ABSTRACT

Rabbit antibodies to the phenobarbital (PB) inducible rat liver microsomal cytochrome P-450s b and e and to 3-methylcholanthrene (MC) inducible P-450c were used to examine the expression of these isozymes in rat lungs. Western blots of total lung microsomes demonstrated that about 40 pmol P-450b/mg protein (and no detectable P-450e) were present in lungs from control or MC treated rats and that pretreatment with PB caused a small but significant (P < 0.05) increase in the expression of P-450b. Microsomes from control and PB treated lung contained minimal levels of P-450c, and MC induced this isozyme to 185 pmol/mg. Immunocytochemistry was used to demonstrate immunoreactivity to these isozymes in specific cell types. Neither P-450b nor P-450c was detectable in endothelial cells from control or PB treated lungs, but MC increased immunoreactivity to P-450c in pulmonary endothelial cells. Type II alveolar cells showed distinct immunoreactivity to P-450b and weak immunoreactivity to P-450c in control or PB treated rats. Individual Clara cells stained for either P-450b or P-450c in control, MC treated, and PB treated rats, and colocalization was observed in some cells. An increase in type II alveolar cells and Clara cell immunoreactivity to P-450c was observed after MC induction. Mast cells, identified by metachromatic Giemsa staining, appeared to react nonspecifically with both antisera. In conclusion, (a) P-450c is highly inducible by MC in rat lung (detected in microsomes by Western blot), specifically in endothelial cells, Clara cells, and alveolar type II cells (as visualized by immunocytochemistry); and (b) P-450b is present in rat lung microsomes, and immunoreactivity to this isozyme is localized in alveolar type II and Clara cells.

INTRODUCTION

The lungs are a target site for xenobiotics because of their large airway epithelial surface area directly contacting the ambient environment. In addition, the lung is the only organ in the body the capillary bed of which receives the total cardiac output. Thus, this organ is uniquely situated to take up and metabolize xenobiotics delivered via either the inhaled air or the blood. The cells of the pulmonary vascular endothelium, in contrast to those of the liver endothelium, are linked by tight junctions. Therefore, selective uptake from the pulmonary circulation is an important function of these cells (1).

Metabolism of xenobiotics has been shown to occur within several pulmonary cell types, and at least three P-450 isozymes have been identified in rabbit lung (2). Cytochrome P-450 containing mixed function oxidase systems is present in nonciliated bronchiolar (Clara) cells (3) and alveolar type II pneumocytes (4). Furthermore, work by Dees et al. (5) suggests that two P-450 forms, both known to be inducible in the rabbit liver by MC, are present in endothelial cells of pulmonary arteries and veins after treatment with an MC-type agent. However, portions of this immunofluorescence work have been questioned (2). There are also conflicting observations regarding the presence of P-450 in alveolar macrophages (4–6).

This paper examines the expression of specific cytochrome P-450 isozymes in rat lung and their induction by MC or PB. In conjunction with immunological quantitation in microsomes from total lung tissue, P-450 isozymes were localized in specific pulmonary cell types at the light microscope level using an immunocytochemical technique with high precision and resolution.

MATERIALS AND METHODS

Animal Treatment and Tissue Preparation. For induction of P-450 isozymes adult male Sprague-Dawley rats were pretreated for 3 days with either MC (25 mg/kg) or PB (100 mg/kg) as described previously (7). On the 4th day rats were killed with Nembutal (MC treated rats) or ketamine (PB treated rats) and exsanguination. The lungs were removed for microsomal preparations or were perfused in situ with heparinized Krebs-ringer bicarbonate solution. Microsomes were prepared by differential centrifugation. Each preparation was a pool of tissue from 4 to 6 rats. For immunocytochemistry the perfused lungs were fixed by tracheal instillation of Bouin's solution at a pressure of 15 cm H2O.

