Cytochrome P450 expression and activities in rat, rabbit and bovine tongue

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Abstract:

Xenobiotic metabolism in the tongue has received little attention in the literature. In the present study, we report a comparative analysis of constitutive cytochrome P450 (CYP) expression and activities in the tongue. First we compared catalytic activities of rabbit, rat and bovine tongue samples using the probe substrates 4-nitrophenol, 1-phenylethanol, caffeine and 7-ethoxycoumarin. Rabbit tongue samples showed the highest activities for all substrates. We then compared the activities in rat and rabbit tongue with those in the rabbit liver, along with the effects of P450 inhibitors on specific activities. Combined, the activity studies indicate that CYP1A1 is active in rabbit tongue cells, but CYP1A2, CYP3A6 and CYP2E1 are below limits of detection. RT-PCR was also used to compare mRNA levels of 11 different rabbit and six different rat P450 isoforms in the tongue to those in the liver of these two species. Only CYP2E1, CYP1A1 and CYP4A4 were detected at significant levels in the rabbit tongue. None of the six rat isoforms probed were observed in the tongue. Although 4-nitrophenol activity was observed in the rabbit tongue samples, the kinetic parameter Km was inconsistent with the involvement of CYP2E1. We suggest that although CYP2E1 is expressed in the tongue, it is rapidly degraded in this organ, and the nitrophenol hydroxylation and caffeine hydroxylation we observe is the result of activity of CYP1A1.

Keywords:

Activity; Bovine; Cytochrome P450; Rabbit; Rat; RT-PCR; Tongue; Microsomes

Article:

1. INTRODUCTION

In humans, the internalization of foreign compounds occurs through three primary routes; absorption through the skin, inhalation and ingestion. Although the skin and nasal mucosa have been studied extensively in terms of their xenobiotic metabolizing potential, metabolism in the oral cavity and in particular, the tongue, is poorly characterized (Duell et al., 1992; Ahmad et al., 1996; Katiyar et al., 2000; Ding and Coon, 1988; Jenner and Dodd, 1988; Nef et al., 1989). Consequently, the extent to which this metabolism may contribute to development of oral diseases such as cancer remains uncertain.

Several clinical studies have examined the risk of developing oral type cancers in relation to the occurrence of various polymorphic CYP genes. For example, Park et al. (1997) observed a higher incidence of oral cancer in individuals with the CYP1A1 exon 7 ile:val polymorphism, implicating $P450_{1A1}$ in detoxification. Likewise, studies involving $P450_{2D6}$ and $P450_{2E1}$ showed similar correlation between mutant CYP alleles and the risk of developing oral cancer (Worrall et al., 1998; Hung et al., 1997). The interpretation of these findings is that CYP enzymes present in the oral cavity have a protective effect against certain unknown oral carcinogens. In contrast, it has been reported that high levels of aromatic hydrocarbon hydroxylase (AHH) activity, and a greater level of inducibility of this activity, have been associated with an increased risk of developing oral carcinoma as a result of exposure to the carcinogenic compounds found in tobacco products (Andreasson et al.,

1985). Furthermore, the inhibition of AHH activity was shown to inhibit cell proliferation in cultured mouse tongue epithelial cells (Gijare et al., 1990).

The identification of a variety of cytochrome P450 enzymes in oral tissue has been addressed using several different techniques. For example, the expression of seven different human cytochrome CYP isoforms were monitored in cultured oral epithelial cells using RT-PCR, and it was shown that CYPs 1A1, 1A2, 2E1, 2B6, 2D6 and 3A were expressed in these cells (Farin et al., 1995). Of the isoforms examined in this study, only 2A6 was absent. A subsequent investigation demonstrated the expression of a similar array of isoforms as well as EROD and MROD activities in cultured human buccal cells (Vondracek et al., 2001). These studies provided strong support for the expression of CYP genes in the oral epithelium, however, they did not specifically address the tongue. The use of CYP2E1-specific antibodies and whole-body autoradiography of rat cross-sections has provided evidence for the presence of CYP2E1 enzyme in the tongue mucosa of ethanol treated rats, but not in control rats (Shimizu et al., 1990). Furthermore, both in vivo and in vitro metabolism of 14C-carbon tetrachloride has been observed in the rat tongue mucosa, and cultured mouse tongue epithelial cells have been shown to display AHH activity characteristic of the 1A family (Tjalve and Lofberg, 1983). Combined, these finding suggest that cytochrome P450 enzymes (1) are present, (2) can be induced and (3) play a direct role in xenobiotic metabolism, in the oral cavity. Still missing, however, is a comprehensive analysis of CYP expression and activity in the tongue.

