Constitutive and Inducible Expression of Cyp1a1 and Cyp1b1 in Vascular Smooth Muscle Cells
Role of the Ahr bHLH/PAS Transcription Factor

J. Kevin Kerzee, Kenneth S. Ramos

Abstract—Ahr is a ligand-activated bHLH/PAS transcription factor involved in cytochrome P450 (CYP) gene regulation and murine susceptibility to atherogenic stimuli. The present studies were conducted to examine constitutive and inducible expression of Cyp1a1 and Cyp1b1 in vascular smooth muscle cells (VSMCs) from Ahr+/+ and Ahr−/− mice. Cyp1a1 mRNA was not expressed constitutively in VSMCs irrespective of Ahr phenotype. Although Cyp1a1 was inducible in Ahr+/+ by 3 μmol/L benzo(a)pyrene, a known hydrocarbon inducer, the protein was uninducible. In contrast, Cyp1b1 mRNA and protein were expressed under constitutive and inducible conditions irrespective of Ahr phenotype or growth status. CYP-encoded aryl hydrocarbon hydroxylase activity was higher in Ahr−/− VSMCs under constitutive conditions and induced by benzo(a)pyrene in Ahr+/+ and Ahr−/− VSMCs. CYP expression was influenced by mitogenic status, because randomly cycling cells consistently exhibited higher levels than growth-arrested counterparts. Actinomycin D (2 μg/mL) or cycloheximide (10 μmol/L) did not inhibit constitutive or hydrocarbon-inducible aryl hydrocarbon hydroxylase activity in VSMCs. These data indicate that in murine VSMCs, expression of Cyp1a1 and Cyp1b1 is differentially influenced by Ahr phenotype and mitogenic status, with patterns that may dictate inherent susceptibility to atherogenic stimuli. (Circ Res. 2001;89:573-582.)

Key Words: Cyp1a1 ■ Cyp1b1 ■ vascular smooth muscle cells ■ aryl hydrocarbon receptor ■ atherogenesis

Cytochrome P450s (CYPs) are membrane-associated proteins that use molecular oxygen and reducing NADPH equivalents to catalyze oxidative, peroxidative, and reductive metabolism of endogenous and exogenous substrates. More than 400 distinct genes encoding CYP-associated activities have been cloned, but their relative expression exhibits remarkable tissue, gender, and developmental specificity. Members are assigned to a family on the basis of sequence homology, with >55% identity required for classification within the same subfamily. CYP expression is regulated at the transcriptional and posttranscriptional levels by a variety of environmental factors, including diet, steroids, drugs, and cigarette smoke.1 A detailed understanding of vascular CYP functions is pivotal in view of the central roles of CYP in bioactivation of arachidonic acid and environmental procarcinogens and steroid biogenesis.

Multiple CYP isoforms have been identified within the vascular wall, including CYP1A1, CYP1B1, CYP2J2, CYP2B6, and CYP3A.2 Vascular CYPs oxidize arachidonic acid to cis-epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET), mid-chain cis-trans-conjugated dienols (5-, 8-, 9-, 11-, 12-, and 15-HETE), or ω-terminal alcohols (16-, 17-, 18-, 19-, and 20-HETE).3−5 The ability of CYP2J2-derived EETs to inhibit leukocyte adhesion, reduce superoxide anion production, and attenuate lipid peroxidation induced by hypoxia-reoxygenation in human coronary endothelial cells6 indicates that CYPs can play a beneficial role in vascular tissue. Irizar and Ioannides7 have shown that dietary administration of 1% cholesterol for 8 weeks to rabbits increases aortic expression of CYP2B and CYP3A protein, but the consequences of this induction are not known. In contrast, vascular CYP1A1 and CYP1B1 catalyze oxygenation of hydrocarbon procarcinogens present in foods and smoke to reactive moieties that adduct cellular macromolecules in vascular smooth muscle cells (VSMCs) and induce oxidative stress.8 CYP1B1 has also been implicated in the formation of genotoxic catechol estrogens.9 The dichotomy of vascular actions among different members of the CYP superfamily requires examination of cell-specific patterns of expression at the mRNA and protein levels.