Antibody Preparation. Cytochrome P-450 (the major form induced in liver by MC) was purified from livers of MC treated rats, and cytochrome P-450b and e (both inducible in liver by PB and exhibiting 97% amino acid sequence homology) were purified from the livers of PB treated rats. The purity of the antigen preparations was demonstrated by the presence of a single protein band following electrophoresis. A complete description of purification techniques and verification of antigen purity has been published (7). Antibodies to P-450c and P-450b were raised by multiple injections of each antigen into New Zealand White rabbits. The IgG fraction was affinity purified on protein A-Sepharose (7). Preimmune serum from the same rabbits produced no detectable staining when incubated with duplicate Western blots.

Quantitation of Individual Cytochrome P-450 Isozymes. A purified, polyclonal antibody raised in rabbits against cytochrome P-450b was used to quantitate immunologically related proteins on Western blots. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (8) with several modifications which were used to optimize the separation of specific P-450 isozymes: (a) the resolving gel was 7% acrylamide (rather than 7.5% as used previously); (b) the upper reservoir buffer was twice the concentration of the lower reservoir buffer; and (c) electrophoresis was run at 30 mA/gel and was continued for 60 min after the dye front had eluted from the gel. Proteins were electrophoretically transferred (Western blotted) to nitrocellulose sheets as described by Guengerich et al. (9) using a Hoeffer (San Francisco, CA) TE51 Transphor apparatus at a setting of 300 mA for 2 h. Visualization of immunoreactive proteins was accomplished by sequential incubation of the sheets with: (a) purified, rabbit antibody raised against either P-450b (40 ug/ml) or P-450c (10 ug/ml); (b) goat anti-rabbit IgG (1/100 dilution); (c) peroxidase/antiperoxidase complex (1/2000 dilution); and (d) H2O2/3,3-diaminobenzidine (9). Phosphate buffered saline containing 0.3% Tween 20 was substituted for the bovine serum albumin-fetal calf serum buffer in all of the washes.

Immunoreactive proteins were quantified by scanning with a Zeineh model SL-504-XL Soft Laser densitometer. Peak areas of the resulting images were then calculated using a Hewlett-Packard digitizer. Standard curves with known amounts of P-450b and e (for anti-P-450b blots) or P-450c (for anti-P-450c blots) ranging from 0.1 to 2.0 pmol were prepared using pooled microsomes.
run on each gel. In addition, for anti-P450b blots, several lanes containing both P-450b and P-450e were run and quantified to ensure that the two forms could be resolved by the densitometer. Modification of SDS-polyacrylamide gel electrophoresis conditions (described above) resulted in sufficient separation of P-450s b and e to enable their individual quantification. Preliminary studies using known amounts of each isozyme demonstrated that our polyclonal preparation of anti-P-450b recognized these two proteins equally well on Western blots (data not shown), and preabsorption of the antibody preparation with purified P-450b eliminated both P-450 bands. For determination of individual proteins in microsomal samples, duplicate lanes with each of three different amounts, ranging from 1 to 100 μg of microsomal protein, were separated and quantified as described above.

Analytical Methods. Total liver P-450 content was determined spectrophotometrically by measuring the absorbance difference for reduced versus oxidized CO bound cytochrome, using an extinction coefficient of 91 mM⁻¹ cm⁻¹ for the 450–490 nm wavelength pair (10). Due to interference from hemoglobin, lung P-450 was determined using the difference absorbance approach from hemoglobin with a 450–490 nm wavelength pair (10). Due to interference from hemoglobin, lung P-450 was determined using the difference absorbance approach.

Table 1 Specific content of cytochrome P-450 isoforms in male Sprague-Dawley rat liver and lung microsomes