The goal of the current study is to provide a comparative analysis of CYP expression and activities in tongue tissue from several different animal models. The substrates chosen for this study included 1-phenylethanol as a general probe, caf feine as a probe of 1A2 and 3A activities, 4- nitrophenol as a CYP2E1 substrate and 7-ethoxycoumarin as a CYP1A probe (Vaz and Coon, 1994; Butler et al., 1989; Gu et al., 1992; Morita et al., 1998; Amato et al., 1998; Raner et al., 2002). We chose the rabbit and rat as models because much is known concerning xenobiotic metabolism in these two species. Furthermore, it is of interest to know whether P450 expression in the cow tongue is similar to that in the more traditional animal models. The results from activity assays obtained for the rat, rabbit and bovine tongues were compared to those for rabbit liver samples prepared in an identical fashion. In addition, expression of six rat CYPs and 11 rabbit CYPs was probed in the tongues of these two species by RT-PCR, in an effort to correlate observed activities with CYP gene expression in the tongue.

2. MATERIALS AND METHODS

2.1. Total RNA isolation and RT-PCR analysis

The SV Total RNA Isolation System protocol (Promega Co., Madison, WI) was used for isolation of total RNA. The protocol involved disruption of cells, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity and finally removal of proteins and degradation of residual DNA by nuclease digestion. Reverse transcription of 10 µg of RNA to a corresponding amount of cDNA was carried out using a First-Strand cDNA Synthesis Kit (Amersham Biosciences Co., Piscataway, NJ).

The PCR reactions were performed in a final volume of 50 ml consisting of 5 ml cDNA, 5 ml 10 x PCR buffer, 200 µM of each deoxynucleotide triphosphate, 2.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Promega Co.) and 50 pmol each of the forward and reverse primers. The primers for the rabbit 2E1 isoform were previously reported (Peng and Coon, 1998). All other rabbit primers were designed with the aid of PRIMER PREMIER software program (Premier Biosoft International). Primer sequences, expected PCR fragment length and annealing temperatures for the remaining rabbit P450 isoforms examined are listed in Table 1. The primers for RT-PCR involving the rat isoforms 1A1, 1A2, 1B1, 3A1, 3A2 and 2E1 were taken from the literature and amplification conditions were as reported (Walker et al., 1999; Gushchin

Table 1
Primer sequences, expected PCR product sizes and annealing temperatures for RT-PCR primers designed for rabbit tongues in this study

Primer[a]	Sequence	Product (bp)	Annealing temperature (°C)
1A1	5′ GGTCACAACTGCCATCTCG 3′ 5′ GTTCTGCCACTGGTTCACG 3′	280	59
1A2	5′ GGGAGCACTATGAGGACTTCG 3′ 5′ GGTTGATTTGCCACTGGTTTAT 3′	441	57
3A6	5′ CCCAATCAATTATCATTCTC 3′ 5′ ATTCATCAGGCTCAGTCC 3′	348	51
2A10	5′ GGGACCGCTTTGACTACGA 3′ 5′ GGTTCCTGCCAATCACTCG 3′	453	57
2A11	5′ CTGACCACGCTCAACCTC 3′ 5′ GTTCCTGCCAATCACTCG 3′	138	54
2B4	5′ CATCACCCAGAGCGTAGA 3′ 5′ ATGACATAGCCTCGGAAC 3′	414	50
2J1	5′ CGCCTTTCTGTTCTTTGC 3′ 5′ TCCTGAATGCGTTCCTCT 3′	405	50
2C1	5′ AGCATGTGATTGGCAGAC 3′ 5′ CTCATAGGAGGGAGGGAC 3′	470	50
4A4	5′ GAACTCCCACTCCTACATC 3′ 5′ CCTCTTCCTCCTGACCTT 3′	226	54
4B1	5′ CCCACTCACTGGCTGTTT 3′ 5′ CGTAGTAGCTGCTGTCCCT 3′	490	55

[a] The primers for the rabbit 2E1 isoform were taken from the literature (Peng and Coon, 1998).

et al., 1999; Yoo et al., 1997). The reaction mixtures were heated at 95 °C for 4 min and then cycled 30 times through a 1 min denaturation step at 94 °C, a 1 min annealing step at a different temperature for respective isoforms, and a 2-min extension step at 72 °C in a Perkin Elmer DNA Thermal Cycler (Wellesley, MA). A 4 min extension time at 75 °C was included at the end of 30 cycles, and this was followed by incubation at 4 °C for an indefinite period. Isolated RNA from rabbit liver cells was used as a positive control in each RT-PCR experiment for the respective CYP isoforms. An aliquot of 20 µl from each reaction was separated on an 8% acrylamide gel and visualized by ethidium bromide staining. All PCR products were of the correct size, indicating that the results reflected amplification of cDNA and not genomic DNA. Where negative results were obtained additional experiments were carried out using 40 cycles rather than 30, and in all cases, results were still negative.