CYP1A1-encoded enzymes are expressed in vascular endothelium and smooth muscle, with considerably higher levels of activity present in endothelium.10 CYP1B1 is preferentially expressed in vascular smooth muscle, with little expression in vascular endothelium.10 Both of these genes encode proteins that catalyze conversion of hydrophobic substrates to hydrophilic metabolites. Cyp1a1 encodes ethoxyresorufin-O-deethylase (EROD), whereas both Cyp1a1
and Cyp1bl encode aryl hydrocarbon hydroxylase (AHH) activities. Several aromatic hydrocarbons, including benzanthracene, benzo(a)pyrene (BaP), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), induce Cyp1al and Cyp1bl mRNA and associated activities. Induction of Cyp1al by benzanthracene and BaP is considerably higher than TCDD, suggesting that this gene is activated through a mechanism preferential for polycyclic aromatic hydrocarbons and distinct from Cyp1al orthologs. Transcriptional activation of Cyp1al and Cyp1bl is believed to involve the aryl hydrocarbon receptor (Ahr), a ligand-activated transcription factor that binds food-derived indole derivatives and environmental planar aromatic hydrocarbons.

Ahr is a member of the large basic helix-loop-helix (bHLH) and PAS homology domain family of transcription factors that includes proteins involved in myoblast differentiation, such as MyoD, the cellular response to hypoxia, such as Arnt (Ahr nuclear translocator) and Hifα, the Drosophila neurogenic protein Sim (single-minded), and the Drosophila circadian rhythm protein Per (period). In response to agonist binding within the PAS domain, the cytosolic Ahr undergoes a conformational change, dissociates from two 90-kDa heat-shock proteins and an unknown 43-kDa protein, and translocates to the nucleus where it dimerizes with Arnt. The Ahr/Arnt heterodimer interacts with Ahr-responsive elements (AHREs) (5′−TNGCGTG−3′) upstream of target genes to activate or repress transcription of target genes. The ligand-activated Ahr regulates expression of several drug-metabolizing enzymes, including CYP1A1, CYP1A2, and CYP1B1, GST-A1 subunit, quinone oxidoreductase, and UDP-glucuronosyltransferase.

Ahr has been implicated in several disorders, including chloracne, immunosuppression, thymic atrophy, teratogenesis, cell-specific hyperplasia, certain types of malignancies, and atherosclerosis. Its role in atherogenesis was proposed on the basis of the observation that 3-methylcholanthrene itself is a ligand for the Ahr and a potent inducer of Cyp1al and Cyp1bl. More recent studies have shown a direct role for the Ahr in vascular development and differentiation, suggesting that Ahr and Ahr-interacting proteins play key roles in the regulation of vascular functions.

In VSMCs, expression of CYP1A1 and CYP1B1 may influence oxidative metabolism of exogenous and endogenous substrates and promote formation of reactive oxygen species and electrophilic intermediates. These effects may shift redox status and culminate in oxidative vascular injury. Evidence is presented in this study that Cyp1al and Cyp1bl are differentially regulated in VSMCs, with constitutive and inducible expression at the mRNA and protein level strongly influenced by Ahr phenotype and mitogenic status.

### Materials and Methods

#### Chemicals

BaP was obtained from Aldrich Chemical Co. Medium 199, antibiotic, and trypsin were purchased from Gibco. FBS was from Atlanta Biologicals. Nylon membranes were purchased from Amer sham. [α-32P]-dCTP was from New England Nuclear. High Prime random-primed labeling kit was purchased from Boehringer Mannheim. Tri-reagent was purchased from Molecular Research Center, Inc. Polymerase chain reaction (PCR) reagents were purchased from Perkin Elmer. CYP1A1 and CYP1B1 antibodies were from Gentest. All other chemicals were from Sigma Chemical Co.

