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Cytochrome P450 and Steroid Hydroxylase Activity in Mouse Olfactory and Vomeronasal Mucosa

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The aims of this study are to identify the sex steroid-metabolizing cytochrome P450 enzymes of the vomeronasal organ (VNO) and to determine the activities of VNO microsomes to metabolize estradiol, progesterone, and testosterone. Several P450 isoforms, including CYP1A2, CYP2A, CYP2B, CYP2C, CYP2G1, and CYP3A, NADPH P450-reductase, and microsomal epoxide hydrolase were detected in mouse VNO, although their expression levels were much lower than those in the main olfactory epithelium. VNO microsomes were active toward the three steroid hormones, producing metabolite profiles similar to those seen with olfactory mucosal microsomes. Thus, the mammalian VNO, a steroid hormone target tissue, contains multiple steroid-metabolizing P450 isoforms and is capable of metabolic disposition of the three major sex steroid hormones. These findings support the proposed roles of olfactory mucosal and VNO microsomal P450 enzymes in maintaining cellular hormonal homeostasis and other perireceptor processes associated with olfactory chemosensory function. © 1999 Academic Press

The main olfactory epithelium (OE) and the vomeronasal organ (VNO) are located in different parts of the nasal cavity and are implicated in different chemosensory functions. Whereas the OE is primarily involved in odorant signal transduction, the VNO is responsible for pheromone detection in many species (1, 2). The OE contains high levels of cytochrome P450 (P450) monooxygenases (3, 4) which are responsible for the remarkably high metabolic activities toward sex steroid hormones found in OE microsomes (5, 6). However, little is known about the expression of P450 enzymes or the steroid hydroxylase activities in the VNO, except for an immunohistochemical study in which CYP2A and CYP2G-related immunoreactivities were detected in rat VNO (7).

Both the OE and the VNO are target tissues for sex steroid actions (8–12). Since P450-catalyzed biotransformation may play a crucial role in maintaining homeostasis of the steroid hormones in a target tissue, we examined the expression in the OE and the VNO of several P450 isoforms known to be capable of metabolizing sex steroids. The ability of the VNO to metabolize the major sex steroid hormones was also examined and compared with the metabolic activity of the OE.

MATERIALS AND METHODS

Microsomes were prepared from the OE and the VNO according to Ding and Coon (13). Mice were decapitated according to an approved animal study protocol. The nasal passages were exposed and the VNOs were removed from 40 female ICR mice (6–11 weeks old, Charles River). The bony capsule surrounding the VNO was removed rapidly over ice. Pooled VNO tissue was divided into two groups (20 in each) and stored at −20°C. OE tissue, collected by scraping the nasal passages, was similarly pooled, divided, and stored frozen until use. Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL) with bovine serum albumin as the standard. Immunoblot analysis was performed with an ECL kit from Amersham as described previously (13). Polyclonal antibodies to mouse CYP2A5, which also recognize CYP2G1, have been described recently (14). Antibodies to rat CYP1A2, CYP2B1, CYP2C11, and CYP3A2, human CYP1B1 and CYP2D6, and rat NADPH-P450 reductase were purchased from Gentest (Woburn, MA). The antibody to rat microsomal epoxide hydrolase (mEH) was a gift from Dr. Charles Kasper of the University of Wisconsin.

Assays for testosterone, progesterone, and estradiol (E_2) metabolism were performed as described previously (6, 15). Steroid metabolites were analyzed by HPLC with use of a Waters (Milford, MA) 4-μm Nova-Pak C_{18} column (150 × 3.9 mm, i.d.) preceded by a Nova-Pak C18 precolumn cartridge. The mobile phase consisted of water, methanol, and acetonitrile. On-line radiometric detection and quantification of metabolites were performed as described previously

