The atherogen 3-methylcholanthrene induces multiple DNA adducts in mouse aortic smooth muscle cells: role of cytochrome P4501B1

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Abstract

Objective: 3-Methylcholanthrene (MC), a polycyclic aromatic hydrocarbon, induces atherogenesis in mice fed an atherogenic diet. In this study, we tested the hypothesis that MC would induce DNA adducts in mouse aortic smooth muscle cells (SMCs) and that cytochrome P4501B1 (CYP1B1) plays an important role in the activation of MC to genotoxic intermediates. Methods: Cultured SMCs were treated with MC or the vehicle dimethyl sulfoxide (DMSO), and DNA was isolated after 24 h. In some experiments, the cells were pre-treated with the CYP1B1 inhibitor 1-ethynylpyrene (EP) prior to exposure to MC. DNA adducts were determined by the \textsuperscript{32}P-postlabeling assay. Aryl hydrocarbon hydroxylase assay was measured by fluorimetry. Results: MC induced formation of 12 DNA adducts that were not observed in DMSO-treated cells. DNA adduct formation was dose-dependent, with maximum response observed at 3 \textmu M. Pre-treatment of cells with EP dramatically suppressed DNA adduct formation by MC. MC treatment caused induction of CYP1B1, but not CYP1A1. Conclusion: The induction of high levels of multiple DNA adducts in SMCs by MC suggests that SMCs have a functional enzymatic machinery capable of metabolically activating MC to genotoxic metabolites. The significant inhibition by EP of MC-induced DNA adduct formation indicated that CYP1B1 was the primary CYP enzyme responsible for formation of genotoxic metabolites that may play a role in the induction of atherosclerosis by MC. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Atherosclerosis, a complex and chronic disease process involving elastic and muscular arteries, is the leading cause of death in the US. A preponderance of evidence suggests that cigarette smoking is potentially the most remedial contributor to cardiovascular mortality and morbidity [1,2]. Polycyclic aromatic hydrocarbons (PAHs) are important constituents of cigarette smoke, and animal and human studies have suggested that PAHs may be involved in the etiology of atherosclerosis associated with exposure to cigarette smoking [3–9]. However, the molecular mechanisms of vascular damage by PAHs are not understood. 3-Methylcholanthrene (MC), a potent PAH carcinogen that induces tumors in a variety of organs in experimental animals, is also a potent atherogen in animals. Penn and Snyder [5] have found that MC administration to cockerels enhances atherosclerosis by 8–16-fold. In addition, MC initiates atherosclerosis in several mouse strains, with Ah-responsive mice being more susceptible to atherosclerosis than Ah-non-responsive mice [6,7]. The Ah-responsiveness refers to the inducibility by PAHs such as MC and benzo[\textit{a}]pyrene (BP) of cytochrome P4501A1 (CYP1A1) [10], which is regulated by the Ah receptor (AHR). The catalytic activities of CYP1A1 are frequently determined by measuring the activity of aryl hydrocarbon hydroxylase (AHH), which catalyzes the conversion of BP to 3-OH-BP [11]. The induction of CYP1A1 involves binding of ligand to the AHR, followed by nuclear translocation and complexing of the ligand–AHR with the AHR nuclear trans-

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locator (ARNT). The ligand–AHR–ARNT complex interacts with specific regulatory elements, i.e., Ah response elements (AhREs) that are located in multiple copies on the CYP1A promoter, eventually leading to augmentation of gene expression [12].

The parent PAHs by themselves are non-toxic; metabolic activation of PAHs by CYP1A1/1A2 leads to the production of reactive metabolites capable of covalently binding to DNA, a key event in the initiation of carcinogenesis and/or atherogenesis [13]. While the liver CYP enzymes play a major role in the PAH metabolism, leading to transport of the metabolites to target organs, extra-hepatic tissues are also known to contain the CYP enzymes that can activate PAHs [14]. In fact, PAH–DNA adduct formation has been demonstrated in several human cancer cell lines and organ explant cultures of extra-hepatic origin [15,16]. However, PAH activation leading to DNA adduct formation in cultured smooth muscle cells (SMCs) of aorta, the target organ for the development of atherosclerosis, has not been demonstrated. Unregulated proliferation of vascular SMCs can be mimicked in vitro in the absence of endothelial cells, macrophages, or other non-cellular factors, suggesting that vascular SMCs play an important role in chemical-induced atherogenesis [17]. Our working hypothesis is that genome alterations involving mutational changes, possibly through DNA adduct formation, in vascular SMCs contribute significantly to atherogenesis.