#### Murine Aortic Smooth Muscle Cell Culture

The primary cell culture procedure was based on methodology described in detail previously.

#### Chemical Treatments

Stock solutions of BaP (40 mmol/L) and TCDD (10 μmol/L) were prepared in dimethyl sulfoxide (DMSO) and kept at −20°C in the dark. Cells were allowed to attach for 24 hours and then growth-arrested. The BaP concentrations tested were chosen on the basis of established gene-inducibility profiles. For reverse transcriptase (RT)-PCR, Northern and Western analyses, and AHH measurements, VSMCs were seeded in 100-mm culture dishes at a density of 150 cells/mm². VSMCs were growth-arrested by serum deprivation in Medium 199 containing 0.1% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/mL amphotericin B for 72 hours or allowed to randomly cycle in Medium 199 containing 10% FBS before BaP challenge. Final DMSO concentrations in the cultures never exceeded 0.075%.

#### Flow Cytometry

VSMCs were harvested and fixed with ice-cold 100% ethanol before staining with propidium iodide (PI) (20 mg/mL PI and 40 mg/mL RNase in PBS) for 30 minutes at room temperature.

#### Northern Blot Analysis

Total RNA was extracted using TRI-reagent according to manufacturer’s specifications as described by Chomczynski and Sacchi and modified. Cyp1al and Cyp1bl probes were directed at nucleotides (1682 to 2129 and 1038 to 1562) of the full-length mRNA, respectively.

#### Western Analysis

Total cellular protein was harvested in NETN in (mmol/L, NaCl 100, Tris 20, and EDTA 1; pH 8.0, 0.5%NP-40) and lysed by sonication. Twenty-five micrograms of protein were electrophoresed on a 7.5% polyacrylamide gel and transferred to a precoated polyvinylidene difluoride membrane. Western analysis was completed as described previously.

#### RT-PCR Analysis

Total RNA (200 ng) was combined with 5 mmol/L MgCl2, 1× reaction buffer, 1 mmol/L dNTPs, 1 U/μL RNase inhibitor, 2.5 U/μL reverse transcriptase, and 50 μmol/L oligo d(T) primer was reverse transcribed using a GenAmp RT-PCR kit (Perkin-Elmer). For PCR reaction, the RT reaction was combined with MgCl2 to a final concentration of 2 mmol/L, 1× reaction buffer, and 0.025 U/μL Taq polymerase and 0.4 μmol/L primers. The PCR products were separated on a 1.2% agarose gel in 1× TBE buffer and stained with ethidium bromide.

#### AHH Activity

VSMCs were processed for measurements of AHH activity as previously described. Protein concentration was determined by the method of Bradford.

#### EROD Activity

VSMCs were processed for measurements of EROD activity as previously described.
Statistics ANOVA was used to assess significance followed by Fisher’s LSD post hoc test. The 0.05 level of probability was accepted as significant. Values represent mean ± SEM.

Results To evaluate the role of Ahr in constitutive and inducible expression of Cyp1a1 and Cyp1b1, (aortic) VSMCs from Ahr<sup>+/+</sup> and Ahr<sup>−/−</sup> mice were challenged with DMSO (control) or 3 μmol/L BaP, a known hydrocarbon inducer. Steady-state mRNA levels were monitored in growth-arrested and randomly cycling cultures after addition of 10% FBS for various times. Serum restriction or hydrocarbon treatment did not compromise cellular viability or induce apoptosis under the experimental conditions examined (not shown).

Cyp1a1 mRNA was not constitutively expressed in growth-arrested or randomly cycling VSMCs of either phenotype (Figure 1). Ahr<sup>−/−</sup> VSMCs were refractory to hydrocarbon challenge, whereas treatment of Ahr<sup>+/+</sup> cells with BaP for up to 5 hours preferentially induced Cyp1a1 mRNA in randomly cycling cells (Figure 1). Thus, Cyp1a1 is influenced by Ahr phenotype and mitogenic status in VSMCs.

