Expression of cytochrome P450 2A3 in rat esophagus: relevance to \(N\)-nitrosobenzylmethylamine

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\(N\)-nitrosobenzylmethylamine (NBzMA) must be metabolically activated to exert its carcinogenic potential and is a potent inducer of tumors in the rat esophagus. The activation is believed to occur in the esophagus. Although the pathways of NBzMA metabolism are well studied, the principal cytochrome P450 enzyme(s) (P450) responsible for catalyzing its activation is unknown. Several preliminary studies have suggested that this enzyme may belong to the P450 2A family. We report here that P450 2A3 expressed in a baculovirus system metabolizes NBzMA, predominantly by methylene hydroxylation. To determine whether or not P450 2A3 is present in the rat esophagus, the relative level of P450 2A3 mRNA was determined by reverse transcriptase–polymerase chain reaction (RT–PCR). The mRNA levels of P450 2A3 were compared with the levels of P450 2A1 and 2A2 mRNA in the esophagus, liver, lung and nasal mucosa. P450 2A3 mRNA was detected in rat nasal mucosa, lung and esophagus, but not in liver, whereas P450 2A1 and 2A2 mRNAs were detected only in the liver. To determine the relative expression of P450 2A3 in each tissue, quantitative RT–PCR with PCR-MIMICS used as internal standards was performed. The expression level in the nasal mucosa was by far the greatest. The expression in the lung and esophagus was 60- and 1600-fold less, respectively. Using antibodies to P450 2A4/5 and P450 2A10/11 a 50 kDa immunoreactive protein was detected in all three tissues by western blot analysis. This is consistent with the expression of P450 2A3 in these tissues. However, the amount of protein detected in the nasal mucosa was much greater than that in the esophagus or lung. The expression of P450 2A protein was similar in the lung and esophagus. The rate of coumarin 7-hydroxylation in cultured rat esophagus was very low. This is a reaction efficiently catalyzed by P450 2A5, 2A6 and 2A10. In summary, our results clearly demonstrate the presence of P450 2A3 protein and mRNA in the esophagus, but the amounts are low and may not be sufficient to account for NBzMA activation in this tissue.

Introduction

The esophagus of the rat is uniquely sensitive to tumor induction by nitrosamines (1,2). Like most carcinogens, nitrosamines require metabolic activation to exert their carcinogenic potential (3). More than 20 years ago, it was suggested that organ-specific tumor induction by nitrosamines was due to organ-specific activation of these carcinogens (4). Data that support the role of esophageal P450s in the metabolism of the esophageal carcinogens, \(N\)-nitrosobenzylmethylamine (NBzMA), \(N\,N\)-nitrosonornicotine (NNN) and \(N\)-nitrosomethylpentylamine have been reported (5–7). However, the enzyme(s) responsible for the metabolism of these nitrosamines in the esophagus has not been identified.

Among the >100 nitrosamines tested, NBzMA is the most potent esophageal carcinogen in the rat (3). It selectively induces esophageal tumors regardless of its route of administration (8). Significant numbers of tumors are not induced in the lung, nasal cavity or any other tissue. Similar to most nitrosamines, the proposed mechanism of metabolic activation of NBzMA is via cytochrome P450 (P450) catalyzed hydroxylation, alpha to the nitroso group (6). \(\alpha\)-Hydroxylation of NBzMA occurs either at the methyl carbon or the methylene carbon of the benzyl moiety (6). Methylene hydroxylation generates a methylating species that reacts with DNA to generate O6-methylguanine. This promutagenic base is thought to be the initiating event in NBzMA induced tumorigenesis (9–11). Consistent with the hypothesis that tissue specific activation is critical to esophageal tumorigenesis, Labuc and Archer reported that rat esophageal microsomes metabolized NBzMA almost exclusively by methylene hydroxylation (6). This metabolism was inhibited >95% by CO and 70% by SKF 525-A, classic P450 inhibitors (6). Therefore, NBzMA methylene hydroxylation is believed to be P450 mediated, but which esophageal P450 catalyzes this reaction remains to be identified.