Several lines of evidence suggest a close parallelism between carcinogenesis and atherogenesis, with both processes involving key steps of initiation and promotion [4,18]. The presence of PAH–DNA adducts in atherosclerotic lesions of humans suggest that DNA adducts contribute to the development of atherosclerosis [8,9]. Although CYP enzymes are known to bioactivate PAHs to DNA-binding derivatives, little progress has been made in understanding the relationship between CYP-mediated PAH metabolic activation and DNA adduct formation in vascular tissue. Evidence to date has shown that BP induces CYP1A1 in rat aortic tissues [19], while MC administration to rabbits enhances formation of phenolic metabolites in aortic homogenates, further suggesting induction of CYP1A1 [20]. While CYP1A1 has been localized in both endothelial cells and SMCs of aorta [21,22], CYP1A1 is under negative regulation in adult quail aortic SMCs [23]. Thus, it appears that other CYP isoforms contribute to PAH metabolism in SMCs.

A number of CYP isoforms are expressed within the vascular wall, including CYP1B1, whose activity can also be determined by measuring AHH [24]. This isoform is preferentially expressed in SMCs [25], as opposed to vascular endothelium, and is co-expressed with CYP1A1 in several extra-hepatic tissues [26]. CYP1B1, which participates in PAH metabolic activation to oxidative intermediates and carcinogenic precursors [27–30], has been implicated in atherogenesis by PAHs, which are potent inducers of the enzyme [31]. On the basis of this, we tested the hypothesis that MC can induce DNA adducts in mouse aortic SMCs and that CYP1B1 plays an important role in the activation of MC to genotoxic metabolites.

Because AHH activity is catalyzed by CYP1A1 and CYP1B1 [23], we used isozyme-selective inhibitors that would discriminate between the two isoforms. To this end, we used (i) ellipticine that selectively inhibits CYP1A1 [32]; (ii) α-naphthoflavone (α-NF) that inhibits both CYP1A1 [33] and CYP1B1 [34,35]; and (iii) 1-ethylpyrene (EP), which is specific for CYP1B1 [35,36], to identify the CYP1 isoform in the vascular SMCs that is responsible for the metabolic activation of MC.

2. Methods

2.1. Chemicals

MC and BP (98% purity) were obtained from Sigma (St. Louis, MO, USA). EP was a kind gift from Dr. William Alworth (Department of Chemistry, Tulane University, New Orleans, LA, USA). Monoclonal antibody against CYP1A1 was a gift from Dr. Paul E. Thomas (Rutgers University, New Brunswick, NJ, USA). Polyclonal antibodies to CYP1B1 were purchased from Gentest (Woburn, MA, USA). All other chemicals were purchased from Sigma unless otherwise noted. BP, MC, EP, ellipticine, and α-naphthoflavone (α-NF) stock solutions were prepared in dimethyl sulfoxide (DMSO).

2.2. Cell culture

Primary cultures of vascular SMCs were isolated from female C57BL/6 mouse aorta and maintained under standard conditions as described by Ramos and Cox [37]. Female mice were used because the development of mouse model(s) of atherogenesis by MC was based on experiments conducted in female mice [6,7]. The investigation conforms with the Guide for Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Cells were grown in Medium 199 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA), 2 mM glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B (Gibco). Subcultures were prepared by trypsinization (Gibco) of subconfluent primary cultures. Cells were seeded at 100 cells/mm² onto 100 mm plates for AHH assays, and 75 cells/mm² onto 150 mm plates for postlabeling experiments, cells were allowed to acclimate for 48 h, then challenged with MC, BP, EP, ellipticine or α-NF for the time periods specified in the figure legends. Concentrations of DMSO in all experiments were <0.15%.
The rationale for using these concentrations was that DMSO did not produce any effect on vascular SMCs.

