Cytochrome P4501A1 and 1A2 Gene Expression in the Liver of 3-Methylcholanthrene- and o-Aminoazotoluene-Treated Mice: A Comparison between PAH-Responsive and PAH-Nonresponsive Strains

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The objective of this study was to investigate cytochrome P4501A1 and 1A2 mRNA, protein, and enzyme activity in the liver of male mice differing in the aryl hydrocarbon receptor (AhR) genotype during treatment with the carcinogenic compounds 3-methylcholanthrene (MC) and o-aminoazotoluene (OAT). The basal levels of the CYP1A1 and CYP1A2 enzyme activities were comparable among the mouse strains examined. Significant interstrain variations were observed after treatment by the inducers: EROD and MROD activities were considerably increased in C57BL and A/Sn mice, but not in AKR, SWR, and DBA mice. Western blot analysis did not detect CYP1A1 in the liver of untreated mice. Treatment of mice with MC or OAT caused CYP1A1 accumulation in the liver of C57BL and A/Sn mice, but not in AKR, SWR, and DBA mice. CYP1A2 was detected in all studied mouse strains in both untreated and inducer-treated livers. The results of multiplex RT-PCR showed that the CYP1A1 mRNA in the liver of untreated mice was hardly detectable while constitutive expression of the CYP1A2 gene was rather high. After treatment with MC and OAT the CYP1A1 mRNA level dramatically increased in all strains examined while the increase in the CYP1A2 mRNA level was not striking. This finding did not correlate with the data on the enzyme activity. Our results demonstrated a discrepancy between the transcription of CYP1A1 and CYP1A2 genes and the inducibility of these enzymes in the liver of mice, suggesting a posttranscriptional mechanism of cytochrome P4501A regulation. This comparison between aromatic hydrocarbon-responsive and -nonresponsive strains could contribute to understanding of cytochrome P4501A gene regulation in the liver under the influence of environmental factors.

Key Words: liver; inbred mouse strains; cytochrome P4501A1 and 1A2; aryl hydrocarbon receptor; 3-methylcholanthrene; o-aminoazotoluene.

Cytochrome P450 is a superfamily of enzymes, which are expressed predominantly within the liver, kidney, lung, and intestine of mammalian species where they play an important role in the oxidative metabolism of both endogenous and exogenous (xenobiotic) compounds. The cytochrome P4501A subfamily (CYP1A1 and CYP1A2 in the human and rat liver, and CYP1A1 and CYP1A2 in the mouse liver) is of major interest due to its activation of many procarcinogens, including polycyclic aromatic hydrocarbons (PAH) and heterocyclic amines. CYP1A1 metabolizes mostly PAH, while CYP1A2 is responsible for metabolism of heterocyclic amines, aromatic amines, and azobenzenes (Nebert and McKinnon, 1994). Although parent xenobiotics are often inert, their metabolism by CYP1A enzymes may lead to highly reactive intermediates. These metabolites can form adducts with DNA, RNA, and cell proteins and cause the mutagenic events responsible for tumor initiation. The amount of an active metabolite formed and the efficiency of its detoxification determine the toxic effect of a carcinogen.

Genetic variations in the inducibility of CYP1A can modify the metabolism pathway of a carcinogen (Daly et al., 1994). The basal expression of CYP1A1 and CYP1A2 proteins in the liver is not high, and the amount of highly reactive metabolites is not significant. However, induction of CYP1A1 and CYP1A2 increasing both the mRNA levels and the enzyme activities, follows treatment by many chemical substances, including PAH, heterocyclic amines, aromatic amines, and azobenzenes. One such substance is o-aminoazotoluene (OAT), which is activated mainly by CYP1A2 through N-dealkylation and induces CYP1A1 and CYP1A2 in the livers of rats (Cheung et al., 1994) and mice (Timofeeva et al., 2000). Furthermore, it was shown that repeated treatment of mice with OAT causes tumors in the liver of a number of mouse strains (C57BL, A/Sn, CBA, C3H/He, A/He, DBA, SWR, DD) but not in others (BALB/c, AKR; Kaledin et al., 1990; Kaledin and Zakharova, 1984). However, the effect of OAT on the mouse CYP1A induction is not described completely. Interestingly, in previous experiments with rats, it was shown that mutagenicity
and CYP1A induction by azobenzenes, including OAT, correlates with their carcinogenicity (Cheung et al., 1994). Therefore, one could expect the same effect in the studies with the different inbred mouse strains. Since the effect of MC on the genotype, but not with the Ah receptor (AhR)-dependent mechanism (Cheung et al., 1994; Dogra et al., 1998; Gonzalez et al., 1993), we have chosen this compound as the standard for comparison of CYP1A1 induction during treatment with OAT. CYP1A1 and CYP1A2 induction by OAT, as well as by PAH, is mediated by an aryl hydrocarbon receptor (AhR)-dependent mechanism (Cheung et al., 1994; Dobria et al., 1998; Gonzalez et al., 1993).