Little is known about the presence and levels of particular P450s in the rat esophagus. There is evidence that P450 1A1, 1A2 and 17 are expressed in rat esophagus (12,13). P450 1A1 and P450 1A2 mRNA was detected using reverse transcriptase–polymerase chain reaction (RT–PCR) (12). In addition, Ahn et al. reported that western blot analysis of rat esophageal microsomes with P450 1A1 antibody revealed detectable levels of this P450 (14). The same authors did not detect P450 2B1, 2E1 or 3A1/2 by immunoblot analysis (14). Shimizu et al. detected P450 2E1 by immunocytochemistry in esophageal tissue from ethanol-treated, but not untreated, rats (15). Evidence that any of these P450s are involved in the esophageal metabolism of NBzMA or any other esophageal carcinogens is limited. The P450 1A1 inducer, 3-methylcholanthrene (3-MC), does not induce the hepatic metabolism of NBzMA (16,17). Similarly, acetone, a P450 2E1 inducer, had no effect on NBzMA hepatic metabolism (17). More recently, Patten et al. reported that P450 2E1 expressed in vaccinia virus does metabolize NBzMA to benzoaldehyde, with a \(K_m\) of 47 \(\mu\)M (18).

Abbreviations: ECL, enhanced chemiluminescence; HPRT, hypoxanthine-guanine phosphoribosyl transferase; NBzMA, \(N\)-nitrosobenzylmethylamine; NNN, \(N\,N\)-nitrosonornicotine; P450, cytochrome P450 enzyme; PMSF, phenylmethysulfonyl fluoride; RT–PCR, reverse transcriptase–polymerase chain reaction; SDS, sodium dodecyl sulfate.
It is our hypothesis that a P450 2A is responsible for the esophageal metabolism of NBzMA. This view is based on the following information. The human liver P450, 2A6, is an efficient catalyst of the 5′-hydroxylation of NNN, a tobacco-specific esophageal carcinogen (19), and P450 2A6 appears to be an equally good catalyst of NBzMA α-hydroxylation (M.A. Morse, J.Lu, L.A.Peterson, S.E.Murphy and G.D.Stoner, submitted for publication). None of the other human liver P450s are similar to P450 2A6 in their ability to catalyze the α-hydroxylation of these two nitrosamines. The rat ortholog of P450 2A6, P450 2A3, is a major P450 in the nasal mucosa, a tissue that is qualitatively similar to the esophagus in its ability to α-hydroxylate nitrosamines (20,21). Also, the P450 2A3 orthologs in the mouse (P450 2A5) and rabbit (P450 2A10) metabolize a number of other nitrosamines. For example, P450 2A5 catalyzes the α-hydroxylation of the esophageal carcinogen nitrosodimethylamine (22) and P450 2A10 α-hydroxylates 4-(methylmethanesulfonamido)-1-(3-pyridyl)-1-butanone (20,23). Therefore, P450 2A3 is a likely candidate for a P450 that would catalyze the methylene hydroxylation of NBzMA. If it does catalyze this reaction, and it is present in the rat esophagus, it may play an important role in nitrosamine-induced esophageal carcinogenesis.

P450 2A3 cDNA was originally isolated from rat lung (24). However, P450 2A3 mRNA levels in the nasal mucosa are much higher than in the lung (25). P450 2A3 is not present in rat liver. This enzyme has not been isolated, but recently it was expressed in a baculovirus insect system and reported to have coumarin 7-hydroxylation activity (26). The 7-hydroxylation of coumarin is catalyzed specifically and efficiently by human P450 2A6 and other closely related P450 2As (24,27). We report here that P450 2A3 catalyzes the methylene hydroxylation of NBzMA equally well. However, if P450 2A3 is important in the organ-specific metabolism and carcinogenicity of NBzMA, it must be expressed in the esophagus at levels high enough to account for the efficient metabolism of NBzMA in this target tissue. Therefore, we have analyzed the levels of both P450 2A3 mRNA and protein expression in the rat esophagus, and compared these levels with those in the nasal mucosa, liver and lung. In addition, we have compared the tissue specificity of P450 2A3 mRNA expression with the expression of two other known rat P450 2As, 2A1 and 2A2.