2.3. Aryl hydrocarbon hydroxylase activity

The methodology described by Nebert and Gelboin [38] with modifications, was used to process samples for measurement of AHH activity. In brief, cells were treated in triplicate with BP, ellipticine, α-NF, or EP. In preliminary dose-range finding studies, cells were treated for 1 and 24 h with ellipticine (0.001–0.1 nM), α-NF (1–100 nM) or EP (1–1000 nM). For metabolism inhibition studies, cells were treated with the CYP inhibitors ellipticine (0.1 nM), α-NF (10 nM), or EP (1000 nM) for 1 h prior to treatment with BP (3 μM) for 24 h. Cells were washed with 1 ml ice-cold Tris–sucrose buffer (0.05 M Tris, 0.2 M sucrose, pH 8.0), scraped from the substrate and centrifuged (1100 rpm, 5 min, 4 °C). Supernatants were decanted and the pellets resuspended in ice-cold Tris–sucrose buffer. One-third of the sample was combined with 0.1 M HEPES (pH 8.0), 0.4 mM NADPH (in 1% sodium hydroxycarbonate) in a borosilicate tube and incubated at 37 °C for 2 min, followed by addition of BP (3 μM in MeOH), protected from light and further incubated at 37 °C for 15 min. The reaction was stopped with ice-cold acetone and hexane, vortexed and the organic (top) layer removed. To the organic layer, 1 M NaOH was added, vortexed and the aqueous (bottom) layer removed for analysis. 3-OH BP standards were prepared in 1 M NaOH and read using a spectrophotometer prior to analysis of samples (396 nm excitation, 522 nm emission). The initial cell sample in Tris–sucrose buffer was analyzed for total protein concentration by the method of Bradford [39].

2.4. Western blotting

CYP1A1 and 1B1 protein expression in the vascular SMCs was determined by Western blotting using CYP1A1 and 1B1-specific antibodies. The cell pellets described above were used as the protein source. Procedures for Western analysis have been described previously [40].

2.5. Cell isolation for 32P-postlabeling

Vascular SMCs were seeded at 75 cells/mm² in three separate 150 mm culture plates per treatment to generate ~1×10⁷ cells. Cells were treated with DMSO (control) or (0.03, 0.3, or 3 μM) MC for 24 h. Some cells were treated with EP (1 μM) alone or pretreated with EP (1 μM; 1 h) prior to MC treatment. At the end of treatment, cells were harvested by trypsinization, centrifuged (1100 rpm, 5 min), resuspended and counted in a hemocytometer. Cells were then pelleted (1100 rpm, 5 min), resuspended in 1 ml TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.65), and stored at −20 °C prior to analysis.

2.6. DNA isolation and 32P-postlabeling

DNA was isolated as reported previously [41]. The nuclease-P1-enhanced version of the 32P-postlabeling assay for MC–DNA adducts was performed as reported previously [41–43]. Briefly, DNA (10 μg) was digested with micrococcal nuclease (MN) (0.04 U/ml) and spleen phosphodiesterase (SPD; 0.4 μg/ml) at pH 6.0 and 37 °C for 3.5 h. The DNA was then treated with nuclease P1 (0.6 μg/ml) at pH 5.0 at 37 °C for 40 min followed by labeling with [γ-32P]ATP (4000 Ci/mmol) and T4 polynucleotide kinase (0.5 U/ml) at pH 9.5 and 37 °C for 30 min. The labeled products were separated by two-dimensional polyethyleneimine (PEI)-cellulose thin-layer chromatography (2D-PEI-TLC) [43]. The 2D maps were exposed to autoradiography with intensifying screens at a typical exposure of 16 h at −80 °C. The adduct spots were quantified by scintillation counting and expressed as relative adduct labeling (RAL) values [42]. In order to quantify spots that were not well separated, we subjected the 32P-postlabeled TLC maps to shorter autoradiographic exposures (4–6 h), which showed clearer demarcation of individual spots, and then used these films as templates for quantification.