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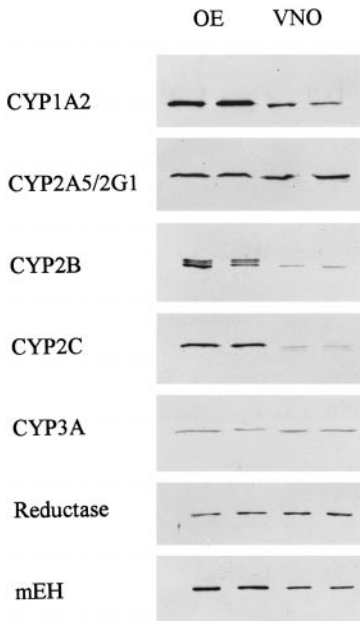

FIG. 1. Expression of P450 and other biotransformation enzymes in mouse OE and VNO. Microsomal proteins from the OE and the VNO were analyzed in duplicate on immunoblots with the following antibodies: goat anti-rat CYP1A2 (CYP1A2), rabbit anti-CYP2A5/2G1 (CYP2A5/2G1), rabbit anti-CYP2B1 (CYP2B), goat anti-CYP2C11 (CYP2C), goat anti-CYP3A2 (CYP3A), rabbit anti-rat P450 reductase (Reductase), and goat anti-rat mEH. The amounts of proteins applied were, for the OE, 0.25 μg for the blot with anti-CYP2A5/2G1 and 1 μg for the other blots, and, for the VNO, 5 μg for the blots with anti-CYP1A2, anti-CYP2A5/2G1, anti-CYP2C, and anti-CYP3A, and 2.5 μg for the other blots.

(15). The contents of reaction mixtures are described in the Table and Figure legends. [6, 7-^{3}H(N)] E_2 and [1, 2, 6, 7-^{3}H(N)] progesterone were from New England Nuclear, [1, 2, 6, 7-^{3}H(N)] testosterone was from Amersham. The sources of purified mouse CYP2A5, CYP2G1, and rabbit hepatic NADPH-P450 reductase have been described elsewhere (14). For inhibition studies, antibodies were added to the reaction mixtures and incubated on ice for 15 min prior to the addition of NADPH.

Total RNA was prepared from pooled VNO and OE of two groups of adult female mice (six per group) with use of TRI Reagent (Molecular Research Center, Cincinnati). RNA concentration was determined spectrally and the integrity of the RNA samples was ascertained by ethidium bromide staining following agarose gel electrophoresis. RNA-PCR was performed essentially as described previously (16), with a GeneAmp RNA-PCR kit from Perkin Elmer Cetus. The identity of PCR products was confirmed by DNA sequence analysis with the PCR primers.

RESULTS

To identify the P450 enzymes expressed in mouse OE and VNO, microsomal preparations were analyzed on immunoblots with antibodies to several P450 isoforms known to be active toward sex steroid hormones. As shown in Fig. 1, immunoreactive proteins with expected sizes were detected with the antibodies to CYP1A2, CYP2A5/CYP2G1, CYP2B1, CYP2C11, and CYP3A2 in both the OE and the VNO. The expression of P450 reductase and mEH was also detected in both tissues. The anti-CYP1A2 antibody cross reacts with both 1A1 and 1A2; however, the band detected in the nasal tissues was confirmed to be 1A2 by comparing its position to the 1A1 and 1A2 bands detected in mouse liver microsomes (data not shown). Two major and one minor bands were detected in the OE with the antibodies to CYP2B1, most likely corresponding to the three known mouse CYP2B isoforms, 2B9, 2B10, and 2B13 (17). However, only one band was detected in the VNO, which appeared to correspond to one of the bands detected in the OE. A single band was detected with the anti-CYP2C11 antibody in the OE, which is consistent with the known occurrence of only a single gene, *Cyp2c29*, in the mouse *Cyp2c* subfamily. Interestingly, two CYP2C-related bands were detected in the VNO, suggesting the potential occurrence of an additional mouse *Cyp2c* gene, which is expressed in the VNO. Only one band was detected in either the OE or the VNO with the anti-CYP3A2 antibody, although three *Cyp3a* genes, *3a11*, *3a13*, and *3a16*, are known in the mouse.

