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Expression and induction of cytochrome P450s in rabbit parotid glands

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Abstract

Earlier, we isolated and purified five different P450 isoforms from rabbit kidney cortex microsomes, three of which are members of the CYP4A subfamily (CYP4A5, CYP4A6, and CYP4A7), with the others being CYP2B4 and CYP1A1. In contrast, P450s in parotid glands were unknown. The fact that the parotid glands bear a marked morphological and functional resemblance to kidney tissue prompted us to investigate P450s in these glands. The present study was undertaken to determine which P450 isoforms are expressed in this tissue. Microsomes from parotid glands of untreated rabbits were found to contain 42.3 pmol of P450/mg protein and to catalyze the ω-hydroxylation of laurate. Administration of di(2-ethylhexyl) phthalate (DEHP) resulted in a 7-fold increase of laurate ω-hydroxylation. This enzyme activity was greatly inhibited by pretreatment with antibodies against CYP4A5. Furthermore, parotid gland CYP4A5, CYP4A6, and CYP4A7 mRNAs were identified by RT–PCR. Moreover, the CYP4A enzymes were demonstrated immunohistochemically to be localized exclusively in the ducts of these glands. In addition to the CYP4A enzymes, immunoblot analysis revealed that CYP2B4 is constitutively present, and that CYP1A1 is induced in these glands by treatment with 3-methylcholanthrene. Taken together, we can conclude that the P450 isoforms expressed in rabbit kidney cortex and parotid glands are identical in composition.

Keywords: Cytochrome P450; ω-hydroxylase; Induction; Localization; Parotid gland; Rabbit

1. Introduction

Although mammalian P450s are present in the liver at the highest levels, most extrahepatic tissues also appear to have particular P450 systems [1]. Earlier studies from this laboratory isolated four distinct fatty acid and prostaglandin ω-hydroxylating P450s (designated CYP4A4, CYP4A5, CYP4A6, and CYP4A7 [2]), from microsomes of rabbit kidney cortex [3,4], lung [5], small intestine [6], and colon [7], and demonstrated that fatty acid ω-hydroxylase is one of the major P450s found in the kidney cortex, small intestine, and colon of untreated rabbits. In addition, prostaglandin ω-hydroxylase was found to be greatly induced in rabbit lung by progesterone treatment or pregnancy. The physiological role of ω-hydroxylation in extrahepatic tissues remains to be completely understood. To date, two hypotheses have been proposed: one is for a role in the catabolic process linked to the β-oxidation pathway, especially in peroxisomes [8]; the other is for a role in the synthesis of 20-HETE, an intracellular signal metabolite [9,10].

The parotid, submandibular, and sublingual glands are salivary glands of the branched acinar or tubular type. The main function of these glands is the secretion of saliva to support the beginning of food digestion. Another important function of these glands is to excrete potassium ions and to reabsorb sodium ions at the striated ducts [11]. This function makes saliva hypotonic and potassium ion rich. The striated ducts possess morphological and functional similarities to the renal tubules in which various P450s are expressed. However, nothing was known about the P450s in salivary glands. This prompted us to determine whether there are ω-hydroxylases in the salivary glands.

In the present paper, we provide substantial evidence for the presence of CYP4A enzymes in rabbit parotid glands. Furthermore, the data indicate that the composition of the P450 isoforms in parotid gland microsomes is identical to that in rabbit kidney cortex.

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Abbreviations: CYP and P450, cytochrome P450; DEHP, di(2-ethylhexyl) phthalate; RT–PCR, reverse transcriptase–polymerase chain reaction; 20-HETE, 20-hydroxyeicosatetraenoate; and PGA_1, prostaglandin A_1.