3. Results

Exposure of SMCs to 0.3 μM MC gave rise to 12 32P-postlabeled DNA adduct spots that were not observed in cells treated with the vehicle DMSO (Fig. 1). In cells treated with 0.03 μM MC, adducts 1,7,10, and 12 were not formed (not shown). In SMCs exposed to 3 μM, the intensities of all adduct spots increased. Quantitative analyses revealed that adduct formation was dose-dependent, with total adduct RAL values increasing from 2.62×10⁵ for 0.03 μM dose to 211.95×10⁵ for the 3 μM dose (Table 1). The most marked increase in adduct values at the highest dose was observed for spots 1, 2, 5, 6, 7, and 9, which displayed 10–15-fold induction compared to cells treated with a 10-fold lower dose (Table 1). Treatment with EP, a specific CYP1B1 inhibitor, followed by MC, resulted in strong inhibition (~90%) of total adducts levels (Fig. 2; Table 2). There were no qualitative changes in MC–DNA adduct profiles in cells pre-treated with EP (Fig. 2); however, formation of all individual adducts was suppressed (25–95%) (Table 2) by EP. EP itself did not induce any adducts (Fig. 2). While adduct patterns were quite reproducible in repeat experiments, we did notice some variation in levels of MC–DNA adducts when quantitative data from independent experiments (Tables 1 and 2) were compared. This was probably due to a combination of inter-experimental variability in cell treatments, DNA isolation, and 32P-postlabeling.

We tested three agents for their ability to inhibit AHH activity, which is catalyzed by CYP1A1 as well as
CYP1B1 [23]. The concentrations used for each inhibitor were based on pilot studies, and concentration ranges of 0.001–0.1, 1–100, and 10–1000 nM were found to be optimal for ellipticine, α-NF, and EP, respectively. Ellipticine (Fig. 3A) did not significantly inactivate enzyme activity. However, α-NF (Fig. 3B) as well as EP (Fig. 3C) caused a modest inhibition of the enzyme activity. We then tested the ability of each of these agents to inhibit aryl hydrocarbon inducible activities, with BP as the AHH inducer, since, like MC, it is a ligand for the AHR and SMCs respond well to induction by BP. As shown in Fig. 4, BP (3 μM) treatment of SMCs caused marked induction of AHH activity. Pre-treatment of cells with ellipticine or α-NF, at the indicated concentrations, followed by BP challenge, did not modulate AHH activity. However, EP pretreatment dramatically inhibited hydrocarbon-inducible AHH activity (Fig. 4).

In order to confirm that MC induced CYP1B1, but not CYP1A1, in vascular SMCs, cells exposed to MC were subjected to Western analyses. As shown in Fig. 5A, uninduced cells showed basal expression of CYP1B1, and MC (0.03 μM) treatment led to induction of CYP1B1. However, at higher MC concentrations (0.3–3 μM), the induction of CYP1B1 was not very pronounced (Fig. 5A). In cells pre-treated with EP (1 μM), followed by MC (0.3 μM), CYP1B1 expression was elevated, compared to those treated with MC (0.3 μM) alone (Fig. 5A).

In contrast, CYP1A1 was not detectable in uninduced cells or in SMCs treated with MC (Fig. 5B). In order to ensure that we are able to detect CYP1A1 in our experimental conditions, as a positive control we simultaneously performed Western analysis of microsomal proteins (5 μg) from livers of rats treated with MC [40], which is known to cause marked induction of CYP1A1. A very intense band corresponding to 54 kDa protein (CYP1A1) was detected (Fig. 5B), strongly suggesting that the experimental conditions we employed were optimal for detecting CYP1A1.