A single band was also detected in both the OE and the VNO with the anti-CYP2A5 antibody. This antibody is known to crossreact with mouse CYP2G1, and the two isoforms are not resolved by SDS-polyacrylamide electrophoresis (14). Thus, RNA-PCR experiments were performed to determine whether both CYP2A5 and CYP2G1 are expressed in the VNO. As shown in Fig. 2, CYP2G1 mRNA was detected in both the OE and the VNO with a pair of PCR primers derived from exons 1 and 4, respectively. No product was detected in negative control reactions in which the RNA sample was omitted during reverse transcription. CYP2A5 mRNA was also detected in both tissues in additional RNA-PCR experiments not shown.

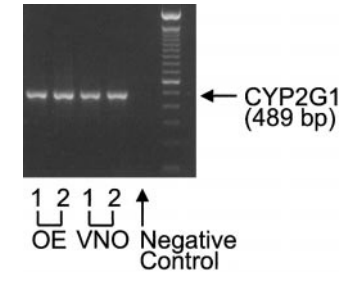

FIG. 2. Detection of CYP2G1 in mouse OE and VNO by RNA-PCR. Total RNA preparations from the OE and the VNO of two groups of mice (1 and 2) were used in reverse transcription reactions with an oligo-d$(T)_{16}$ primer. First strand cDNAs were amplified with a set of primers that produce a 489-bp DNA fragment containing exons 1–4 of the mouse *Cyp2g1* gene (forward: 5′-gccacatttcagtcct-tccagaagc-3′; reverse: 5′-gggcttgctcatctccacaaagc-3′). PCR products (10 μl/lane) were analyzed by electrophoresis in a 1.5 % agarose gel and visualized by staining with ethidium bromide. RNA was omitted in negative control reactions. A 100-bp DNA marker (BRL) were included for size determination.