<table>
<thead>
<tr>
<th>Tissue and treatment</th>
<th>P-450b (pmol P-450/mg microsomal protein)</th>
<th>P-450c (pmol P-450/mg microsomal protein)</th>
<th>Total (pmol P-450/mg microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4 ± 2 (12)</td>
<td>20 ± 5 (12)</td>
<td>8 ± 6 (12)</td>
</tr>
<tr>
<td>PB</td>
<td>974 ± 147 (8)</td>
<td>473 ± 170 (8)</td>
<td>8 ± 6 (3)</td>
</tr>
<tr>
<td>MC</td>
<td>2 ± 1 (3)</td>
<td>12 ± 5 (3)</td>
<td>894 ± 150 (8)</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>42 ± 7 (4)</td>
<td>&lt;1 (4)</td>
<td>2 ± 1 (3)</td>
</tr>
<tr>
<td>PB</td>
<td>64 ± 15 (4)</td>
<td>&lt;1 (4)</td>
<td>1 ± 1 (3)</td>
</tr>
<tr>
<td>MC</td>
<td>41 ± 8 (4)</td>
<td>&lt;1 (4)</td>
<td>185 ± 20 (6)</td>
</tr>
</tbody>
</table>

* Determined by densitometry of Western blots.
* Determined spectrophotometrically by the method of Johannesen and Depierre (11).
* Mean ± SD.
* Numbers in parentheses, number of determinations of tissue samples pooled from 4 to 6 rats.

RESULTS

Quantitation of Specific Cytochrome P-450 Isozymes. P-450e was consistently quantitated in the livers of untreated rats at about 20 pmol/mg microsomal protein (±3% of total P-450) whereas P-450b was expressed only occasionally and at lower levels (Table 1) than the former isoform. This is in agreement with two recent reports (17, 18), which used a monoclonal antibody (17) and a specific oligonucleotide probe (18) to respectively detect P-450e protein and mRNA. In liver microsomes both proteins were substantially induced by PB, with P-450b expressed at twice the level of P-450e. Consistent with recent publications (18, 19), P-450e was never detected in lung microsomes. Pretreatment with PB resulted in a small but significant (P < 0.05) increase in pulmonary expression of P-450b (Table 1).

Although antibodies to cytochrome P-450c are known to partially cross-react with cytochrome P-450d, a second MC inducible hepatic isozyme (7, 20), titration curves of Western.
blots using our protein A-Sepharose purified IgG preparation exhibited an approximately 10-fold greater affinity for P-450c compared to P-450d. Quantitative data for cytochrome P-450d are not shown in Table 1 since the reduced affinity of the antibody preparation made quantitation of P-450d unreliable. P-450c and P-450d were clearly separated on SDS gels, eliminating any interference with the quantitation of P-450c. Microsomes from the livers and lungs of untreated rats contained minimal levels of P-450c (Table 1). MC treatment resulted in substantial increases in expression of this protein in both tissues. Cytochrome P-450d was a major protein in both control and MC liver microsomes. In contrast a protein with mobility equal to that of P-450d was barely detectable in lung microsomes, in agreement with recent findings (21), and this minimal pulmonary level of P-450d was not increased by MC treatment.

**Cellular Localization of P-450 Isozymes.** The above quantitative results were supported by immunocytochemical findings. Specifically, among untreated rats P-450b-like immunoreactivity was present in (a) a large fraction of Clara cells (Table 2; Figs. 5 and 6b) and (b) a large fraction of type II pneumocytes (Table 2; Fig. 4). The lung immunoreactivity for P-450b appeared unchanged by PB or MC treatment. Successful PB induction was verified by a 250-fold P-450b increase in liver microsomes (Table 1) and a strong hepatocytic immunoreactivity in treated rats compared to weak or absent hepatocytic immunoreactivity among controls.

In agreement with the quantitative data, with immunocytochemistry, P-450c-like immunoreactivity among control rats was of weak intensity and was localized to (a) a small fraction of Clara cells (Fig. 6a) and (b) a very small fraction of type II pneumocytes. The lungs of 4 of the 10 control rats did not show any P-450c immunoreactivity, but the lungs of all MC treated rats displayed immunoreactivity patterns indicative of P-450c induction.

P-450-like immunoreactivity was not observed in vascular endothelium from control rats (Figs. 4, 6a and b). However, with MC treatment, but not PB treatment, endothelium of arteries, arterioles, veins, venules, and septal capillaries demonstrated strong immunoreactivity for P-450c (Figs. 1a, 2, 3, and 7a).