In contrast to Cyp1a1, Cyp1b1 mRNA was detected in both Ahr<sup>+/+</sup> and Ahr<sup>−/−</sup> VSMCs under serum-restricted (0.1% FBS) and randomly cycling conditions (10% FBS) (Figure 2A). The larger 5.2-Kb mRNA encodes for CYP1B1 protein, whereas the smaller 4.3-Kb product does not. Cyp1b1 mRNA levels were higher in Ahr<sup>+/+</sup> VSMCs compared with Ahr<sup>−/−</sup> counterparts. Long-term serum restriction reduced constitutive levels of Cyp1b1 mRNA in Ahr<sup>−/−</sup> VSMCs, indicating that this gene can be regulated by mitogenic status in an Ahr-independent manner. To additionally evaluate profiles of Cyp1b1 inducibility, growth-arrested cultures were challenged with 10% FBS to stimulate cell-cycle transit or allowed to cycle randomly in the presence of BaP or DMSO for 1, 3, and 5 hours (Figures 2B and 2C). Cyp1b1 mRNA levels were reduced in growth-arrested cultures relative to randomly cycling counterparts (compare Figures 2B and 2C) but inducible by BaP irrespective of growth conditions or Ahr phenotype. Randomly cycling cells of either phenotype were induced to a greater extent than growth-arrested cells, with inducible expression in growth-arrested Ahr<sup>−/−</sup> VSMCs greatly reduced relative to wild-type cells (Figure 2C). The kinetic profile of hydrocarbon inducibility in randomly cycling VSMCs was influenced by Ahr phenotype, because maximal induction in Ahr<sup>+/+</sup> was seen by 3 hours versus 5 hours in Ahr<sup>−/−</sup> cells. Next, the effects of Ahr antagonists on Cyp1b1 induction in growth-arrested Ahr<sup>+/+</sup> VSMCs challenged with BaP in the presence of 10% FBS for 1, 3, and 5 hours were examined (Figure 3). Ellipticine (ellip) and α-naphthoflavone (α-NF) did not influence Cyp1b1 mRNA levels but inhibited hydrocarbon inducibility.

Figure 1. Cyp1a1 mRNA expression in Ahr<sup>+/+</sup> VSMCs challenged with BaP. VSMCs were serum-deprived for 72 hours to growth-arrest the cells or allowed to cycle randomly before BaP (3 μmol/L) challenge in the presence of serum for 1, 3, and 5 hours. β-tubulin mRNA was analyzed to control for RNA loading and transfer efficiency. Data are representative of duplicate experiments.

Figure 2. Cyp1b1 mRNA expression in Ahr<sup>+/+</sup> and Ahr<sup>−/−</sup> VSMCs challenged with BaP. A, Ahr<sup>+/+</sup> and Ahr<sup>−/−</sup> VSMCs were incubated in media with 10% or 0.1% FBS for 24, 48, and 72 hours. Ahr<sup>+/+</sup> and Ahr<sup>−/−</sup> VSMCs were serum-deprived for 72 hours to growth-arrest the cells (B) or allowed to cycle randomly before BaP (3 μmol/L) challenge in the presence of serum for 1, 3, and 5 hours (C). β-tubulin mRNA was analyzed to control for RNA loading and transfer efficiency. Data are representative of duplicate experiments.
mRNA expression profiles do not consistently correlate with changes at the protein level. This was the case in VSMCs where CYP1B1 protein levels, as measured by Western blot, were higher in Ahr\(^{+/+}\) VSMCs than Ahr\(^{-/-}\) counterparts irrespective of growth conditions (Figure 4). Hydrocarbon treatment did not induce CYP1B1 protein in growth-arrested cells of either Ahr phenotype. CYP1B1 protein was selectively induced in randomly cycling Ahr\(^{+/+}\) VSMCs treated with BaP, indicating that posttranscriptional control of this gene involves Ahr-regulated events. CYP1A1 protein was not detectable under any of the experimental conditions examined.