4. Discussion

The central hypothesis of this study is that aortic SMCs possess the capability to bioactivate PAHs such as MC to genotoxic intermediates and that CYP1B1 plays a major role in the metabolic activation of MC. The observation (Fig. 1) showing formation of multiple DNA adducts in DNA of SMCs exposed to MC indicates that MC is a potent genotoxic agent in these cells and that the individual adducts are derived from interaction of different ultimate

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**Table 1**

<table>
<thead>
<tr>
<th>Adduct No.</th>
<th>MC (0.03 μM)</th>
<th>MC (0.3 μM)</th>
<th>MC (3 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N.D.</td>
<td>4.5±0.05</td>
<td>55.90±6.7</td>
</tr>
<tr>
<td>2</td>
<td>0.57±0.06</td>
<td>1.5±0.001</td>
<td>26.80±3.1</td>
</tr>
<tr>
<td>3</td>
<td>0.38±0.04</td>
<td>4.3±0.05</td>
<td>20.95±2.2</td>
</tr>
<tr>
<td>4</td>
<td>N.D.</td>
<td>4.9±0.06</td>
<td>4.8±0.51</td>
</tr>
<tr>
<td>5</td>
<td>0.24±0.06</td>
<td>0.89±0.09</td>
<td>7.2±0.55</td>
</tr>
<tr>
<td>6</td>
<td>0.56±0.07</td>
<td>6.2±0.07</td>
<td>56.1±6.2</td>
</tr>
<tr>
<td>7</td>
<td>N.D.</td>
<td>0.25±0.003</td>
<td>4.4±0.50</td>
</tr>
<tr>
<td>8</td>
<td>0.28±0.03</td>
<td>6.0±0.07</td>
<td>13.8±1.42</td>
</tr>
<tr>
<td>9</td>
<td>0.17±0.009</td>
<td>1.1±0.002</td>
<td>11.1±1.22</td>
</tr>
<tr>
<td>10</td>
<td>N.D.</td>
<td>1.0±0.12</td>
<td>6.3±0.56</td>
</tr>
<tr>
<td>11</td>
<td>0.42±0.05</td>
<td>2.1±0.23</td>
<td>2.3±0.31</td>
</tr>
<tr>
<td>12</td>
<td>N.D.</td>
<td>3.9±0.40</td>
<td>2.3±0.28</td>
</tr>
</tbody>
</table>

Adduct values represent the means of three independent experiments and are expressed as mean±S.E. of RAL×10³. MC treatment of cells, DNA isolation, and ³²P-postlabeling were carried out as described in Methods.
genotoxic intermediates with DNA at different sites [44]. Individual MC–DNA adducts have not been structurally characterized in SMCs or other cell types, but Osborne et al. [45] have reported that formation of syn- and anti-9,10-diol epoxides as ultimate carcinogenic metabolites of MC in V79 cells. We reported previously [41] that MC administration to rats leads to production of multiple DNA adducts in liver and other tissues. DNA adduct formation represents a key event in initiation of carcinogenesis [13], and, if unrepaired, could lead to gene mutations and development of tumors. Because there is a parallelism between carcinogenesis and atherogenesis [18], it is likely that DNA adduct formation in SMCs contributes to atherogenesis by PAHs. In fact, DNA synthesis and DNA repair in SMCs treated with PAHs has recently been reported [46].

The formation of DNA adducts in SMCs indicates the presence in these cells of a functionally active enzymatic system that activates PAHs. Because CYP1 enzymes play important roles in the metabolic activation of PAHs, we conducted experiments to determine the nature of CYP1 enzymes in SMCs. For this, we studied the effect of inhibitors of AHH activities and DNA adduct formation. As shown in Fig. 3A, ellipticine, a specific inhibitor for CYP1A1 [32], did not inhibit AHH activities in SMCs. This finding, in conjunction with the modest, but significant inhibition by EP (Fig. 3C), a specific CYP1B1 inhibitor [36], and α-NF (Fig. 3B), which also inhibits CYP1B1, suggests the CYP1B1 is the major CYP1 enzyme responsible for PAH metabolism in SMCs. The marked inhibition of BP-inducible AHH activity by EP (Fig. 4), further supports the hypothesis that CYP1B1 is the major PAH-inducible CYP enzyme present in SMCs.