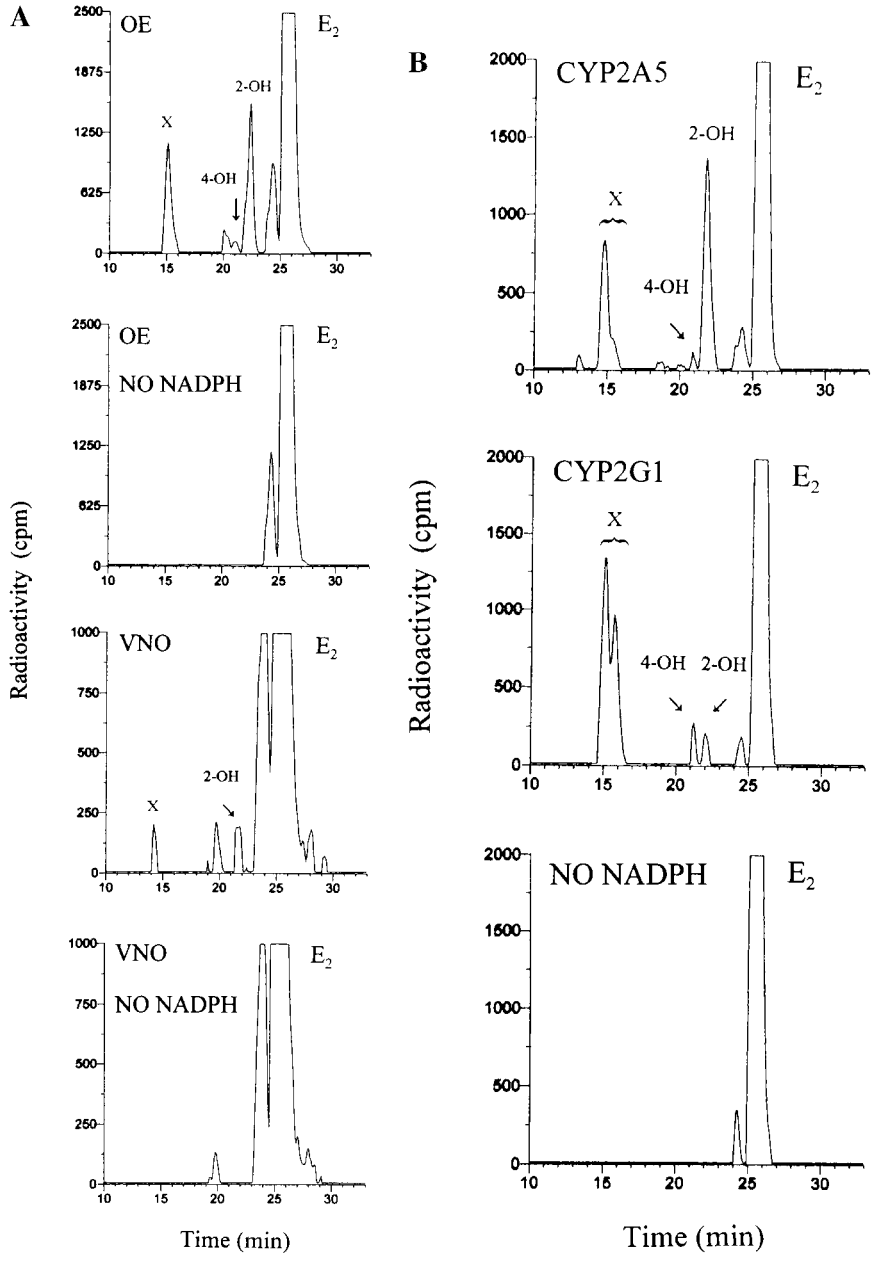

FIG. 3. Estradiol metabolites generated by OE and VNO microsomes (A) and by CYP2A5 and CYP2G1 in a reconstituted system (B). The complete reaction mixtures contained 10 μM [6,7-^{3}H(N)] E_2 (2 Ci/mmol), 50 mM phosphate buffer, pH 7.4, 0.15 mg/ml microsomal protein (A) or a reconstituted system containing 0.2 μM purified P450, 0.3 μM NADPH-P450 reductase, 30 μg/ml dilauroylphosphatidylcholine (B), and 1 mM *L*-ascorbic acid in a final volume of 125 μl. The reactions were initiated by the addition of NADPH to a final concentration of 1 mM and were carried out at 37°C for 15 min, except for reactions with VNO microsomes, in which an NADPH-generating system (containing 100 mM phosphate buffer, pH 7.4, 1.3 mM NADP, 3.3 mM $MgCl_2$, 3.3 mM glucose-6-phosphate, and 0.4 unit/ml glucose-6-phosphate dehydrogenase) was used and the mixtures were incubated for 150 min. NADPH or the NADPH-generating system was omitted in control reactions. The metabolites were analyzed using radiometric HPLC as described under Materials and Methods.

The relative band intensities were determined by densitometric analysis of the immunoblots. The immunoreactive proteins were much more abundant in the OE than in the VNO. The difference between the two tissues in the levels of expression of the various P450 isoforms varied from almost 30-fold (for CYP2C) to about 5-fold (for CYP3A) higher in the OE than in the VNO. Notably, the tissue difference in the expression level of the reductase was only about 2.5-fold.

Both OE and VNO microsomes were active in the metabolism of E_2 although the OE was much more active than the VNO (Fig. 3A and Table 1). Three NADPH-dependent metabolite peaks were detected in reactions with the OE. Two of the peaks were identified

TABLE 1

Microsomal Metabolism of Testosterone, Progesterone, and Estradiol in Mouse OE and VNO

Microsomal preparation	Rate of total metabolite formation (pmol/min/mg protein)		
	Testosterone	Progesterone	Estradiol
OE	1160 (1290, 1030)	910 (1000, 820)	130 (140, 120)
VNO	7.6 (8.9, 6.2)	10.2 (12.8, 7.6)	1.6 (1.9, 1.2)

Note. Reactions were carried out as described in the legends to Figs. 3 and 4, with the substrates at 10 μM. The rates of formation of all metabolites were determined; no efforts were made to correct for the potential loss of ^{3}H label in the unidentified products, which would result in an underestimation of the rates of product formation. The values presented are the averages of two experiments, with the individual values shown in parentheses; each microsomal sample was prepared from pooled tissues of 20 mice.