2.2. Preparation of tongue microsomes

Bovine tongues were purchased from Randolph Packing Company (Ashville, NC), where they were removed from the cow, immediately placed on dry ice and transported to the lab. Rabbit and rat tongues, along with rabbit and rat livers, were purchased from Pel-Freez Biologicals Inc. (Rogers, AR). The bovine microsomes were prepared from the anterior region of a single tongue using a variation of a published protocol (Guengerich, 1994). Tissue samples were cut into small 1-cm cubes and homogenized in 0.1 M tris-acetate buffer (pH 7.4) containing 0.1 M KCl, 1.0 mM EDTA and 5 mg/l BHT, with a hand held electric Bio Homogenizer (Fisher Scientific). Samples were kept on ice throughout the homogenization procedure. Cell debris was removed by centrifugation at 10 000 x g. The supernatant was then centrifuged at 100 000 x g for 1 h at 4 °C. The resulting pellet was re-suspended in 1.0 M potassium phosphate buffer (pH 7.4) containing 1.0 mM EDTA and 5 mg/l BHT, and centrifuged once again at 100 000 x g for 1 h. The pellet at this point was suspended in 10.0 mM tris-acetate buffer (pH 7.4) containing 1.0 mM EDTA and 5 mg/ml BHT. For preparation of rat and rabbit tongue microsomes, 100 rat (or 35 rabbit) tongues were homogenized in the 0.1 M tris-acetate buffer described above, using a Waring blender. A procedure that was identical to that for the bovine tongue microsomes was then followed thereafter. Rabbit liver microsomes were also prepared, for control reactions, by the same method.

2.3. Catalytic assays

The substrates, caffeine and 4-nitrophenol, along with the reaction products, acetophenone, 4-nitrocatechol, 1,3,7-trimethyluric acid, 1,3-dimethyluric acid, 1,7-dimethyluric acid and 3,7-dimethyluric acid, were purchased from Sigma Chemical Co. (St. Louis, MO), as was NADPH. 1-Phenylethanol was purchased from Fluka Chemie (Switzerland) and purified according to a published procedure (Vaz and Coon, 1994). For the analysis of 4-nitrophenol oxidation, tongue microsomes were incubated with 4-nitrophenol concentrations ranging from 0.1 to 50 mM as reported in the literature (Larson et al., 1991). For the bovine microsomes, reactions were carried out for 2.5 h at 37 °C, whereas rat and rabbit assays were performed for 40 min at 37 °C. HPLC analysis using a Hai-Sil C18 column and an isocratic mobile phase consisting of 35 acetonitrile and 65% H2O, both containing 0.1% TFA, was effective in the separation of the product 4-nitrocatechol from substrate.

Caffeine metabolism was monitored using a published procedure, with slight modification (Morita et al., 1998). Tongue microsomes were incubated with caffeine concentrations ranging from 1.0 to 60.0 mM at 37 °C in 100 mM phosphate buffer, pH 7.4, containing 1.0 mM NADPH. All incubations with tongue microsomes were carried out for 2 h prior to the addition of 0.4 vol. 6% perchloric acid to quench the reaction. No extraction of the products was required and HPLC conditions were as described previously (Morita et al., 1998). Detection of the 8-hydroxy product was at 285 nm, while the N-demethylated products were detected at 270 nm. The HPLC system used has been described previously (Raner et al., 2000). The reaction times for each assay were selected on the basis of the linearity of the reaction over that time period. The oxidation of 1- phenylethanol by rabbit and bovine tongue microsomes was performed essentially as described in the literature, except reactions times were 40 min and 2.5 h for the rabbit and bovine tongue microsomes, respectively (Vaz and Coon, 1994). The 7-ethoxycoumarin O-deethylase activity (ECOD) was measured as described (Raner et al., 2002).

2.4. CO inhibition and O_2 dependence

With rabbit tongue microsomes, the dependence of the observed catalytic activities on O_2 and inhibition of this activity by CO were determined. Reactions involving 1-phenylethanol and 4-nitrophenol as substrates were set up as described above, only the mixtures were placed in screw-cap vials with silicone sealed tops. The reactions were degassed and purged with CO prior to the addition of a degassed sample of NADPH using a gas-tight syringe. Reactions in which the vial remained open during the incubation, and no CO was added, were also carried out as controls.

2.5. Inhibition of catalysis by ketoconazole and disulfiram

The inhibitors ketoconazole and disulfIram were purchased from Sigma Chemical Co. Control reactions involving the substrates caffeine and 4- nitrophenol were carried out as described above with rabbit and bovine tongue microsomes along with rabbit liver microsomes. Caffeine concentrations were chosen that were in the range of the value of K_m for that particular type of microsomal sample, as determined in this study. The caffeine concentrations selected were 20 mM for the rabbit liver microsomes, 35 mM for the bovine tongue microsomes and 50 mM for the rabbit tongue microsomes. The rat tongue microsomes showed very low activity in this assay and were not used in the inhibition study. Disulfiram concentrations ranging from 20 to 500 µM were added to a second identical set of reaction mixtures, and activities were compared to those of the control reactions. A similar set of reactions was carried out using the same range of disulfiram concentrations, but with 4-nitrophenol as the substrate. Substrate concentrations were chosen that were near the measured Km for those microsomes. These concentrations were 0.050 mM for rabbit liver, 12 mM for cow tongue, 10 mM for rabbit tongue and 4.0 mM for rat tongue.