MC treatment resulted in higher intensity of P-450c-like immunoreactivity present in greater numbers in both Clara cells (Table 2; Fig. 7a) and in type II pneumocytes (Table 2; Fig. 2). In addition, with MC treatment, type II pneumocytes with P-450c-like immunoreactivity were more numerous than those with P-450b-like immunoreactivity, whereas approximately the same number of Clara cells was positive with either antisera. Type II pneumocytes, immunoreactive to either antisera, frequently displayed lateral processes extending along the luminal surface of the interalveolar septa (Fig. 2).

Through the use of two techniques, (a) comparing P-450b-like and P-450c-like immunoreactivity in adjacent sections and (b) double staining for both isozymes in the same section, it was shown that P-450b and P-450c were colocalized in a small subset of Clara cells (Figs. 6 and 7), whereas two other subsets immunoreacted with either P-450b or P-450c alone.

Alveolar type I pneumocytes and alveolar macrophages did not immunoreact with either P-450b or P-450c forms.

---

**Table 2 Immunoreactivity in pulmonary cell types among controls and rats treated with PB or MC**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vascular endothelium</th>
<th>Clara cells</th>
<th>Type II pneumocytes</th>
<th>Alveolar macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10)*</td>
<td>b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>c</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>PB (3)</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>MC (8)</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
</tr>
</tbody>
</table>

*<sup>a</sup> b and c, antibodies to P-450 isozyme.
*<sup>b</sup> Numbers in parentheses, number of rats.
*<sup>c</sup> -, No immunoreactivity; +, positive immunoreactivity; (+), low immunoreactivity; *, enhanced by induction.
However, mast cells appeared to display nonspecific immuno-reactivity using both antisera, because preabsorption with either antigen did not cause satisfactory blocking of immunoreactivity using the PAP or immunofluorescence methods, and staining was achieved when omitting the primary antibody or using preimmune serum.

DISCUSSION

The results presented in this paper allow a comparison of imunoquantitation (Western blots) of P-450 isozymes from whole lung microsomes with immunolocalization (immunocytochemistry) in specific pulmonary cells types. P-450b has been shown to be the predominant P-450 isozyme in microsomes from untreated rat lungs. In lung sections from untreated rats P-450b-like immunoreactivity was observed in Clara cells and alveolar type II pneumocytes; this P-450b-like immunoreactivity was not noticeably changed by PB or MC treatment. Antibody to P-450b also reacts with liver P-450e. However, since P-450e was below the limit of detection using the Western blot method, cellular immunoreactivity was probably attributable to P-450b. This lack of detectable P-450e is consistent with a recent Western blot analysis of rat lung P-450 (19). Quantification of mRNA levels in rat lung also indicated the presence of P-450b mRNA but P-450e mRNA was not detected (18).

As is evident in Table 1, the sum of the amounts of immunochemically quantifiable P-450 isozymes can significantly exceed the amount of total spectrophotometrically detectable P-450, particularly in the lung. Other investigators have noted similar discrepancies (9). One group has presented evidence that this difference may be due to the presence of pools of inactive apocytochrome P-450 in extrahepatic tissues (22). Further work is required to resolve this discrepancy.

Serabjit-Singh et al. (3) previously found P-450 form 2 (analogous to rat P-450b) in rabbit Clara cells, and Dees et al. (5) noted immunoreactivity to this rabbit P-450 form 2 in cells resembling type II pneumocytes. The latter group further suggested the presence of P-450 form 2 in rabbit alveolar type I

Fig. 5. Cytochrome P-450b-like immunoreactivity in Clara cells of large airway of control rat. × 400.

Fig. 6. Adjacent lung sections from a control rat showing cytochrome P-450c-like immunoreactivity of weak intensity in Clara cells of a small bronchiole (a) colocalized in a subset of Clara cells (arrows) with cytochrome P-450b-like immunoreactivity (b). Another subset of P-450c positive cells did not react with anti-P-450c (*). Note that in control rats vascular (Va) endothelium does not show immunoreactivity to either antiserum. × 400.