To determine if changes at the gene or protein level correlate with changes in enzymatic activity, EROD and AHH activities were measured in growth-arrested and randomly cycling Ahr\(^{+/+}\) and Ahr\(^{-/-}\) VSMCs in the presence or absence of BaP for various times. AHH and EROD are enzymatic activities encoded by members of the CYP1 family.\(^{15}\) TCDD was included as a positive control for Ahr-mediated inducibility.\(^{13}\) No EROD activity was detected in VSMCs at any of the hydrocarbon concentrations examined (not shown). Low levels of AHH activity were measured in Ahr\(^{+/+}\) and Ahr\(^{-/-}\) VSMCs irrespective of growth conditions, although levels of enzymatic activity in Ahr\(^{-/-}\) VSMCs under constitutive conditions were generally higher than in Ahr\(^{+/+}\) counterparts (Figure 5). AHH activity increased in both growth-arrested and randomly cycling VSMCs of either phenotype within 1 hour after treatment with BaP in the presence of serum (Figures 5A and 5B). Induction of enzymatic activity was concentration dependent under growth-arrested conditions, with Ahr\(^{-/-}\) VSMCs induced to a greater extent than Ahr\(^{+/+}\) counterparts. Basal and hydrocarbon-inducible activities were higher in randomly cycling cultures than growth-arrested VSMCs. Similar responses were observed in hydrocarbon-treated cultures after 3 hours (Figures 5C and 5D) and 5 or 24 hours (not shown). TCDD did not induce AHH activity significantly irrespective of Ahr phenotype or duration of treatment. Treatment of Ahr\(^{+/+}\) VSMCs with ellip (0.001 to 0.1 nmol/L) or \(\alpha\)-NF (1 to 100 nmol/L) partially inhibited AHH activity by 15.3\(\pm\)4.1 to 35.4\(\pm\)0.8\%, respectively (\(n=3\) to 6). Thus, regulation of Cyp1b1-encoded enzymatic activity involves mechanisms independent of the Ahr itself but is influenced by Ahr phenotype.

Although previous studies of CYP1B1 have described cell specificity regarding the role of Ahr,\(^{16}\) Ahr-independent
regulation of Cyp1b1 in murine VSMCs was unexpected. To investigate this relationship, growth-arrested and randomly cycling VSMCs were incubated with BaP for 5 hours and subsequently challenged with actinomycin D (ActD) to inhibit de novo synthesis of mRNA. Steady-state mRNA levels were measured semiquantitatively by RT-PCR from 5 to 20 hours. An extended time course was used to correlate mRNA levels and AHH activity in cells treated with BaP for longer periods after depletion of mRNAs by ActD treatment.

Long-term challenge with BaP induced Cyp1a1 mRNA in growth-arrested and randomly cycling Ahr−/− VSMCs (Figure 6A, panels 1 and 5). As expected, no induction was seen in Ahr+/− VSMCs irrespective of growth conditions (panels 3 and 7). After challenge of Ahr+/− VSMCs with ActD, Cyp1a1 induction by BaP was inhibited. BaP induced Cyp1b1 mRNA at all time points in Ahr+/− VSMCs, and this response was preferentially observed in randomly cycling cells (compare panels 2 and 6). A time-dependent decrease in basal and hydrocarbon-inducible Cyp1b1 mRNA was observed in growth-arrested and randomly cycling VSMCs of either phenotype in the presence of ActD (Figure 6A).