Inbred mouse strains are widely used as a model for investigating mechanisms of chemical carcinogenesis. In the mouse, the strain-dependent variation in the response to aromatic hydrocarbon inducers of drug metabolism is well established as a polymorphism of the AhR locus (Chang et al., 1993; Gielen et al., 1972). Considerable increase of CYP1A1 and CYP1A2 mRNAs and enzyme activities has been shown in the liver of mice with the Ah<sup>+</sup>/Ah<sup>+</sup> or Ah<sup>−</sup>/Ah<sup>−</sup> (PAH responsive mice, Ah<sup>+</sup> genotype), but not with the Ah<sup>+</sup>/Ah<sup>−</sup> (PAH nonresponsive mice, Ah<sup>−</sup> genotype; Nebert, 1989).

For rat and human CYP1A1 7-ethoxyresorufin (ER) is considered to be a specific substrate, while 7-methoxyresorufin (MR) is specific for CYP1A2 (Burke et al., 1994). However, it was shown that microsomes from CYP1A2 (MR) knockout mice did not have immunodetectable CYP1A2 protein, but methoxyresorufin-O-demethylase (MROD) activity was high after induction with 2,3,7,8-tetrachlordibenzo-p-dioxin (TCDD; Hamm et al., 1998). Studies with cDNA-expressed microsomal and purified CYP1A1 and CYP1A2 proteins in C57BL mice showed that the substrate specificity of CYP1A1 overlaps CYP1A2 (Tsyrliv et al., 1993). Thus, it is quite difficult to estimate the inducibility and activities of CYP1A isozymes. This difficulty can be alleviated by using highly specific antibodies.

The purpose of this study was to estimate and compare CYP1A1 and CYP1A2 expression in the liver of 3-methylcholanthrene- or o-aminobenzotriazene-treated mice, differing in the Ah receptor genotype. We used C57BL, A/Sn (Ah<sup>−</sup> genotype, aromatic hydrocarbon responsive) and AKR, DBA, SWR (Ah<sup>−</sup> genotype, aromatic hydrocarbon nonresponsive) mouse strains.

**Materials and Methods**

**Experimental animals.** Male mice (10–12 weeks of age) of C57BL, AKR, DBA, A/Sn, and SWR strains were supplied by the Institute of Cytology and Genetics (Novosibirsk, Russia). Mice were housed under standard vivarium conditions in a pathogen free environment and had free access to food and water. Induction of microsomal monoxygenases in the mouse liver was performed by ip injection of OAT or MC (225 mg/kg or 80 mg/kg body weight in 0.3 ml of corn oil, respectively; Sigma, St. Louis, MO). Control mice received corn oil (25 ml/kg). Twenty and 72 h after injection (for mRNA quantitative analysis and for enzyme assays, respectively), the animals were sacrificed; their livers were rapidly removed.

**Enzyme assays.** The microsomal liver fraction was isolated from freshly excised livers by standard differential centrifugation (Burke et al., 1985). The protein concentrations in microsomes were measured according to Lowry et al. (1951). The cytochrome P450 content in the liver was determined as described by Omura and Sato (1964). The rates of O-dealkylation of 7-ethoxy- and 7-methoxyresorufins were assessed according to Burke and Mayer (1974).

**Western blotting and immunodetection.** SDS-polyacrylamide gradient (7.5–15%) gel electrophoreses of microsomal proteins (60 μg) were carried out as described by Laemmli (1970). Electrophoretic transfer of the proteins to 0.45-μm nitrocellulose sheets (Schleicher & Schuell, Germany) was performed as described by Towbin et al. (1979). The immunobassay was normally done by blocking the transfer membrane with a 5% nonfat milk powder to prevent further nonspecific binding of proteins; this was followed by incubation of the membrane in a diluted 1% antibody solution (Grishanava and Lyakhovich, 1992), washing of the membrane, incubation in diluted alkaline phosphatase-conjugated rabbit-anti-mouse IgG, further washing, and the colorimetric detection using bromo-chloro-indolyl phosphate (BCIP) and Nitro-blue-tetrazolium (NBT) salts.