as 2- and 4-hydroxyE_2, respectively, on the basis of co-elution with standard metabolites formed by heterologously expressed human CYP1B1, which is known to produce 4-hydroxyE_2 as a major product and 2-hydroxyE_2 as a minor product (18), and by GC-MS analysis of the purified products (data not shown). The other metabolite peak (designated X) has not been identified and the shape of the peak in the reactions with OE microsomes suggests that it may represent two unresolved metabolites. This peak, which was not formed by cDNA-expressed human CYP1B1, did not comigrate with available E_2 standards including 2-, 4-, 6α-, and 16α-E_2 (not shown). In reactions with VNO microsomes, only 2-hydroxyE_2 and one unidentified product were detected (Fig. 3A). The rates of total E_2 metabolite formation in VNO microsomal reactions were about 80 times lower than those in the OE (Table 1).

Purified CYP2A5 and CYP2G1 were both active toward E_2 in a reconstituted system, producing 2- and 4-hydroxyE_2 and two additional, not-well-resolved peaks with similar retention time as the X peak detected in microsomal reactions (Fig. 3B). However, the two isoforms differed since the metabolite profile generated by CYP2A5 was very similar to that generated by OE microsomes, with 2-hydroxyE_2 as the major metabolite and 4-hydroxyE_2 barely detectable, while CYP2G1 primarily produced the unidentified products, with much lower levels of either catechol estrogen.

A peak with a retention time of about 24 min was formed in all reactions, with or without NADPH addition. It probably represents a degradation product formed during the incubation since it was also generated in incubations with the purified P450s. An additional, non-NADPH-dependent product (with a retention time of about 20 min) was detected in the 150-min incubations with VNO microsomes, but not in the 15-min incubations with OE microsomes. This may also represent a degradation product because it was detected following 150-min incubations with the purified enzymes in the absence of NADPH (not shown).

VNO microsomes were also active toward testosterone and progesterone (Fig. 4 and Table 1). The major metabolites of testosterone in VNO microsomal reactions were 15α- and 2β-hydroxytestosterone (not shown), as previously found for testosterone metabolism by OE microsomes (15). With progesterone, two metabolite peaks (P1 and P2) were detected in VNO microsomal reactions, which had the same retention times as those of the two metabolites formed by OE microsomes (Fig. 4). These progesterone metabolites have not been identified; however, as reported in the previous study with OE microsomes (15), they did not comigrate with available standards, including 2α-, 6α-, 6β-, 11α-, 11β-, 12α-, 15α-, 15β-, 16α-, 17α-, 18-, 19-, 20α-, 20β-, and 21-hydroxyprogesterone; 6-keto, 11-keto-, 16-keto, and 19-keto-progesterone; and 6β,21-dihydroxyprogesterone. No product was detected in the absence of NADPH. The rates of total metabolite formation from testosterone and progesterone were about 150 and 90 times, respectively, higher in OE than in VNO microsomal reactions (Table 1). With either tissue, the microsomal activities toward testosterone and progesterone were much higher than those toward E_2.

Microsomal metabolism of the three sex steroid hormones is most likely catalyzed by one or more of the P450 isoforms detected in the OE and the VNO. In experiments not shown, the anti-CYP2A5/2G1 IgG, added at 4 mg IgG/nmol P450, completely inhibited all metabolite formation from testosterone and progesterone in OE microsomes. The same antibody also completely inhibited the formation of the unidentified metabolites (Peak X) from E_2, but only caused a 20% inhibition of 2- and 4-hydroxyE_2 formation, suggesting that other isoforms may be primarily responsible for the catechol estrogen formation. However, CYP1A2 does not seem to play an important role in OE metabolism of E_2 because no difference was found in the rates of metabolite formation between *Cyp1a2(−/−)* mice (19) and *Cyp1a2(+/+)* mice. The relative contributions of CYP2A5 and CYP2G1 to OE microsomal metabolism could not be determined because an isoform-specific inhibitory antibody or chemical inhibitor has not been identified. The roles of individual P450 isoforms in VNO microsomal metabolism of the steroid substrates were not determined due to a lack of sufficient sensitivity of the assays.