Next, AHH activity was monitored as a measure of metabolic capacity in growth-arrested and randomly cycling Ahr+/− and Ahr−/− VSMCs challenged with BaP in the presence of serum and ActD after 5 hours. As shown previously, challenge with BaP was associated with time-dependent induction of AHH activity irrespective of growth conditions or Ahr phenotype. Growth-arrested and randomly cycling Ahr+/− and Ahr−/− VSMCs generally exhibited comparable profiles of AHH activity (Figures 7A through 7D), indicating that early kinetic differences between Ahr+/− and Ahr−/− subside as a function of time (compare Figures 5 and 7). AHH activities in randomly cycling cells were consistently higher than in growth-arrested counterparts. Despite decreases in Cyp1a1 and Cyp1b1 mRNAs in VSMCs challenged with BaP in combination with ActD (Figure 6A), AHH activity remained elevated in both Ahr+/− and Ahr−/− VSMCs with few exceptions (Figures 7A through 7D). It should be noted, however, that growth-arrested Ahr−/− VSMCs displayed a time-dependent decrease in AHH activity on addition of ActD (Figure 7A), whereas Ahr+/− counterparts displayed an initial decrease followed by full recovery of activity by 20 hours (Figure 7C). Differential effects of ActD in growth-arrested versus randomly cycling cultures are consistent with mitogen-dependent regulation of CYP gene expression in VSMCs.

mRNA processing and protein stabilization have been proposed as significant AHH regulatory mechanisms. Therefore, Cyp1a1 and Cyp1b1 mRNA expression profiles were monitored by RT-PCR in growth-arrested and randomly cycling Ahr+/− and Ahr−/− VSMCs challenged for 3 hours with BaP alone or in the presence of 10 µmol/L cycloheximide (ChX) to inhibit protein synthesis. As expected, BaP selectively induced Cyp1a1 mRNA in Ahr+/− VSMCs irrespective of growth conditions (Figure 6B, panels 1 and 5). ChX alone did not influence gene expression, but cotreatment with BaP superinduced Cyp1a1 in randomly cycling Ahr+/− VSMCs (Figure 6B, panel 5). ChX treatment downregulated basal and inducible Cyp1b1 expression in growth-arrested Ahr+/− VSMCs and reduced mRNA levels in randomly cycling Ahr+/− VSMCs cotreated with BaP (Figure 6B, panels 2 and 6). BaP induced Cyp1b1 in Ahr−/− VSMCs, and ChX did not influence this response (Figure 6B, panels 4 and 8).

The influence of ChX on AHH activity in growth-arrested and randomly cycling Ahr+/− and Ahr−/− VSMCs was also examined (Figure 8). No significant differences in AHH activity were observed between BaP alone or in combination with ChX irrespective of Ahr phenotype or mitogenic status. Growth-arrested Ahr−/− VSMCs exhibited the lowest levels of inducible AHH activity of any of the treatment groups. It should be noted, however, that some inhibition of AHH activity was observed in growth-arrested Ahr−/− VSMCs at 5 hours. The highest level of BaP-inducible AHH activity by 24 hours was observed in randomly cycling Ahr+/− VSMCs. Thus, the overall profile of BaP-inducible AHH activity

![Figure 5. AHH activity in Ahr+/− and Ahr−/− VSMCs challenged with BaP. Growth-arrested (A and C) and randomly cycling (B and D) VSMCs were challenged with BaP (0.3 or 3 µmol/L) or TCDD (1 nmol/L) for 1 (A and B) and 3 hours (C and D). AHH analysis was performed as described in Materials and Methods. Solid bars represent Ahr+/− and open bars represent Ahr−/−. Error bars=SEM for 3 separate measurements. *P<0.05.](http://circres.ahajournals.org/ Downloaded from kerzeeramos.com)
paralleled protein expression and was not affected by decreased protein synthesis.

Conclusions

A role for Ahr in atherogenesis was first proposed based on the observation that 3-methylcholanthrene causes more severe atherosclerotic vascular lesions in mice expressing the high affinity form of the Ahr.17 Subsequent studies linked Ahr signaling to CYP-mediated bioactivation of procarcinogens to intermediates that modulate redox status and bind covalently to DNA.8 Evidence is presented in this study that CYP1A1 and CYP1B1 are differentially regulated in mouse VSMCs, with constitutive and inducible expression at the level of mRNA, protein, and enzyme activity strongly influenced by Ahr phenotype and mitogenic status.