Materials and methods

Metabolism of coumarin by rat esophageal explants

Seven- to nine-week-old male F344 rats were killed by exposure to CO2, and their esophagi were removed, stripped of the underlying submucosa, split longitudinally and cultured as described previously (28). Tissues, one esophagus per dish, were incubated in 2.5 ml of PFMR-4 medium containing 0, 0.3 or 3.0 µM coumarin for 4 h at 37°C. Aliquots of 50 µl media were filtered through acridine and analyzed for 7-hydroxycoumarin by reversed-phase HPLC (29). The HPLC system consisted of a Rhodyne model 7010 injection valve, an ESA model 580 solvent delivery module fitted with a 5 µ Burdick and Jackson C18 (4.6x250 mm) column, and a Waters model 470 scanning fluorescence detector. The mobile phase was 1% acetic acid and 30% methanol, pH 7.5. The column temperature was maintained at 30°C and the flow rate was 1.0 ml/ min. 7-Hydroxycoumarin was eluted isocratically and detected fluorometrically (excitation at 376 nm, emission at 460 nm).

Metabolism of [9H-benzyl]NBzMA

Rat P450 2A3, obtained from a baculovirus/insert expression system (provided by Dr XinXin Ding, Wadsworth Center, New York Department of Health), was incubated for 10 min with 5 µM (1.4 µCi/mmol) [9H-benzyl]NBzMA (provided by Dr Lisa A.Peterson, University of Minnesota Cancer Center, MN), as described previously (19). After terminating the reaction and precipitating the protein with barium hydroxide and zinc sulfate, the supernatant was analyzed by HPLC with radioflow detection as previously described (21). Semiarcibizde was included in the reaction mixture to trap benzaldehyde. The identity of the benzaldehyde semicarbazone and the benzyl alcohol was confirmed by co-elution with standards in two HPLC systems. One was an isocratic system with 75% ammonium acetate (25 mM, pH 4.0):25% methanol (20 mM, pH 7.0):40% methanol over 50 min.

Semi-quantitative RT–PCR and sequence analysis

The expression of P450 2A1, 2A2 and 2A3 in rat nasal mucosa, esophagus, lung and liver tissues was determined by RT–PCR analysis of mRNA extracted from these tissues. Nasal mucosa, esophagus, lung and liver were harvested from untreated 7- to 8-week-old F-344 rats (Harlan Sprague–Dawley, Indianapolis, IN) after they were killed. Esophagus tissue was stripped of underlying mucosa, as previously described (6), and consisted primarily of epithelial tissue. RNA was extracted using TRIzol (Gibco BRL, Gaithersburg, MD) according to the manufacturer’s directions. All samples were analyzed for integrity of 18S and 28S rRNA by ethidium bromide staining of 1 µg of RNA resolved on a 1% agarose electrophoresis gel. Reverse transcription and PCR were performed as described previously for rat P450 2A1, 2A2, 2A3 and a housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT) (30). Primer sequences and PCR conditions are summarized in Table 1. The amplification cycle number was determined to keep amplification in the linear range to avoid the ‘plateau effect’ associated with increased numbers of PCR cycles. Primer sequences were designed using Primer Premier software (Premier Biosoft, Palo Alto, CA) for P450 2A1 and 2A2, and an OLGIO primer analysis program (National Biosciences, Plymouth, MN) for P450 2A3. Primers for the HPRT gene were described previously (31). Sequencing products were analyzed using the Perkin Elmer ABI 377 Prism Automated DNA Sequencer. Two primer sets were utilized to sequence the cDNA (1327 bp) of the PCR products obtained from P450 2A3 primer set 6. The sequences obtained were compared with the published P450 2A3 cDNA sequence (24).

Northern blot analysis

Ten micrograms of total cellular RNA isolated from F344 rat nasal mucosa, lung, esophagus and liver was electrophoresed through a 1.2% agarose gel and blotted onto a nylon membrane. The membrane was subjected to hybridization with 32P-labeled rat P450 2A3 cDNA (generously provided by Dr Frank Gonzalez, National Cancer Institute, Bethesda, MD) using a random primed labeling kit (Boehringer Mannheim, Indianapolis, IN). The blots were washed in 2x, 1x, 0.5x, 0.1x, 0.05x sodium dodecyl sulfate (SDS), at increasing stringency until a low background was obtained. To assess equivalent RNA loading, the membranes were stripped and reprobed with a 32P-labeled mouse 18S cDNA probe (Ambion, Austin, TX).