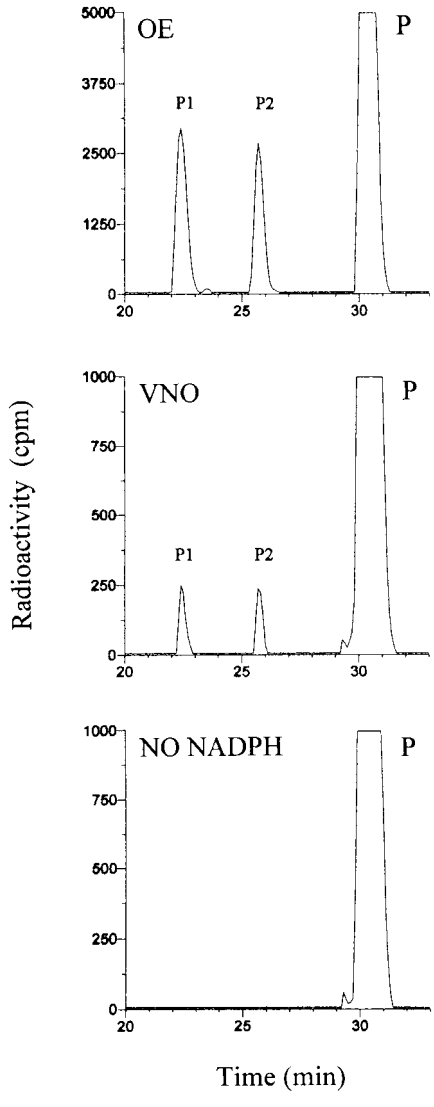

FIG. 4. Progesterone metabolites generated by OE and VNO microsomes. The complete reaction mixtures contained 10 μM [1,2,6,7-^{3}H(N)] progesterone (2 Ci/mol), 50 mM phosphate buffer, pH 7.4, 0.15 mg/ml microsomal proteins, and 1 mM *L*-ascorbic acid in a 125-μl final volume. The reactions with OE microsomes were initiated by the addition of NADPH to a final concentration of 1 mM and were carried out at 37°C for 15 min; an NADPH-generating system was used with VNO microsomes, and the reactions were carried out for 150 min.

DISCUSSION

Several P450 isoforms were previously known to be expressed in mouse OE, including CYP1A2, 2A5, 2E1, 2G1, and 3A (14, 20). In this study, we have further detected CYP2B and CYP2C in mouse OE and demonstrated for the first time that most P450 isoforms found in the OE, including CYP1A2, 2A5, 2B, 2C, 2G1, and 3A, are also expressed in the VNO. Furthermore, VNO microsomes were active in the metabolism of the three major sex steroid hormones.

P450-catalyzed oxygenation represents one of the first steps in the inactivation and disposition of the sex steroid hormones. Both the OE and the VNO have been shown to contain estrogen receptors and are potential target organs for this and the other sex steroid hormones (8–12). The sex steroid-metabolizing P450 isoforms could play an important role in regulating intracellular hormonal availability and thus modulating hormonal function in the target tissue. The remarkably high steroid hydroxylase activity of the OE as demonstrated here and in previous studies (5, 6) supports such a regulatory role.

The steroid hydroxylase activity in VNO microsomes is low; however, the steroid-metabolizing enzymes could be localized in selected cell types and some of the P450 isoforms could be inducible by exposure to endogenous or exogenous compounds. Immunohistochemical studies of several OE microsomal P450s, including CYP1A, 2A, 2B, 2G, and 4B, indicated that they are expressed in non-neuronal cells, particularly in the sustentacular cells in the epithelium and the Bowman's glands in the submucosa (7, 21–24). In rat VNO, CYP2A and CYP2G-related immunoreactivities were detected in intraepithelial duct cells, in granules at the apical surfaces of the duct cells, and in the mucus layers above the sensory and non-sensory epithelia, and were also associated with secretory granules of acinar cells of vomeronasal glands (7).