Inhibition of caffeine and 4-nitrophenol oxidation by ketoconazole was also examined. In these experiments, either 5.0 or 0.50 µM ketoconazole was included in the reaction mixtures described above, and all other reaction conditions remained

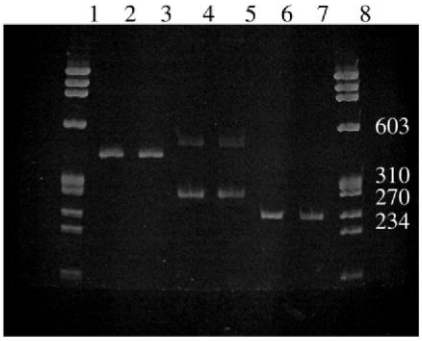

Fig. 1. Acrylamide gel (8%) showing RT-PCR products for P450s 1A1, 4A4 and 2E1 in rabbit tongue and liver. Lanes 1 and 8: molecular weight standards; Lane 2: 2E1 expression in the rabbit liver; Lane 3: 2E1 expression in rabbit tongue; Lane 4: 1A1 expression in rabbit liver; Lane 5: 1A1 expression in the rabbit tongue; Lane 6: 4A4 expression in the rabbit liver; Lane 7: 4A4 expression in the rabbit tongue. Expected PCR fragment lengths were 410, 280 and 226 bp for 2E1, 1A1 and 4A4, respectively.

the same. Once again, since rat tongue microsomes showed very little activity with caffeine as a substrate, they were omitted from this study.

3. RESULTS

3.1. RT-PCR analysis of rabbit and rat tongues

A total of six different rat (1A1, 1A2, 2E1, 1B1, 3A1 and 3A2) and 11 rabbit (1A1, 1A2, 2A10, 2A11, 2B4, 2C1, 2E1, 2J1, 3A6, 4A4 and 4B1) CYP isoforms were probed in the rat and rabbit tongues using RT-PCR. Expression of all 6 rat and 8 of the 11 rabbit isoforms (1A1, 1A2, 2B4, 2C1, 2E1, 3A6, 4A4 and 2J1) was observed in the livers of these two species; however, in tongue samples, only the rabbit 2E1, 1A1 and 4A4 were detected. Fig. 1 shows the polyacrylamide gel electrophoresis analysis of the RT-PCR products from the 2E1, 1A1 and 4A4 specific reactions, in both rabbit tongue and liver. The predicted fragment sizes of 410, 280 and 226 bp for the 2E1, 1A1 and 4A4 isoforms, respectively, are consistent with the data obtained. The reaction involving primers for the 1A1 isoform were not as definitive as those for 2E1 and 4A4. A band was observed in the gel for both liver and tongue samples that appeared to correspond to the correct fragment size; however, a larger size fragment was also observed. The larger fragment, which presumably resulted from non-specific primer binding, could not be eliminated by changing the annealing conditions.

3.2. ECOD activity

Rabbit liver microsomes catalyzed the efficient conversion of 7-ethoxycoumarin to 7-hydroxycoumarin, as did rabbit tongue samples. The activities followed the Michaelis–Menten kinetic model with calculated values of 0.07 and 0.04 nmol/min/mg for V_{max} and 4.0×10^{-4} and 9.0×10^{-4} mM for Km in the liver and tongue, respectively (data not shown). Activities in the rat and bovine were not observed.

3.3. Metabolism of 1 -phenylethanol

Both the bovine and rabbit tongue microsomes catalyzed the oxidation of 1-phenylethanol to acetophenone at a substrate concentration of 10 mM. This activity was NADPH and time-dependent, and was significantly higher in the rabbit tongue than in the bovine tongue. For example, under identical conditions, rabbit tongue microsomes catalyzed the oxidation at a rate of 0.033 nmol/min/mg, whereas this value was 0.011 nmol/min/mg for the bovine microsomes. The parameters K_m and V_{max} were not determined for this activity.

3.4. Metabolism of 4-nitrophenol

Bovine tongue microsomes catalyzed the NADPH-dependent hydroxylation of 4-nitrophenol; however, the activity did not follow simple Michaelis–Menten kinetics (Fig. 2). The reaction was linear for nearly 4.0 h at 37 °C, using 1.0

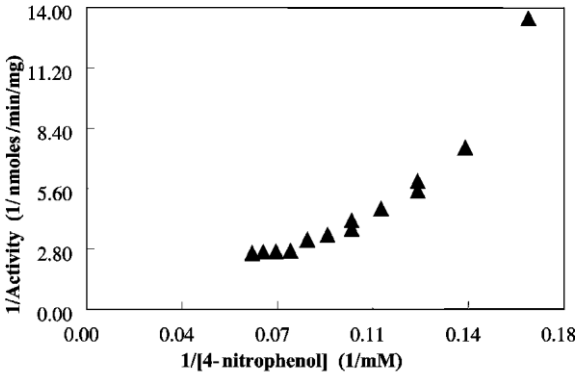

Fig. 2. Double reciprocal plot for the hydroxylation of 4-nitrophenol by bovine tongue microsomes.