Quantitative RT–PCR using P450 2A3 PCR MIMICs

To analyze the relative amounts of P450 2A3 mRNA expressed in nasal mucosa, lung, esophageal and tissue, competitive PCR was performed (32). Reactions were carried out using one concentration (0.1 µg) of reverse-transcribed mRNA, and six 2-fold dilutions of P450 2A3 MIMICS (Clontech, Palo Alto, CA). PCR MIMICS are internal standards representing non-homologous DNA fragments with primer templates that are recognized by a pair of P450 2A3 specific primers. The concentration ranges used for each of the tissues were as follows: nasal mucosa, 50·10−2 to 1.56·10−2 attomol; lung, 5·10−2 to 1.56·10−3 attomol; esophagus, 5·10−2 to 1.56·10−3 attomol. The six 2-fold dilutions of HPRT MIMICS were used in the range 5·10−3 to 1.56·10−2 attomol. The samples were run on a 1.8% agarose gel after 35 cycles of PCR. Reaction conditions were 19.2 mM Tris–HCl (pH 8.4), 48 mM KCl, 2.3 mM MgCl2, 0.2 µM each of dATP, dGTP, dCTP and dTTP, 0.25 µM each 5′ and 3′ P450 2A3 primers (primer set 5), 0.625 U Taq polymerase and 0.625 U Taq Start antibody. The reaction volume was 25 µl. Products were stained with ethidium bromide and the gel photographed. The peak areas corresponding to the tissue mRNA for P450 2A3 and P450 2A3 MIMICS were determined using an AlphaImager Imaging System (Alpha Innotech, San Leandro, CA). The ratios of the P450 2A3 target peaks were compared with P450 2A3 MIMICS peak areas and plotted against the reciprocal of the molar amount of P450 2A3 MIMICS added to the PCR reaction. Linear
rabbit IgG, respectively, for 1 h. The immunoreactive proteins were detected followed by incubation with peroxidase-labeled anti-chicken IgG and anti-P450 2A10 antibodies were diluted 1:7500 and 1:4500 in P450 2A5 recognizes P450 2A3 effectively. Antibody against P450 2A10 had from rat P450 2A3 by only 23 amino acids (24,33), we assumed that anti-donated by Dr Matti Lang, Lyon, France) and anti-rabbit P450 2A10 (kindly
Western blot analysis was performed using anti-mouse P450 2A5 (kindly
Fifteen micrograms of nasal mucosa and 30 µg of lung and esophageal microsomes were used. Baculovirus-expressed P450 2A3 protein was also run in parallel with the samples to determine the specificity of the antibody. The proteins were transferred to a nitrocellulose membrane and blocked in 1 µg/ml polyvinyl alcohol, 25 mM Tris (pH 7.4) and 150 mM NaCl for 1 min. Western blot analysis was performed using anti-mouse P450 2A5 (kindly donated by Dr Matti Lang, Lyon, France) and anti-rabbit P450 2A10 (kindly donated by Dr Xinxin Ding) antibodies. Since the mouse P450 2A5 differs from rat P450 2A3 by only 23 amino acids (24,33), we assumed that anti-P450 2A5 recognizes P450 2A3 effectively. Antibody against P450 2A10 had been used previously to detect P450 2A3 in nasal mucosa (25). P450 2A5 (20 mg/ml) and P450 2A10 antibodies were diluted 1:7500 and 1:4500 in phosphate-buffered saline with Tween-20 and incubated overnight at 4°C, followed by incubation with peroxidase-labeled anti-chicken IgG and anti-rabbit IgG, respectively, for 1 h. The immunoreactive proteins were detected using an ECL detection kit (Amersham Life Sciences, Arlington Heights, IL).