**RNA isolation and cDNA synthesis.** Total RNA was extracted from liver tissue by the SDS/phenol method (ChattoPadhyay et al., 1993). Purity of the RNA was assessed by the ratio of the optical densities at 260 and 280 nm, and the integrity examined by electrophoresis on a 1% agarose gel containing ethidium bromide. RNA was assessed by the ratio of the optical densities at 260 and 280 nm, and the integrity examined by electrophoresis on a 1% agarose gel containing of ethidium bromide-stained agarose gels and to remain within the exponential phase of the amplification curve (data not shown).

**Multiplex PCR.** The oligonucleotide primers for CYP1A1, CYP1A2, and RPL30 were synthesized according to sequences chosen using PCR primer design software GeneRunner (www.generunner.com) to ensure specific and efficient coamplification of target sequences. The sequences of oligonucleotide primers were:

**CYP1A1 U, 5′-CAGAAGCTTAATCTCTTTCCCTGGATGCC-3′; CYP1A1 R, 5′-GCACTGCAAGGTTGTTACAGTACATGA-3′; CYP1A2 U, 5′-CAGAAGCTTTCAAGACGAGAAGAAC-3′; CYP1A2 R, 5′-CGAGTACCGGTTCTTGTGATGCGCCA-3′; RPL30 U, 5′-ATGGTGGCGTCGAAAGAAAGAC-3′; RPL30 R, 5′-GTATCTACTCCACGAAGAATG-3′.**

A sequence of PCR reactions using each primer pair was performed initially to determine the number of cycles required to barely visualize these PCR products on ethidium-bromide-stained agarose gels and to remain within the exponential phase of the amplification curve (data not shown).

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RESULTS

EROD and MROD activities of CYP1A1 and CYP1A2 activities were measured in mouse liver microsomes. We did not observe any significant interstrain differences in the EROD and MROD activities in the liver of untreated mice (Table 1). The constitutive levels of CYP1A1 and CYP1A2 activities in the mouse liver microsomes were almost the same among the strains studied. Treatment with MC or OAT caused an increase of total amount of P450 as well as a significant increase EROD and MROD in the liver of mice with Ah<sup>−</sup>Ah<sup>−</sup> and Ah<sup>+</sup>Ah<sup>+</sup> genotypes (C57BL and A/Sn), and the fold induction with OAT was almost the same as by induction with MC. In the liver of mice with the Ah<sup>−</sup> Ah<sup>−</sup> genotype (SWR, AKR, and DBA), treatment with MC or OAT did not result in significant alterations of total cytochrome P450 content or CYP1A1 and CYP1A2 catalytic activities. These results confirm data from previous studies of CYP1A1 and CYP1A2 induction in the liver of mice differing in the Ah receptor genotype (Nebert, 1984). The CYP1A2 mRNA level differed only slightly among the mouse strains examined except for SWR mice, where the level was 3–4-fold higher than in untreated C57BL, A/Sn, AKR, and DBA mice.

To estimate the induction of CYP1A1 and CYP1A2 mRNAs the multiplex RT-PCR method was applied. The “housekeeping” gene, ribosomal protein L30, was chosen as an endogenous internal control to which PCR amplification products of genes studied were normalized. The results of multiplex RT-PCR for CYP1A1 and CYP1A2 are presented in Figure 2. The CYP1A1 mRNA level in the liver of untreated mice was demonstrated to be hardly detectable while constitutive expression of CYP1A2 gene was rather high. These data are consistent with the results of Western blot analysis as well as with the results of previous studies (Kimura et al., 1986; Tukey and Nebert, 1984). The CYP1A2 mRNA level differed only slightly among the mouse strains examined except for SWR mice, where the level was 3–4-fold higher than in untreated C57BL, A/Sn, AKR, and DBA mice.