Cyp1a1 was not constitutively expressed in VSMCs irrespective of Ahr phenotype or growth conditions. The gene was induced by hydrocarbon treatment in randomly cycling Ahr<sup>+/+</sup> VSMCs (Figure 1), but protein levels remained uninducible. The profiles of gene induction and ActD sensitivity (Figures 2A and 6A) are consistent with the ontogeny of Cyp1a1 in VSMCs and its dependence on Ahr-mediated transcription.13 ChX did not enhance constitutive mRNA levels irrespective of growth conditions but superinduced Cyp1al in randomly cycling Ahr<sup>+/+</sup> VSMCs treated with hydrocarbon (Figure 6B). Thus, as described in other species,33,34 cytodifferentiation programs that repress Cyp1a1 are conserved in murine VSMCs.

Cyp1b1 mRNA was highly expressed in VSMCs irrespective of Ahr phenotype or growth conditions (Figure 2). Expression of Cyp1b1 mRNA in Ahr<sup>+/+</sup> VSMCs is consistent with previous studies showing that assembly of enhancer complexes in the 5′-untranslated region of Cyp1b1 after

Figure 6. A, RT-PCR analysis of Cyp1a1 and Cyp1b1 in Ahr<sup>+/+</sup> and Ahr<sup>−/−</sup> VSMCs challenged with BaP alone or in combination with ActD. Ahr<sup>+/+</sup> and Ahr<sup>−/−</sup> VSMCs were allowed to cycle randomly or growth-arrested before BaP (3 μmol/L) challenge in the presence of 10% FBS. After 5 hours of BaP challenge, ActD (2 μg/mL) was added to inhibit RNA synthesis. B, RT-PCR analysis of Cyp1a1 and Cyp1b1 in Ahr<sup>−/−</sup> and Ahr<sup>+/+</sup> VSMCs challenged with BaP alone or in combination with cycloheximide. Three hours before RNA extraction, ChX (10 μmol/L) was added to inhibit protein synthesis.34 RNA extraction and analysis were performed as described in Materials and Methods. Cyp1a1 and Cyp1b1 mRNA were amplified as described in Materials and Methods. α-smooth muscle actin was coamplified for semiquantitative purposes. Cyp1a1 (lower 5′-CCAGGATGCTCACCAGACCAG-3′; upper 5′-ATGTAGGGTGAGAGGTGC-3′) yielded a 448-bp fragment and Cyp1b1 (lower 5′-GGCGTGTGGAATGGTGACAGG-3′; upper 5′-GGCGTGTGGAATGGTGACAGG-3′) yielded a 525-bp fragment. NA indicates not applicable.
hydrocarbon treatment of Hepa 1 cell variants is independent of Ahr/Arnt heterodimers. Enhanced constitutive expression of Cyp1b1 in Ahr<sup>+/+</sup> cells relative to wild-type counterparts (Figure 2) suggests that Ahr, or Ahr-interacting proteins, can mediate negative regulation of the gene. This interpretation is consistent with the ability of Arnt to repress Cyp1b1 in Hepa 1 cells<sup>15</sup> and Ahr to participate in negative regulation of GST-A1 in VSMCs.<sup>28</sup> Ryu and Hodgson<sup>18</sup> have shown that Cyp1b1 expression in liver is only seen in mice that express the low affinity form of the Ahr. Interestingly, Cyp1b1 expression exhibits cell-type specificity, because primary bone marrow stromal cells from Ahr-null mice constitutively express functional Cyp1b1, whereas fibroblasts do not.<sup>29</sup> An AHRE located at −833 relative to the major start site is essential for constitutive expression of CYP1B1 in MCF-7 cells.<sup>32</sup> This AHRE functions by enhancing accessibility of transcription factors to the enhancer region, although a direct role of AHR in transregulation has not been described. If AHRE is important for constitutive Cyp1b1 expression in VSMCs, then proteins other than Ahr must interact with the sequence. Two factors distinct from Ahr are known to interact with AHREs in liver cells.<sup>36</sup>
Randomly cycling cells of either phenotype exhibited greater Cyp1b1 mRNA inducibility than growth-arrested counterparts (compare Figures 2B and 2C). Thus, like Cyp1a1, Cyp1b1 expression in murine VSMCs is influenced by mitogenic status. Although Ahr was not required for expression of Cyp1b1 mRNA, Ahr antagonists effectively blocked induction by BaP (Figure 3). Cyp1b1 expression in both Ahr+/+ and Ahr−/− involves a transcriptional component, because ActD inhibited both basal and inducible gene expression (Figure 6A). Posttranscriptional mechanisms are also important, because ChX also influenced gene expression (Figure 6B). ChX may interfere with cellular processing of Cyp1b1 mRNA, given that tissue-specific alternative 3′-processing generates an unstable mRNA that does not accumulate to detectable levels.32 Additional experiments were conducted to evaluate how the absence of Ahr influences the Cyp1a1 and Cyp1b1 response. These experiments tested the hypothesis that increased BaP-inducible AHH activity in the absence of Ahr is mediated by protein stabilization of CYP1B1 protein, as described previously for fibroblasts.31 Western analysis showed that CYP1A1 protein is not detectable in VSMCs, whereas CYP1B1 protein was constitutively expressed irrespective of Ahr phenotype and regulated by hydrocarbon treatment only in randomly cycling AHR+/+ cells (Figure 4). Interestingly, the pattern of Cyp1b1 mRNA, protein, and AHH activity were not always consistent, particularly in Ahr−/− VSMCs under constitutive conditions (compare Figures 2 and 4 with Figure 6). Instead, BaP increased AHH activity irrespective of Ahr phenotype and growth conditions, with early kinetic differences subsiding over time. It was also observed that Ahr antagonists only partially inhibit AHH activity in VSMCs. Collectively, these findings indicate that (1) AHH activity in VSMCs is likely encoded by CYP1B1; (2) protein
stabilization is not responsible for increased AHH activity under growth-arrested conditions in Ahr−/− VSMCs relative to wild-type cells; and (3) enzymatic activity is regulated by complex mechanisms influenced by Ahr phenotype and growth status but independent of the Ahr itself.