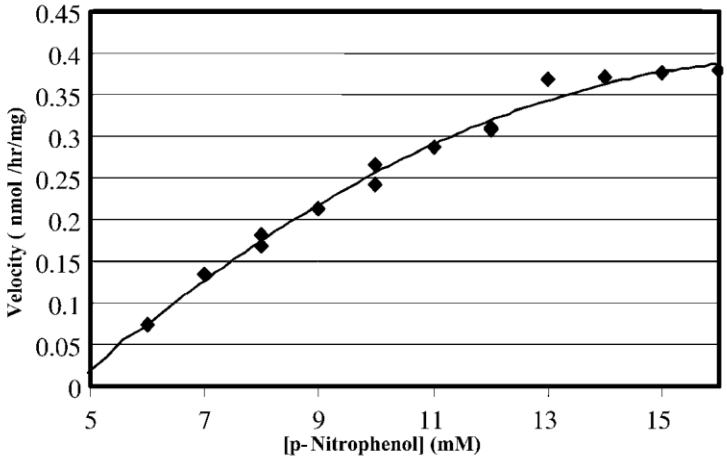

Fig. 3. Plot of activity vs. 4-nitrophenol concentration for bovine tongue microsomes. Reactions were carried out for 2.5 h at 37 °C in the presence of 1.0 mM NADPH, at pH 7.4.

mM NADPH. Saturation did occur, however, at substrate concentrations above 12 mM, and under these conditions, the microsomes had an apparent V_{max} of 0.010 nmol/min/mg protein (Fig. 3). Half maximal activity occurred at a substrate concentration of 8.0 mM. Both rabbit and rat tongue microsomes gave linear Lineweaver–Burk plots, in contrast to the bovine microsomes. Furthermore, reactions with the rat and rabbit microsomes were linear only up to 40 min, at which point the reaction rate began to decrease. Table 2 compares the kinetic parameters observed in the hydroxylation of 4-nitrophenol, catalyzed by rabbit, rat and bovine tongue microsomes, in addition to those for the rabbit liver microsomes. Values for V_{max} in the tongue microsomes ranged from 0.010 nmol/min/mg in the bovine to 0.058 nmol/min/mg in rabbit. Furthermore, K_m values were high, ranging from 0.72 mM in rat to 8 mM in the bovine tissue. This is in contrast to the rabbit liver microsomes, for which V_{max} and K_m values were determined to be 10.3 nmol/min/mg protein and 0.040 mM, respectively. No activity was observed in any of the tongue samples when 4-nitrophenol concentrations below 200 μM were used.

3.5. Caffeine metabolism

The 8-hydroxylation of caffeine was observed in the bovine, rat and rabbit tongue microsomes, as well as in rabbit liver microsomes. In the bovine, rabbit and rat tongue, none of the N-demethylated products (theophylline, paraxanthine or theobromine) were produced enzymatically. The formation of 1,3,7-trimethyluric acid was linear for up to 4 h at 37 °C in the bovine microsomes, but activity in the rabbit tongues decreased beyond 40 min. Activities were, therefore, monitored at the 2.5 h time point for bovine microsomes, and at 40

min for the rabbit microsomes. Lineweaver–Burk plots for all reactions were linear, giving V_{max} and K_m data as summarized in Table 3. As

Table 2
Kinetic parameters for oxidation of 4-nitrophenol

Microsomes	K_m[a] (mM)	V_{max}[a] (nmol/min/mg)
Rabbit liver	0.040	10.3
Rabbit tongue	15.7	0.058
Rat tongue	0.72	0.011
Bovine tongue	~8[b]	0.010[b]

[a] Numbers of K_m and V_{max} are means from two independent trials (duplicates were within 5% of the mean value).
[b] Values was estimated based on saturation behavior.

Table 3
Kinetic parameters for the oxidation of caffeine

Microsomes	K_m[a] (mM)	V_{max}[a] (nmol/h/mg)
Rabbit liver	25.0	198
Rabbit tongue	77.4	3.9
Rat tongue	N.D.[b]	N.D.[b]
Bovine tongue	63.3	1.3

[a] Numbers of K_m and V_{max} are means from two independent trials (duplicates were within 5% of the mean value).
[b] N.D. indicates activity was too low to measure.