Treatment with MC or OAT caused a 3–4-fold induction of the CYP1A2 mRNA level in the livers of A/Sn, C57BL, and AKR mice. There was no significant increase in CYP1A2 mRNA expression in SWR mice, but there was a modest, but

<table>
<thead>
<tr>
<th>Strain</th>
<th>AhR genotype</th>
<th>Treatment</th>
<th>Total P450 content</th>
<th>EROD</th>
<th>MROD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWR</td>
<td>Ah&lt;sup&gt;−&lt;/sup&gt;Ah&lt;sup&gt;−&lt;/sup&gt; (Ah&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Control</td>
<td>0.47 ± 0.04</td>
<td>126 ± 25</td>
<td>109 ± 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MC</td>
<td>0.45 ± 0.03</td>
<td>173 ± 30</td>
<td>148 ± 22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OAT</td>
<td>0.42 ± 0.08</td>
<td>178 ± 19</td>
<td>165 ± 30</td>
</tr>
<tr>
<td>AKR</td>
<td>Ah&lt;sup&gt;−&lt;/sup&gt; Ah&lt;sup&gt;−&lt;/sup&gt; (Ah&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Control</td>
<td>0.6 ± 0.04</td>
<td>89 ± 25</td>
<td>100 ± 19</td>
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<tr>
<td></td>
<td></td>
<td>MC</td>
<td>0.58 ± 0.1</td>
<td>75 ± 28</td>
<td>115 ± 14</td>
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<tr>
<td></td>
<td></td>
<td>OAT</td>
<td>0.55 ± 0.12</td>
<td>69 ± 15</td>
<td>125 ± 21</td>
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<tr>
<td>DBA</td>
<td>Ah&lt;sup&gt;−&lt;/sup&gt; Ah&lt;sup&gt;−&lt;/sup&gt; (Ah&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Control</td>
<td>0.45 ± 0.11</td>
<td>144 ± 14</td>
<td>182 ± 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MC</td>
<td>0.5 ± 0.11</td>
<td>185 ± 70</td>
<td>220 ± 41</td>
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<tr>
<td></td>
<td></td>
<td>OAT</td>
<td>0.47 ± 0.08</td>
<td>178 ± 20</td>
<td>192 ± 27</td>
</tr>
<tr>
<td>A/Sn</td>
<td>Ah&lt;sup&gt;−&lt;/sup&gt; Ah&lt;sup&gt;−&lt;/sup&gt; (Ah&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Control</td>
<td>0.55 ± 0.05</td>
<td>90 ± 11</td>
<td>82 ± 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MC</td>
<td>1.1 ± 0.14 (2.0)*</td>
<td>1695 ± 148 (18.83)*</td>
<td>1934 ± 200 (23.59)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OAT</td>
<td>1.2 ± 0.22 (2.18)*</td>
<td>1878 ± 320 (20.86)*</td>
<td>2194 ± 250 (26.76)*</td>
</tr>
<tr>
<td>C57BL</td>
<td>Ah&lt;sup&gt;−&lt;/sup&gt; Ah&lt;sup&gt;−&lt;/sup&gt; (Ah&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Control</td>
<td>0.78 ± 0.01</td>
<td>153 ± 20</td>
<td>144 ± 34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MC</td>
<td>1.5 ± 0.07 (1.92)*</td>
<td>2472 ± 190 (16.16)*</td>
<td>3682 ± 301 (25.57)*</td>
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<tr>
<td></td>
<td></td>
<td>OAT</td>
<td>1.36 ± 0.04 (1.74)*</td>
<td>2911 ± 580 (19.03)*</td>
<td>3261 ± 428 (22.65)*</td>
</tr>
</tbody>
</table>

*Significantly different from the control group by Student’s t-test (*p < 0.05).
statistically significant ($p < 0.05$) increase in the expression of CYP1A2 mRNA in DBA mice during treatment with MC and OAT.

After treatment with MC and OAT, the CYP1A1 mRNA level increased in all the strains examined. This finding does not correlate with the data on enzyme activity and was unexpected, since SWR, AKR, and DBA mice (Ah$^+$Ah$^+$ genotype) are traditionally considered to be PAH noninducible (Nebert, 1989). Among the mouse strains examined, the CYP1A1 mRNA level was approximately two-fold higher in the livers of A/Sn and C57BL mice (Ah responsive), than in SWR, AKR, and DBA mice (Ah nonresponsive).