**Results**

The rate of NBzMA methyl and methylene hydroxylation by P450 2A3 expressed in baculovirus was determined by radioflow HPLC analysis of benzaldehyde and benzyl alcohol. These two metabolites were formed in a ratio of 10:1 and accounted for >90% of the products (data not shown). The rate of benzaldehyde formation from 5 µM NBzMA was 5 nmol/min/nmol P450. Liu et al. determined the rate of coumarin 7-hydroxylase activity for this baculovirus-expressed P450 2A3 to be 0.2 nmol/min/nmol P450 (26). The ability of the rat esophagus to metabolize coumarin was determined by incubating rat esophageal explants with coumarin and analyzing the media for 7-hydroxycoumarin by HPLC with fluorescence detection. 7-Hydroxycoumarin was detected when esophageal explants were incubated with coumarin (Table II). The amount of 7-hydroxycoumarin formed by the esophagus was concentration dependent. Esophagi incubated with 0.3 µM coumarin produced 0.038 pmol 7-hydroxycoumarin/ml media and those incubated with 3 µM coumarin produced 0.401 pmol/ml media.

These results provide evidence that P450 2A3 does metabolize NBzMA and that a P450 2A6-related enzyme, such as P450 2A3, may exist in the rat esophagus. Therefore, to investigate whether P450 2A3 mRNA is expressed in the esophagus, several primers, listed in Table I, were synthesized. RT–PCR was carried out using primer sets 1–6. A number of primers were used to amplify different regions of the cDNA to assure the specificity of the PCR products. This was particularly important since there are several examples of P450 2A genes which only differ by a few amino acids, i.e. mouse 2A4 and 2A5, and rabbit 2A10 and 2A11 (33,34). In addition, to confirm the specificity of our primers as well as the tissue specificity of P450 2A3 mRNA expression, a set of primers for the amplification of both P450 2A1 and 2A2 (primer sets 7 and 8) were also synthesized. Using these primers, the relative expression of P450 2A1, 2A2 and 2A3 was determined in liver, lung, nasal mucosa and esophagus. The expected PCR products of 299 bp for P450 2A1 and 411 bp for P450 2A2, were detected with liver mRNA (Figure 1, lanes 10–12). No P450 2A1 or 2A2 mRNA was detected in the esophagus, nasal mucosa and lung (Figure 1, lanes 1–9). In contrast, as shown in Figure 1, PCR amplification using primer set 5, which is

**Table I. Primers used for semi-quantitative PCR amplification of P450 2A3 gene**

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Exons</th>
<th>Start location</th>
<th>Primer sequence</th>
<th>Size (bp)</th>
<th>PCR conditions</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2A3)</td>
<td>1–4</td>
<td>U-3477</td>
<td>5'-CACGTTGCTTCTCTTGAGTGC-3'</td>
<td>454</td>
<td>95°C for 30 s, 95°C for 15 s, 64°C for 30 s</td>
<td>28</td>
</tr>
<tr>
<td>(2A3)</td>
<td>3–5</td>
<td>U-4826</td>
<td>5'-GACGTTGAGGGCTGTAAGT-3'</td>
<td>383</td>
<td>95°C for 30 s, 95°C for 15 s, 62°C for 30 s</td>
<td>28</td>
</tr>
<tr>
<td>(2A3)</td>
<td>5–7</td>
<td>U-6965</td>
<td>5'-CATAACCAAGAAGTGGAAC-3'</td>
<td>322</td>
<td>95°C for 30 s, 95°C for 15 s, 62°C for 30 s</td>
<td>30</td>
</tr>
<tr>
<td>(2A3)</td>
<td>5–8</td>
<td>U-7000</td>
<td>5'-TGAGGCTTCTCCTGATGTC-3'</td>
<td>444</td>
<td>95°C for 30 s, 95°C for 15 s, 64°C for 30 s</td>
<td>27</td>
</tr>
<tr>
<td>(2A3)</td>
<td>5–8</td>
<td>U-7000</td>
<td>5'-TTAGGACAGGCTGTAAGT-3'</td>
<td>374</td>
<td>95°C for 30 s, 95°C for 15 s, 62°C for 30 s</td>
<td>27</td>
</tr>
<tr>
<td>(2A3)</td>
<td>1–10</td>
<td>U-3491</td>
<td>5'-AGTTGCTTCTCTCCTGTCC-3'</td>
<td>1327</td>
<td>95°C for 30 s, 95°C for 15 s, 64°C for 30 s</td>
<td>30</td>
</tr>
<tr>
<td>(2A3)</td>
<td>1–12</td>
<td>U-11200</td>
<td>5'-AGAATTCTTCTGGTAAGTGTGTGAA-3'</td>
<td>299</td>
<td>95°C for 30 s, 95°C for 15 s, 71°C for 30 s</td>
<td>26</td>
</tr>
<tr>
<td>(2A3)</td>
<td>1–12</td>
<td>U-11200</td>
<td>5'-AGAATTCTTCTGGTAAGTGTGTGAA-3'</td>
<td>411</td>
<td>95°C for 30 s, 95°C for 15 s, 74°C for 30 s</td>
<td>23</td>
</tr>
</tbody>
</table>

