic acid cascade. In this context, we have previously reported that CYP1A1 and CYP1B1 gene expression was induced in the hypertrophied heart of isoprenaline-treated rats (Zordoky *et al.*, 2008).

With regard to the CYP2 family, no significant change was observed for CYP2B1 and CYP2B2 in the hearts of 3-MC- and BaP-treated rats. The current results are in agreement with our previous data showing no significant alteration in the expression of these genes during isoprenaline-induced cardiac hypertrophy. On the other hand, CYP2E1 was found to be significantly induced in rat hearts treated with 3-MC and BaP. Arachidonic acid is metabolized by CYP2E1 to 18- and 19-HETEs (Laethem *et al.*, 1993), and CYP2E1 was found to be significantly induced in the heart, upon exposure to the AhR ligand (Sinal *et al.*, 1999).

Other members of the CYP2 family: CYP2C11, CYP2C13 and CYP2J3 gene expression were not altered by 3-MC and BaP treatments in rat hearts. Similar to the current results, we previously shown that CYP2J3 gene expression is not altered during isoprenaline-induced cardiac hypertrophy (Zordoky *et al.*, 2008). However, the expression of CYP2C11 was significantly lower in the hypertrophic heart than the control hearts (Zordoky *et al.*, 2008). In this context, it is important to mention that the CYP2C and CYP2J subfamilies are important epoxygenase enzymes that are involved in arachidonic acid metabolism leading to the formation of EETs (Jin *et al.*, 2006; Ng *et al.*, 2007).

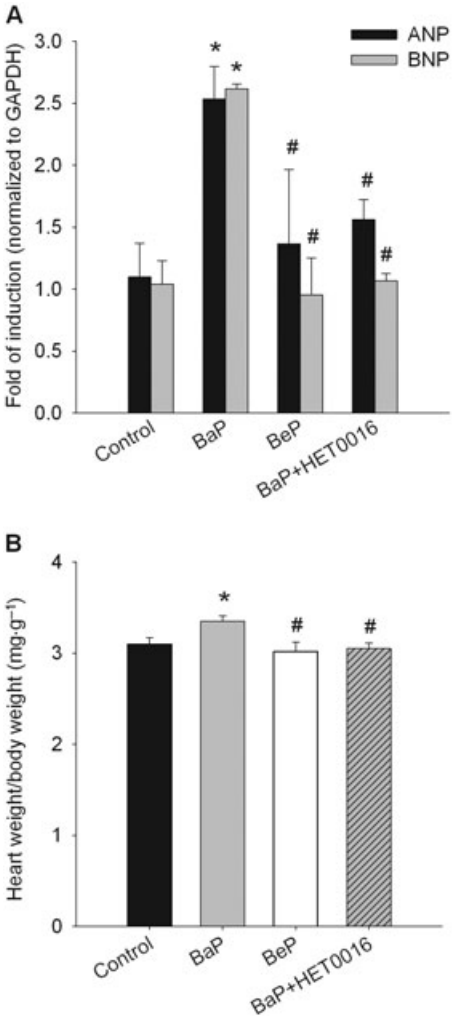

Figure 8 Effect of benzo(e)pyrene (BeP) and HET0016 treatments on the hypertrophic markers. Sprague Dawley rats received daily injections of benzo(a)pyrene (BaP) (20 mg·kg^{-1}), BeP (20 mg·kg^{-1}), combined treatment of BaP (20 mg·kg^{-1}) and HET0016 (0.1 mg·kg^{-1}) for 7 days while weight-matched controls received the same volume of corn oil and/or saline. (A) Gene expression of the hypertrophic markers, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) was determined in the heart. (B) Heart to body weight ratio (mg·g^{-1}) was determined for each animal after seven daily i.p. injections of BaP, BeP or combined treatment of BaP and HET0016 or vehicles. Results are presented as mean ± SEM (n = 6). *P < 0.05 compared with control, $^{\#}P$ < 0.05 compared with BaP.

Arachidonic acid is metabolized by P450 ω-hydroxylases leading to the formation of significant levels of 20-HETE, and CYP4A and CYP4F are the major enzymes that catalyse the ω-hydroxylation of arachidonic acid (Kroetz and Xu, 2005). In the current study, CYP4A3 was induced only in the heart of 3-MC-treated rats, while CYP4F4 and CYP4F5 were induced in both 3-MC- and BaP-treated rats. Moreover, 3-MC and BaP treatments did not alter the gene expression of CYP4F1 or CYP4F6 in the heart. We had previously demonstrated the induction of CYP4A3 in another model of the hypertrophic heart (Zordoky *et al.*, 2008).

In the current study, we demonstrated for the first time the capability of 3-MC and BaP to induce sEH gene expression in rat hearts, comparable to that found in isoprenaline-induced cardiac hypertrophy (Zordoky *et al.*, 2008), and in spontaneously hypertensive rats with heart failure (Monti *et al.*, 2008). The sEH enzyme is a crucial determinant of the levels of EETs because it catalyses the conversion of EETs to DHETs, thus abolishing their biological activity (Jin *et al.*, 2006).

The active translation of P450 and sEH mRNA levels into functional protein was also examined in the current study. Our results show that the protein level of CYP1A1, CYP1B1, CYP2E1 and sEH were significantly increased in heart microsome from 3-MC- and BaP-treated rats. This confirmed the translation of the P450 and sEH mRNA levels to active proteins.