Fig. 7. Adjacent lung sections from a MC treated rat showing cytochrome P-450c-like immunoreactivity in Clara cells of a large bronchiole (a) colocalized in a subset of Clara cells (arrows) with cytochrome P-450b-like immunoreactivity (b). Another subset of P-450c positive cells did not react with anti-P-450b (*). Capillary endothelium (bent arrow) displays immunoreactivity to P-450c but not to P-450b. × 400.
Biochemical evidence of such induction has been provided with chlorodibenzo-p-dioxin. Our results demonstrate that MC in combination with P-450 forms 4 and 6 after treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Jones et al. (24) reported P-450 form I, also equivalent to rat P-450b, in isolated Clara cells and type II pneumocytes from normal rabbits, but not in intact alveolar epithelium.

Minimal levels of P-450c were present in lung microsomes from untreated rats. However, MC caused an approximately 100-fold elevation in the expression of this isozyme. In agreement with the immunoquantitative data, weak P-450c-like immunoreactivity was observed in a small fraction of Clara cells and type II pneumocytes from some control rats, a finding that has not previously been reported in any species. Enhanced immunoreactivity was noted in these cells following MC treatment. In particular, the vascular endothelium, which was negative in untreated rats, displayed intense immunoreactivity after induction with this polycyclic aromatic hydrocarbon, contributing to approximately one-half of the P-450c which was quantitated. The remaining P-450c was attributed to the increased numbers and cellular content (higher immunoreactive intensity) of type II pneumocytes and Clara cells. Dees et al. (5) found immunoreactivity to P-450 form 6 in rabbit lung parenchyma, bronchioles, and bronchi after treatment with the MC-like inducing agent, 2,3,7,8-tetrachlorodibenzo-p-dioxin, but did not identify specific cell types. The rabbit P-450 form 6 is analogous to rat P-450c (2). Dees' group previously showed that the endothelium of arteries and veins of rabbits immunoreacted with P-450 forms 4 and 6 after treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Our results demonstrate that MC induces P-450c in endothelial cells throughout the entire pulmonary vasculature including arterioles, venules, and capillaries. This suggests that under certain conditions, endothelial cells may participate in the metabolism of xenobiotics and may become a target for cytochrome P-450c mixed function oxidase activated pulmonary toxins.

We report here, for the first time, colocalization of P-450b and P-450c in a subset of Clara cells, suggesting that individual Clara cells are capable of synthesizing more than one isozyme. The differential expression of the P-450 isoforms (individual cells displayed immunoreactivity to either or both isoforms) indicates that there are three subsets of Clara cells. This differential expression of P-450 among Clara cells was observed in controls as well as in MC or PB treated rats. A fourth subset of Clara cells not reacting with either antibody remains as a possibility but was not demonstrated here. The mechanism which controls this differential expression of P-450 cytochromes is unknown. Possibly each of the subgroups of Clara cells may represent a phase in the temporal sequence of genetic expression.

In addition, we verified the enhanced P-450 immunoreactivity noted in Clara cells by Ishimura et al. (23) after MC treatment and determined that this was due specifically to P-450c. Biochemical evidence of such induction has been provided by Jones et al. (24).

Our approach combines two complementary immunological methods. Western blots of lung microsomes provide quantification of P-450 isoforms, whereas immunocytochemistry provides evidence of their cellular localization. In order to further quantify our findings, we are currently performing Western blot analyses of enriched cell preparations.

These results strengthen the concept that at least three cell types, Clara cells, type II pneumocytes, and endothelial cells, may participate in pulmonary P-450 mediated metabolism of xenobiotics, primarily involving the cytochrome P-450c isozyme.

ACKNOWLEDGMENTS

The authors wish to thank Robert Kalwinsky for technical assistance.

REFERENCES

Immunological Identification and Effects of 3-Methylcholanthrene and Phenobarbital on Rat Pulmonary Cytochrome P-450

Ingegerd M. Keith, E. Burt Olson, Jr., Neil M. Wilson, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/7/1878

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/47/7/1878.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.