**Table II. 7-Hydroxylation of coumarin by rat esophageal explants**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Coumarin (µM)</th>
<th>Esophagus</th>
<th>7-Hydroxycoumarin (pmol/ml media)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>absent</td>
<td>n.d.</td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>absent</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>present</td>
<td>n.d.</td>
</tr>
<tr>
<td>4, 5</td>
<td>0.3</td>
<td>present</td>
<td>0.038b</td>
</tr>
<tr>
<td>6, 7</td>
<td>3.0</td>
<td>present</td>
<td>0.401b</td>
</tr>
</tbody>
</table>

a Rat esophagi were incubated with 2.5 ml of PFMR-4 medium with either 0, 0.3 or 3.0 µM coumarin for 4 h at 37°C. The presence of 7-hydroxy coumarin in the media was determined by HPLC.
b Average of two independent determinations. n.d., not detected.
Fig. 1. Semi-quantitative RT–PCR of P450 2A1, 2A2 and 2A3 gene expression in nasal mucosa (lanes 1–3), esophagus (lanes 4–6), lung (lanes 7–9) and liver (lanes 10–12). Lanes 13 and 14 are minus RT and water control. PCR products were prepared and analyzed as described in Materials and methods.

Fig. 2. Relative expression of P450 2A3 mRNA. Northern blot analysis of total mRNA (10 µg) from nasal mucosa (lanes 1–3), lung (lanes 4–6), esophagus (lanes 7–9) and liver (lanes 10–12) was carried out using either a P450 2A3 cDNA probe, 4 h exposure (A) and 48 h exposure (B), or 18S probe (C).

Discussion

This is the first study to attempt to quantify the expression of a rat esophageal P450. We report here that P450 2A3 mRNA and protein (or a closely related protein) are expressed in F344 rat esophagus. The level of mRNA expression was low relative to the rat nasal mucosa and lung, but was specific for P450 2A3. The P450 2A family consists of at least three genes in the rat, P450 2A1, 2A2 and 2A3 (27,35). We did not detect P450 2A1 or P450 2A2 in rat esophagus, nasal mucosa and lung, consistent with previous reports that these are liver-specific enzymes (27,35). The cDNA for P450 2A3 was isolated from rat lung in 1989 (24). However, nothing was

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known about P450 2A3 substrate specificity until it was expressed in a baculovirus Sf9 insect system (26). The baculovirus expressed protein was characterized by Liu et al. and reported to have overlapping but unique substrate specificity compared with P450 2A6 (26). Results from the same laboratory demonstrated that P450 2A3 is a major P450 in the rat nasal cavity and that little P450 2A3 is expressed in the lung (25). The high levels of P450 2A3 that we detected in the nasal mucosa and the low levels in the lung are consistent with the previous data.

P450 2A3 is extrahepatic and its amino acid sequence is 71 and 73% identical to P450 2A1 and 2A2, respectively (30). P450 2A3 is the rat ortholog of mouse P450 2A5, rabbit P450 2A10 and human P450 2A6, from which it differs by 23, 71 and 74 amino acids, respectively (33,34,36). We detected a related P450 in the esophagus and lung by western blot analysis using antibodies to P450 2A5 and 2A10. However, the amount of protein in each of these tissues is much lower than in the nasal mucosa. Previously, Su et al. reported the presence of significant P450 2A3 in the rat nasal mucosa, but they did not detect any P450 2A3 in the lung by western blot analysis of lung microsomes with P450 2A10 antibodies (25). The discrepancy between our results and theirs may be explained by the fact that we analyzed 15 times more microsomal lung protein. We detected similar amounts of P450 2A protein in the esophagus and lung, even though the amount of P450 2A3 mRNA in esophagus was 60-fold lower than in the lung. One explanation for this difference is that the antibodies used are reacting with a P450 2A protein distinct from 2A3, whereas the RT–PCR analysis is specific for P450 2A3.