**DISCUSSION**

Our results demonstrated a discrepancy between the transcription of CYP1A1 and CYP1A2 genes and the inducibility of these enzymes in the livers of mice. On the one hand, after treatment with inducers, the CYP1A1 and CYP1A2 mRNA levels increased in all the strains examined, excluding the CYP1A2 mRNA level in SWR mice. On the other hand, the induction of CYP1A1 and CYP1A2 proteins and their catalytic activities were not observed in the livers of mice with the Ah$^+$ genotype. This finding suggests participation of posttranscriptional mechanisms of CYP1A regulation.

Distinctions in the CYP1A1 and CYP1A2 inducibility between C57BL (Ah responsive) and DBA (Ah nonresponsive) mice during induction with MC have been demonstrated (Nebert and Gelboin, 1969). Further investigations have shown that PAH induction is mediated by AhR (Dogra et al., 1998; Gonzalez et al., 1984, 1993). The AhR is a protein that positively regulates CYP1A1 and CYP1A2 gene expression. The mechanism underlying the difference in responsiveness has not been completely clarified. It has been reported, however, that the low responsiveness of DBA mice to aryl hydrocarbon is due to a low binding affinity of AhR to ligands because of amino acid substitution and elongation of the C-terminus in the AhR (Ema et al., 1994).

Previously, it was shown by Gonzalez et al. (1984) that MC induces both proteins in C57BL/6N (Ah responsive) but not DBA/2N (Ah nonresponsive) mice, whereas sufficiently high doses of TCDD induce both CYP1A1 and CYP1A2 in both inbred mouse strains. In C57BL/6N mice, transcriptional rates of the CYP1A1 and CYP1A2 genes increased dramatically after MC treatment; in contrast, no increase in either gene is found in MC-treated DBA/2N mice. Following TCDD administration, both CYP1A1 and CYP1A2 gene transcription rates were elevated in DBA/2N mice (Gonzalez et al., 1984). Authors have assumed that affinity of TCDD to the Ah-receptor is so high that, despite of defective receptor, the complex formed...
is stable enough to initiate transcription of CYP1A1 and CYP1A2 genes. One can notice these data contradict the results observed in our study, where treatment with MC or OAT increased the CYP1A1 mRNA level in all the strains examined, even in SWR, AKR, and DBA mice (Ah-nonresponsive). We suggest the possible reason for this could be the posttranscriptional mechanisms of CYP1A regulation and probably the defects in the protein translation but not the defects of the Ah receptor.

At present, many reports point to contradictions between the Ah-receptor genotype and the inducibility of CYP1A1 and CYP1A2. These results provided basis for hypothesizing AhR-independent mechanisms of induction for CYP1A1 and CYP1A2. Such cases are known for CYP1A2. For example, acenaphthylene and related tricyclic hydrocarbons induce CYP1A2. These results provided basis for hypothesizing AhR-receptor transcriptional mechanisms of CYP1A regulation and probably the posttranscriptional stabilization of the enzyme-inducer complex. Some xenobiotics can influence CYP1A1 and CYP1A2 gene expression without binding to the Ah-receptor as a ligand, confirming the presence of an AhR-independent pathway. For example, phenobarbital does not bind the Ah-receptor with high affinity but induces in liver cells the expression of cytochrome P4501A. Experiments using both wild type and AhR knockout C57BL mice (AhR–/– genotype) showed a lack of responsiveness to the classical Ah-receptor ligand MC of both CYP1A1 and CYP1A2 genes in the liver of knock out animals. However, phenobarbital induced hnRNA, mRNA, and protein for the CYP1A2 gene, irrespective of the presence of the Ah-receptor (Corcos et al., 1998). It is apparent that in the absence of the Ah-receptor, the other transcription factors are capable of binding with special DNA sequences (drug response elements) followed by the initiation of CYP1A2 gene transcription. However, such a mechanism for CYP1A1 has not been shown so far.

The discrepancies found in our study between the increase of mRNA level and the Ah-receptor genotype could be explained by activation of an AhR-independent signal transduction pathway. Other possible mechanisms of regulation can not be excluded; however, lack of protein accumulation during active transcription of the correspondent genes could indicate defects in translation of CYP1A1 and CYP1A2. To reveal the exact mechanism of this phenomenon, a more detailed study of the molecular genetics of Ah-receptor as well as further investigations of posttranscriptional mechanisms of CYP1A1 and CYP1A2 gene regulation in the liver of different inbred mouse strains are required.

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REFERENCES