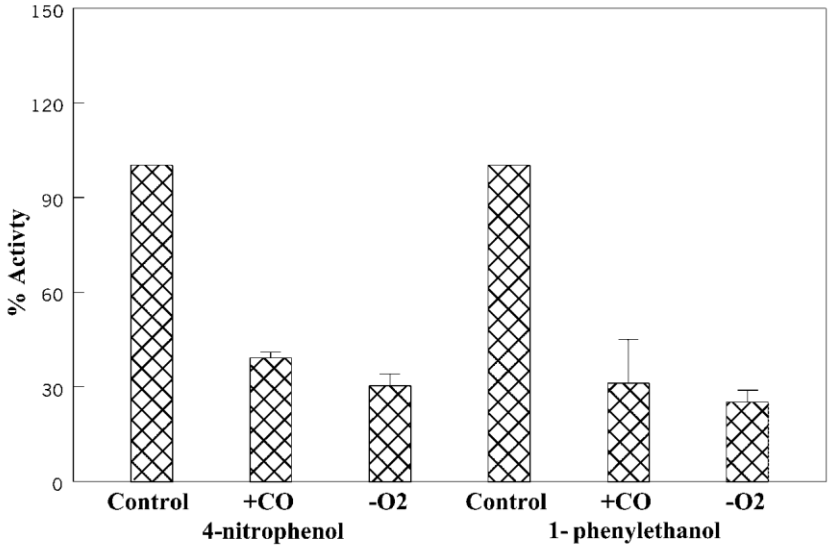

Fig. 4. Bar graph showing the effects of CO and oxygen depletion on the oxidation of 4-nitrophenol and 1-phenylethanol by rabbit tongue microsomes. The catalytic activities of rabbit tongue microsomes in metabolizing 10 mM 4-nitrophenol and 10 mM 1-phenylethanol was inhibited by 61.6±1.1% and 69.1±14.5%, respectively, with addition of CO (Student's *t*-test; $P<0.05$). Values = mean ± average deviation for two independent experiments.

with 4-nitrocatechol oxidation, maximum rates for caffeine hydroxylation, catalyzed by tongue microsomes, were low, ranging from 1.3 nmol/h/mg in the bovine tongue to 3.9 nmol/h/mg in the rabbit. K_m also varied, with 77 mM representing the highest value in rabbit and 63 mM the lowest, in bovine. The corresponding parameters for rabbit liver microsomes were 198 nmol/h/mg protein and 25 mM for V_{max} and K_m, respectively.

3.6. Dependence on O_2 and inhibition by CO

Experiments were carried out using rabbit tongue microsomes in order to further confirm the involvement of cytochrome P450 enzymes in these processes. Rabbit tongue microsomes appeared to be the most active of the three species examined so they were chosen for this study. The effect of CO on the oxidation of 4-nitrophenol and 1- phenylethanol by rabbit tongue microsomes is shown in Fig. 4. Approximately 60% of the 4- nitrophenol

hydroxylation and 70% of the caffeine hydroxylation activities were lost in the presence of saturating CO, relative to reactions in which CO was not present. Purging the sample with N_2 also had an inhibitory effect on the catalytic rate, illustrating the dependence of the reaction on molecular oxygen.

3.7. Inhibition of activities by disulfiram

Disulfiram inhibited the oxidation of 4-nitrophenol by tongue and liver microsomes at concentrations ranging from 20 to 500 μM as shown in Table 4 (Kharasch et al., 1993). In the oxidation of 4-nitrophenol, rabbit liver microsomes were inhibited to a much greater extent than the rabbit, rat or bovine tongue microsomes. For example, 20 μM disulfiram had no inhibitory effect at all on this activity in rabbit tongue, and inhibited rat tongue microsomes by only 15%. In contrast, 51% of the activity was inhibited in the rabbit liver microsomes. At 500 μM disulfiram, inhibition ranged from 52% in the rat tongue to 85% in the rabbit liver. Caffeine hydroxylation was also inhibited by disulfiram, with nearly 100% inhibition of the bovine and rabbit tongue microsomes, along with rabbit liver microsomes, in the presence of 500 μM inhibitor. The inhibition of caffeine hydroxylation in rabbit liver microsomes over a range of disulfiram concentrations matched very well with the corresponding inhibition pattern observed in the rabbit tongue microsomes.

3.8. Inhibition of activities by ketoconazole

Ketoconazole has been described as a specific inhibitor of CYP3A and was, therefore, chosen to

Table 4
Effect of disulfiram on caffeine and 4-nitrophenol (*p*NP) oxidation by bovine, rabbit and rat tongue and rabbit liver microsomes

Microsomes	[Disulfiram] (μM)	[Caffeine] (mM)	Percentage inhibition[a]	[*p*NP] (mM)	Percentage inhibition[a]
Rabbit liver	20	20	19.8	0.050	51.1
	100	20	75.4	0.050	82.1
	500	20	100.0	0.050	87.9
Rabbit tongue	20	50	22.0	10.0	–
	100	50	72.6	10.0	28.8
	500	50	100	10.0	74.4
Rat tongue	20	–	–	4.0	15.1
	100	–	–	4.0	17.4
	500	–	–	4.0	52.0
Bovine tongue	20	35	49.7	12.0	14.5
	100	35	86.6	12.0	37.7
	500	35	100.0	12.0	76.5

[a] Numbers are means from two independent trials (duplicates were within 5% of the mean value).

probe the involvement of this isoform in caffeine metabolism in the tongue (Wohcikowski et al., 2003). Although 5.0 μM ketoconazole inhibited caffeine hydroxylation by approximately 60% in the rabbit liver samples, inhibition of this activity in the rabbit tongue microsomes was not observed, as indicated in Table 5. In contrast, 4-nitrophenol hydroxylase activity was inhibited in the rabbit and bovine tongue microsomes to nearly the same extent as in the liver microsomes (32% in liver, and 23 and 21% in bovine and rabbit tongues, respectively), and to a slightly greater extent (46%) in rat tongue microsomes.