In a previous report, we proposed that a P450 enzyme closely related to P450 2A6 and 2A10 (i.e. a coumarin 7-
hydroxylation of 10 metabolites. The extent of methylene metabolism by esophageal explants was carried out in our laboratory under the same conditions as the coumarin metabolism study reported here (28). The earlier study of NBzMA metabolism by esophageal explants was carried out in part by the Ohio State Cancer Center Grant, P30-CA16058, and in part by the OSU Cancer Center. The expression level of P450 2A3 in the rat esophagus is low, as is the level of coumarin 7-hydroxylase. Yet, rat esophageal explants efficiently metabolize NBzMA (28). The earlier study of NBzMA metabolism by esophageal explants was carried out in our laboratory under the same conditions as the coumarin metabolism study reported here (28). The extent of methylene hydroxylation of 10 μM NBzMA was 2.5 nmol/ml medium in the earlier study whereas in the present study the extent of 7-hydroxylation of 3 μM coumarin was 0.40 pmol/ml medium. More recently, we compared the rates of coumarin and NBzMA metabolism in esophageal microsomes and have found an even greater difference (unpublished data). Therefore, if, as the available data suggest, P450 2A3 is an efficient coumarin 7-hydroxylase, there must be an enzyme other than P450 2A3 responsible for activating of NBzMA in the rat esophagus.

Mouse P450 2A4 (testosterone 15α-hydroxylase) and 2A5 differ by only 11 amino acids, with Val117, Phe209 and Met65 being important for enzymatic function (33). A single amino acid mutation (Phe209→Leu209) in P450 2A5 is sufficient to alter the substrate specificity for coumarin 7-hydroxylase activity to the characteristic high steroid 15α-hydroxylase activity of P450 2A4 (37). Furthermore, Val117 appears to be located at a particularly critical position in the active site for coumarin 7-hydroxylase activity (37). Similarly, rabbit P450 2A10 and 2A11 differ by only eight amino acids but their catalytic properties are quite different. P450 2A10 catalyzes the 7-hydroxylation of coumarin at a rate 10 times faster than P450 2A11 (38). Leu104 and Val117 located in the proposed substrate recognition regions in P450 2A10 have been suggested to contribute to this difference (38). Human P450 2A6 and 2A7 exhibit 94% amino acid sequence similarity (39). The cDNA-expressed P450 2A7 does not possess coumarin 7-hydroxylase activity; the catalytic activities of P450 2A7 are currently unknown (39). Thus, it is plausible that the unidentifed enzyme that catalyzes the hydroxylation of NBzMA in the rat esophagus could be a member of the P450 2A subfamily that is highly homologous to P450 2A3. This P450 2A may only differ from P450 2A3 by a few specific amino acid differences similar to those observed for other coumarin 7-hydroxylase/steroid hydroxylase pairs. The presence of another P450 2A enzyme would explain the relative difference observed between P450 2A3 mRNA levels in the esophagus and lung compared with the similar levels of P450 2A protein present in these two tissues. Although the sequences of the cDNAs obtained by RT–PCR analysis of esophageal P450 2A3 code for one amino acid change in the 448 amino acids coded, it seems most likely that this represents an allelic variant from the same locus of the P450 2A3 gene.

In summary, P450 2A3 does metabolize NBzMA by methylene hydroxylation and it is present in the rat esophagus. However, the low levels of protein expression and the small amount of esophageal coumarin-7-hydroxylation activity suggest that P450 2A3 is probably not the primary enzyme catalyzing the metabolism of NBzMA. Other unidentified esophageal P450s must play a significant role in NBzMA metabolism. The identification of these enzymes and their expression levels in the rat esophagus is critical to understanding the role of organ-specific metabolism in NBzMA tumorigenesis. Therefore, further studies to identify other esophageal P450s are being carried out.

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