The major P450 isoforms responsible for testosterone and progesterone metabolism in the OE were found to be CYP2A5 and CYP2G1, which were also important for the formation of a major metabolite from E_2. Both isoforms are expressed in the VNO and are likely important for local sex steroid metabolism. In addition, these and other VNO P450 isoforms may participate in pheromone metabolism and/or modulation of the proliferation of VNO receptor cells (6, 7, 25).

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REFERENCES

1. Halpern, M. (1987) *Annu. Rev. Neurosci.* **10,** 325–362.
2. Meredith, M. (1998) *Chemical Senses* **23,** 463–466.
3. Dahl, A. R., and Hadley, W. M. (1991) *CRC Crit. Rev. Toxicol.* **21,** 345–372.
4. Ding, X., and Coon, M. J. (1993) *Handbook Expt. Pharmacol.* **105,** 351–361.
5. Brittebo, E. B., and Rafter, J. J. (1984) *J. Steroid Biochem.* **20,** 1147–1151.
6. Ding, X., and Coon, M. J. (1994) *Arch. Biochem. Biophys.* **315,** 454–459.

7. Chen, Y., Getchell, M. L., Ding, X., and Getchell, T. V. (1992) *Neuroreport* **3,** 749–752.
8. Balboni, G. C. (1967) *Arch. Ital. Anat. Embriol.* **72,** 203–223.
9. Segovia, S., and Guillamón, A. (1982) *Develop. Brain. Res.* **5,** 209–212.
10. Kaba, H., Rosser, A. E., and Keverne, E. B. (1988) *Neuroscience* **24,** 93–98.
11. Getchell, M. L., Kulkarni-Narla, A., Marcinek, R., and Getchell, T. V. (1996) *Soc. Neurosci. Abstracts* **22,** 1594.
12. Guo, J., and Moss, R., L. (1998) *Association for Chemoreception Sciences Abstract* **20,** 166.
13. Ding, X., and Coon, M. J. (1990) *Mol. Pharmacol.* **37,** 489–496.
14. Gu, J., Zhang, Q. Y., Genter, M. B., Lipinskas, T. W., Negishi, M., Nebert, D. W., and Ding, X. (1998) *J. Pharmacol. Expt. Ther.* **285,** 1287–1295.
15. Hua, Z., Zhang, Q. Y., Su, T., Lipinskas, T. W., and Ding, X. (1997) *Arch. Biochem. Biophys.* **340,** 208–214.
16. Su, T., Sheng, J. J., Lipinskas, T. W., and Ding, X. (1996) *Drug Metab. Dispos.* **24,** 884–890.
17. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) *Pharmacogenetics* **6,** 1–42.
18. Hayes, C. L., Spink, D. C., Spink, B. C., Cao, J. Q., Walker, N. J., and Sutter, T. R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9776–9781.
19. Liang, H. C., Li, H., McKinnon, R. A., Duffy, J. J., Potter, S. S., Puga, A., and Nebert, D. W. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1671–1676.
20. Genter, M. B., Liang, H. C., Gu, J., Ding, X., Negishi, M., Mckinnon, R. A., and Nebert, D. W. (1998) *Biochem. Pharmacol.* **55,** 1819–1826.
21. Voigt, J. M., Guengerich, F. P., and Baron, J. (1985) *Cancer Lett.* **27,** 241–247.
22. Adams, D. R., Jones, A. M., Plopper, C. G., Serabjit-Singh, C. J., and Philpot, R. M. (1991) *Am. J. Anat.* **190,** 291–298.
23. Zupko, K., Poria, Y. and Lancet, D. (1991) *Eur. J. Biochem.* **196,** 51–58.
24. Getchell, M. L., Chen, Y., Ding, X., Sparks, D. L., and Getchell, T. V. (1993) *Annals Otol. Rhinol. Laryngol.* **102,** 368–374.
25. Weiler, E., McCulloch, M. A., and Farbman, A. I. (1999) *Eur. J. Neurosci.* **11,** 700–711.