4. DISCUSSION

Although the liver is a major site within the body for P450 enzymes, there are various forms of cytochrome P450 expressed in other organs and tissues at significant levels such as skin, nasal mucosa and gastro-intestinal tract (Guengerich and Mason, 1979; Duell et al., 1992; Ding and Coon, 1988; Lechevrel et al., 1999). Previous studies using rats as models have provided evidence for CYP-dependent activities in the tongue tissue, but to our knowledge, this is the first time that the CYP-dependent activities in rabbit and bovine tongues have been reported, and the first report of CYP mRNA detection in this organ (Tjalve and Lofberg, 1983; Gijare et al., 1990; Shimizu et al., 1990).

Our results from RT-PCR suggest that mRNA for CYP1A1, CYP2E1 and CYP4A4 were produced in rabbit tongue. The mRNA transcripts for rabbit CYPs1A2, 2B4, 2C1, 2J1 and 3A6 were detected in the liver, but were not found in the

Table 5
Effect of ketoconazole on caffeine and 4-nitrophenol (*p*NP) oxidation by bovine, rabbit and rat tongue and rabbit liver microsomes

Microsomes	[Ketoconazole] (μM)	[Caffeine] (mM)	Percentage inhibition[a]	[*p*NP] (mM)	Percentage inhibition[a]
Rabbit liver	0.50	20	25.9	0.050	–
	5.0	20	59.4	0.050	31.9
Rabbit tongue	0.50	50	0	10.0	7.4
	5.0	50	0	10.0	20.5
Rat tongue	0.50	–	N.D.[b]	4.0	12.0
	5.0	–	N.D.[b]	4.0	46.2
Bovine tongue	0.50	35	7.4	12.0	–
	5.0	35	11.7	12.0	22.6

[a] Numbers are means from two independent trials (duplicates were within 5% of the mean value).
[b] N.D. indicates activity was too low to measure.

tongue, consistent with a lower basal level of these isoforms in the tongue. Interestingly, no CYP transcripts could be detected in the rat tongue. This was not due to the use of ineffective primers, as the primers were tested using rat liver samples and were found to amplify transcripts in this tissue. However, one could make the argument that the rat primers were less efficient than the rabbit primers, which could raise the limit of detection.

Based on previous studies, the compound, 1- phenylethanol, has been shown to be metabolized to acetophenone by six different purified rabbit liver CYP isoforms; 1A1, 1A2, 2E1, 3A6, 2B4 and 2C3 (Bestervelt et al., 1995). Therefore, it would appear that this compound represents a very good general probe for cytochrome P450 activity. Bovine and rabbit tongue microsomes showed significant activity in the oxidation of 1-phenylethanol, and this activity was dependent on NADPH. In addition, CO was an inhibitor of this activity, which suggested strongly that the observed activity was CYP-dependent. Activity in the rat tissue was below the level of detection, consistent with the mRNA data.

CYP2E1 has been detected in several extrahepatic tissues of different species such as human lung and rat testis (Nishimura et al., 2003; Jiang et al., 1998). To date, CYP2E1 is the only isoform that has been positively identified in ethanol- induced rat tongue epithelial tissue using CYP2E1 specific antibodies (Shimizu et al., 1990). In the present study, we have shown for the first time that the CYP2E1 mRNA is expressed in rabbit tongue at significant levels by RT-PCR technique. Moreover, incubation of the rat, rabbit and bovine tongue microsomes with 4-nitrophenol resulted in the time dependent formation of the product 4- nitrocatechol. Notably, the activity for bovine tongue microsomes did not follow simple Michaelis–Menten kinetics which might be due to the involvement of more than one isoform. The Km values obtained for the tongue microsomes ranged from 0.72 mM for rat to 15.7 mM for rabbit. For the rabbit tongue, this represents roughly a 400- fold increase in Km relative to rabbit liver microsomes, which had a Km of 0.040 mM. In fact, at 4-nitrophenol concentrations that saturated 2E1 in the liver microsomes, 200 μM, no activity at all was seen in any of the tongue microsomes. The kinetic data suggest that the 4-nitrophenol hydroxylation activities did not involve CYP2E1, rather at high substrate concentration, other isoforms can take part in this hydroxylation. This interpretation is consistent with immunohistochemical results reported by Shimizu et al. showing that CYP2E 1 enzyme was not present in control rat tongues; the 2E1 enzyme could only be detected after ethanol treatment in the tongue mucosa (Shimizu et al., 1990).