In order to investigate whether alterations of P450 gene expression and protein levels induced by 3-MC and BaP would alter arachidonic acid metabolism, we incubated heart microsomes from control, 3-MC- or BaP-treated rats with arachidonic acid. Thereafter, arachidonic acid metabolites formations were determined by LC-ESI-MS. Our results showed a significant decrease in 5,6-, 8,9-, 11,12-, 14,15-EET formation in microsomes from both 3-MC- or BaP-treated rats, in comparison with control. Other AhR ligands, including TCDD, were found to decrease EETs formation in liver microsomes of rats (Capdevila *et al.*, 1990), mice (Lee *et al.*, 1998), rabbits (Oliw *et al.*, 1982), guinea pigs (McCallum *et al.*, 1996) and chicks (Gilday *et al.*, 1998).

The decrease in EET formation was accompanied by a significant decrease in 5,6-, 11,12-, 14,15-DHET formation. Although both EETs and DHETs were significantly decreased, the decrease in EET formation was much higher than that of DHETs. By calculating the diol : epoxide ratio, we found a significant increase in 8,9-, 11,12-, 14,15-DHET : EET ratios in 3-MC- and BaP-treated rats. The increase in DHETs : EETs ratio reflects the increase in sEH activity that was confirmed by the induction of sEH gene expression and protein level in the heart. Although, EET formation was significantly decreased during 3-MC and BaP treatment, no significant alteration in CYP2C11, CYP2C13 and CYP2J3 were observed. These results suggest that the decrease in EET may be attributed to higher activity of sEH and/or decrease in total P450 content.

Furthermore, our results demonstrated that 20-HETE formation was significantly lower in heart microsomes of 3-MC- and BaP-treated rats. It had been previously reported that the AhR ligand, TCDD, suppresses 20-HETE formation (Diani-Moore *et al.*, 2006). Moreover, 20-HETE formation was decreased in rat and mouse liver upon treatment with TCDD and other AhR ligands (Capdevila *et al.*, 1990; McCallum *et al.*, 1996; Lee *et al.*, 1998). In the current study, the 20-HETE : total EETs ratios were significantly higher in 3-MC- and BaP-treated rats, suggesting that the formation of 20-HETE is higher than that of total EETs. The relative increase in 20-HETE formation could be attributed to the increased expression of CYP1A1, CYP1B1, CYP4F4 and CYP4F5 in 3-MC- and BaP-treated rats.

To explain the mechanism by which 3-MC and BaP decreased the formation of EETs, DHETs as well as 20-HETE, despite the induction of several CYP enzymes at the mRNA and protein levels, we measured the total P450 content. Our results demonstrated that both 3-MC and BaP significantly decreased the total P450 content. To investigate the

mechanism by which 3-MC and BaP decrease the total P450 content we measured the HO-1 mRNA level as well as total HO-1 activity. In the current study, HO-1 gene expression and catalytic activity were significantly induced by 3-MC and BaP treatments. HO-1 is the enzyme catalysing oxidative degradation of the haem component of both P450 epoxygenases and P450 ω-hydroxylases, leading to significant inhibition of the catalytic activities of these enzymes and subsequently the decrease of EETs and 20-HETE formation respectively.

In order to confirm the cause–effect relationship between the AhR activation and the development of cardiac hypertrophy, we investigated whether BeP, an isomer of BaP that is a poor ligand for the AhR, could induce cardiac hypertrophy (Sharma *et al*., 2008). In the current study, BeP treatment caused a fourfold induction of *CYP1A1* gene expression that was much lower than the 150-fold induction caused by BaP. Interestingly, BeP did not cause any significant induction of the hypertrophic markers or the heart to body weight ratio. These data confirm the involvement of the AhR in the development of cardiac hypertrophy by AhR ligands. However, the mechanism by which this AhR activation leads to cardiac hypertrophy is yet to be elucidated. In the current study, we have demonstrated a relative increase in the formation of 20-HETE over the cardioprotective EETs. Increased 20-HETE levels have been linked to the development and/or progression of cardiac hypertrophy (Zordoky *et al*., 2008). Therefore, we used the ω-hydroxylase inhibitor, HET0016, to inhibit the formation of 20-HETE in BaP-treated rats. Interestingly, HET0016 treatment significantly reversed the BaP-induced cardiac hypertrophy. These data support the role of 20-HETE in AhR ligands-induced cardiac hypertrophy.

In conclusion, this paper provides mechanistic explanations for the role of the AhR ligands, 3-MC and BaP, in the development of cardiac hypertrophy. 3-MC and BaP induce cardiac hypertrophy by increasing the ratio of DHETs : EETs and/or the ratio of 20-HETE : EETs. In addition, our results suggest that 3-MC and BaP alter arachidonic acid metabolism by the induction of several CYP enzymes, increasing sEH activity and/or inhibiting the total P450 content through HO-1.

Acknowledgements

This work was supported by a grant from the Heart and Stroke Foundation of Alberta, NWT, and Nunavut to AOSE. MEA and BNMZ are the recipients of Egyptian Government Scholarships. The authors are grateful to Dr Vishwa Somayaji for his excellent technical assistance with LC-ESI-MS.

Conflict of interests

There are no conflicts of interests.

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