Ahr−/− VSMCs showed some decreases in AHH in the presence of ActD, but in general ActD did not influence enzymatic activity (Figure 7). BaP regulation of the enzyme is not controlled at the transcriptional level, although a role for transcription cannot be completely ruled out, because growth-arrested VSMCs exhibited modest decreases in activity after ActD treatment. Growth-arrested Ahr−/− VSMCs exhibited the lowest levels of AHH activity after extended hydrocarbon exposure (Figure 7), suggesting that Ahr-dependent mechanisms also participate in regulation of AHH activity. The effects of ChX are complex, because alterations present in human atherosclerotic lesions.37 Human lesions oxygenated linoleic acid derivatives and oxysterols, are paramount. CYP-derived peroxidation byproducts, such as metabolism and redox homeostasis within the vascular wall is erosclerosis. As such, study of genes that control oxidative VSMCs.

Macromolecular oxidation is a prominent feature of atherosclerosis. As such, study of genes that control oxidative metabolism and redox homeostasis within the vascular wall is paramount. CYP-derived peroxidation byproducts, such as oxygenated linoleic acid derivatives and oxysterols, are present in human atherosclerotic lesions.37 Human lesions also contain high levels of isoprostanes and peroxidation products of arachidonic acid.38,39 The formation of oxidative byproducts in VSMCs and the leakage of superoxide during repeated catalytic cycles may be influenced by constitutive and inducible expression of CYP1B1. In cases of environmental stress, CYP1B1 expression may sustain futile cycles of injury and repair that initiate and promote atherosclerotic lesion occurrence. Although quiescent populations of VSMCs can bioactivate endogenous and exogenous substrates, mitogenically active, less-differentiated cells are likely the most sensitive to vicious cycles of redox stress and genotoxic injury. Thus, in murine VSMCs, cellular mechanisms involving actions of the Ahr govern the expression of CYPs involved in atherogenesis.

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References


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