To further explore this activity we evaluated the CYP2E1-specific inhibitor, disulfiram, as an inhibitor of 4-nitrophenol oxidation in the tongue microsomes. As expected, disulfiram inhibited 4-nitrophenol oxidation in the control rabbit liver microsomes by 50% at low (20 μM) inhibitor concentration. In contrast, no inhibition

was observed in tongue microsomes with the same concentration of disulfiram. There is evidence that post-transcriptional and post-translational mechanisms play a major role in the regulation of CYP2E 1 and that this enzyme is rapidly degraded in many tissue types (Moncion et al., 2002; Zhukov and Ingelman-Sundberg, 1999). Consequently, the observed transcription of CYP2E1 in rabbit tongues may not translate to observable levels of 2E1 enzyme. This is significant in relation to potential interactions in the oral cavity involving ethanol consumption, since protein stabilization of 2E1 by alcohol is known to occur, and CYP2E1 is a major activator of carcinogenic nitrosamines found in tobacco product (Tsuneoka et al., 1992).

An additional substrate, caffeine, was chosen in an attempt to further probe the catalytic properties of tongue microsomes. It has been reported that caffeine can be used as a marker for CYP1A2- and CYP3A-related activities (Butler et al., 1989; Gu et al., 1992; Morita et al., 1998). CYP1A2 catalyzes N-demethylation at positions 1, 3 or 7 from the purine ring of caffeine, whereas 3A enzymes have been associated with 8-hydroxylation of caffeine in rats and humans. The inability of the bovine, rabbit and rat tongue microsomes to catalyze caffeine N-demethylation further support the RT-PCR data that 1A2 isoform was not expressed constitutively in this tissue. Interestingly, the product 1,3,7-trimethyluric acid (8-hydroxy caffeine) was formed when caffeine was incubated with tongue microsomes and NADPH. This was the case with rat, rabbit and bovine samples. Catalytic activities in the tongue microsomes were much lower than in rabbit liver microsomes, the rat tongue being the lowest, and the Km values in the tongue were 2–3-fold higher than in the rabbit liver. Moreover, 5 μM ketoconazole did not inhibit this activity in rabbit tongue, whereas close to 60% inhibition was observed in the rabbit liver. All these observations are consistent with our conclusion that the CYP3A6 isoform was present in the rabbit liver and not in the rabbit tongue. The lack of correlation between the observed K_m in rabbit tongue vs. rabbit liver may be an indication that another isoform present in the tongue (other than 3A6) is responsible for the observed caffeine hydroxylation.

RT-PCR results suggested that mRNA for CYP1A1, a very important isoform with respect to the bioactivation of many procarcinogens, was expressed in the rabbit tongue. Aryl hydrocarbon hydroxylase activity has been observed in primary embryonal mouse tongue epithelial cells, an activity associated with the CYP1A subfamily (Gijare et al., 1990). In this previous study, the polycyclic aromatic hydrocarbon benzo[a]pyrene was used as an inducer of the activities. Our studies confirmed the presence of active P4501A in the rabbit tongue as determined by ECOD activity. This activity is consistent with our interpretation of the RT-PCR data, which indicates CYP1A1 is expressed in the rabbit tongue. We also propose that the observed 4-nitrophenol hydroxylation and caffeine oxidation by the rabbit tongue is related to CYP1A1. This could explain the very large differences in Km values reported between liver and tongue samples. In addition, we can rationalize the inhibition data in the same manner, assuming both ketoconazole and disulfiram can inhibit the 1A1 isoform. There are reports in the literature that suggest disulfiram and ketoconazole are not specific for 2E1 and 3A4, respectively, rather both compounds are inhibitors of CYP 1A 1 (Lakshmi et al., 1997; Spatzenegger et al., 2000; Sai et al., 2000). Still puzzling is the total lack of observed P450 expression in the rat tongue as determined by RT-PCR. In light of these PCR data, we conclude that 1A1 is not expressed in rat tongue, rather the very low activities for nitrophenol and caffeine oxidation are the result of oxidation by another CYP isoform.

The other isoform we found in rabbit tongue was CYP4A4. This isoform has been previously reported to be expressed at low basal levels in adult rabbit lungs (McCabe et al., 2001). CYP4A4 is a hormonally induced isoform which metabolizes arachidonic acid to ω-hydroxylated products (Aitken et al., 2001). The significance of its expression in the tongue is unclear. However, there have been suggestions that one of the important functions of cytochrome P450 enzymes in extrahepatic tissues could be physiological and it may involve the metabolism of endogenous effecter ligands (Nebert, 1994).

In conclusion, clearly the tongue is an organ that receives extensive environmental exposure, and as such may represent, in part, the body's first line of defense against foreign chemical species. Our RT-PCR results indicate the expression of CYP1A1, CYP2E1 and CYP4A4 mRNA in the rabbit tongue, but failed to identify any isoforms in the rat tongue. Although RT-PCR was able to detect mRNA for CYP2E1, catalytic studies did not

confirm its presence. The results from RT-PCR do suggest that ethanol may have a significant potential for interacting with other xenobiotics in this organ. CYP1A1 activity was observed in support of the RT-PCR data, indicating AHH is a constitutive activity in the tongue. Finally, CYP4A4 was expressed according to RT-PCR data, and we believe the nature of this enzyme as a fatty acid hydroxylase indicates a role in metabolism of endogenous compounds in the tongue. The extent to which CYP metabolism may contribute to oral activation or deactivation of carcinogenic compounds in the tongue is an area that needs to be addressed further.

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