The dramatic inhibition of MC–DNA adduct formation by EP lent further credence to the hypothesis that CYP1B1 played a pivotal role in the metabolic activation of MC to genotoxic metabolites. In fact, the most convincing evidence for the role of CYP1B1 is the Western blotting experiments showing induction of CYP1B1 (Fig. 5A), but not CYP1A1 (Fig. 5B), after treatment of SMCs with MC (0.03 μM). Surprisingly, the induction was not very pronounced when cells were exposed to higher MC concentrations (Fig. 5A). This could have been due to the fact that we analyzed CYP1B1 expression in the SMCs 24 h after treatment with MC, and by this time it is possible

Table 2
Effect of EP on MC–DNA adduct levels

<table>
<thead>
<tr>
<th>Adduct No.</th>
<th>MC (0.3 μM)</th>
<th>EP+MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.75±2.7</td>
<td>0.63±0.71</td>
</tr>
<tr>
<td>2</td>
<td>0.59±0.07</td>
<td>0.19±0.23</td>
</tr>
<tr>
<td>3</td>
<td>14.3±1.5</td>
<td>1.35±0.14</td>
</tr>
<tr>
<td>4</td>
<td>8.8±0.92</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>5</td>
<td>2.01±0.02</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>6</td>
<td>14.86±1.7</td>
<td>1.23±0.02</td>
</tr>
<tr>
<td>7</td>
<td>2.2±0.31</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>8</td>
<td>5.8±0.62</td>
<td>0.42±0.04</td>
</tr>
<tr>
<td>9</td>
<td>2.98±0.03</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>10</td>
<td>1.5±0.02</td>
<td>0.22±0.03</td>
</tr>
<tr>
<td>11</td>
<td>3.29±0.04</td>
<td>0.32±0.04</td>
</tr>
<tr>
<td>12</td>
<td>3.66±0.04</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>Total</td>
<td>71.74±7.6</td>
<td>5.3±0.72</td>
</tr>
</tbody>
</table>

Adduct values represent the means of three independent experiments and are expressed as mean±S.E. of RAL×10⁸. MC treatment of cells, DNA isolation, and ³²P-postlabeling were carried out as described in Methods.
Fig. 4. Effect of CYP inhibitors on arylhydrocarbon-inducible AHH activities. SMCs treated with DMSO, ellipticine (0.1 nM), α-NF (10 nM), or EP (1 μM) for 1 h, followed by exposure to DMSO alone or to BP (3 μM) for 24 h. AHH activity was estimated in SMCs as described in Methods. Values represent the means ± S.E. of three independent experiments. *, Statistically significant differences at P < 0.01.

[23] that CYP1A1 is under negative regulation in vascular SMCs.

The contribution of DNA adducts to the development of atherogenesis is not well understood. The mutation theory of atherogenesis [48] suggests that DNA adduct formation represents the initiation step of atherogenesis. While, there is close parallel between carcinogenesis and atherogenesis, the lack of mutations in the K-ras codon 12 and 13 in the DNA of SMCs isolated from abdominal aorta of human atherosclerotic lesions suggested that K-ras is not a candidate gene that contributes to atherogenesis [49], although mutations in this gene are observed in human cancers [50], and in lung tumors of mice exposed to MC [51]. Thus, DNA adducts may contribute to atherogenesis, and if so there may be differences in target genes that control the processes of carcinogenesis and atherogenesis.

One of our laboratories has demonstrated that c-Ha-ras activation by BP is dependent on the metabolism of PAHs by CYP and that this gene contributes to atherogenesis by BP [52]. In the light of these studies, it is tempting to speculate that genetic and epigenetic changes involving c-Ha-ras and other critical mutational targets contribute to atherosclerotic plaque formation. Further studies in this area are needed to study the specific mechanistic contributions of DNA adducts in PAH-induced atherogenesis.

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Fig. 5. Representative Western blot showing effect of MC on CYP11B1 (A) or CYP1A1 (B) protein expression in vascular SMCs. SMCs were treated with DMSO or MC at the indicated concentrations and Western blotting using CYP1A1 or 1B1 antibody was performed using total cellular protein (20 μg), as described in Methods. In order to study the effect of EP, cells were treated with 1 μM EP 1 h prior to treatment with MC (0.3 μM), and Western analysis was performed. As shown in the figure, basal and inducible CYP1B1 (A), but not CYP1A1 (B), was detectable. P.C., Positive control. A 5-μg amount of microsomal protein from livers of rats treated with MC was applied on the same gel in order to ensure that our experimental conditions were optimal for detecting CYP1A1. M, Molecular weight marker.

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