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2. Materials and methods

2.1. Materials

PGA_1 and the "ADAM reagent" (9-anthryldiazomethane) were purchased from Funakoshi Chemicals; DEHP was obtained from Nacalai Tesque, and the RT–PCR kit with r*Tth* DNA polymerase from Toyobo. Oligonucleotide primers for RT–PCR were synthesized by the Espec Oligo Service based on the specific sequence of each mRNA. CYP1A1 was prepared from kidney cortex microsomes of rabbits treated with 3-methylcholanthrene as described by Ogita *et al.* [12]; CYP2B4, from liver microsomes of rabbits treated with phenobarbital as described by Imai *et al.* [13]; and CYP4A5, from kidney cortex microsomes of rabbits treated with DEHP as described by Yoshimura *et al.* [14]. Guinea pig polyclonal antibodies against CYP1A1, CYP2B4, and CYP4A5 were prepared as described previously [15]. The antibodies against CYP1A1 and CYP4A5 cross-reacted with CYP1A2 and other CYP4A proteins (CYP4A4, CYP4A6, and CYP4A7), respectively (data not shown). Goat anti-guinea pig IgG (heavy and light chain specific) was obtained from ICN Pharmaceuticals (Cappel Products).

2.2. Animal treatment and preparation

Male rabbits (2.5 kg) were fed a commercially prepared diet containing 2% DEHP for 14 days. Other rabbits were injected intraperitoneally with phenobarbital (40 mg/kg) daily for 4 days, or with 3-methylcholanthrene (10 mg/kg) daily for 3 days. Rabbit parotid gland microsomes were prepared in a manner similar to that described previously for rabbit kidney cortex microsomes [12]. Total RNA was isolated from both rabbit parotid glands and kidneys by the acid guanidinium thiocyanate-phenol-chloroform method [16].

2.3. Activity assays

To determine laurate and arachidonate ω-hydroxylase activities, parotid gland microsomes (0.2 mg protein) were incubated with potassium phosphate buffer (pH 7.5, 10 μmol), NADPH (0.2 μmol), and laurate (10 nmol) or arachidonate (5 nmol), in a total volume of 0.1 mL, at 37° for 20 min. ω-Hydroxylation products were separated from substrates using an ODS-120A HPLC column (0.46 × 25 cm) (Tosoh) after treatment with the ADAM reagent, and were measured as described by Sawamura *et al.* [17]. To determine PGA_1 ω-hydroxylase activity, the microsomes (0.3 mg protein) were incubated with sodium phosphate buffer (pH 7.4, 30 μmol), glucose-6-phosphate (12 μmol), glucose-6-phosphate dehydrogenase (1 U), NADP (0.4 μmol), and PGA_1 (20 nmol), in a total of 0.3 mL, at 37° for 20 min, and determined as described previously [17]. 7-Ethoxycoumarin *O*-deethylase activity was determined by the method of Greenlee and Poland [18]. The concentration of P450 was determined by the method of Omura and Sato [19].

2.4. Analytical methods

Proteins (50 μg) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and reacted with anti-sera. Western immunoblotting was carried out as described by Towbin *et al.* [20].

CYP4A5, CYP4A6, and CYP4A7 mRNAs were detected by RT–PCR using three primer pairs: 5′-CTGAATGCCCATCTTCCTGCCC-3′ and 5′-AAGTCTCACCCAGAGCCCTGGA-3′ for CYP4A5; 5′-GACCTGTCTGTCTACTGTCCGC-3′ and 5′-CAGGGTGCCAGGAACAGATCTG-3′ for CYP4A6; 5′-TACTTTCCTCCAGGATGCTGACC-3′ and 5′-ACAGGATGACTTTGGCGTAGTCC-3′ for CYP4A7. Total rabbit RNA (4 μg) was mixed with the four dNTPs (15 nmol each), manganese acetate (125 nmol), RNase inhibitor (20 U), oligonucleotide primers (20 pmol each), r*Tth* DNA polymerase (5 U), and appropriate buffer in a total of 50 μL. The reverse transcriptase reactions were performed at 60° for 30 min with r*Tth* DNA polymerase, and the resultant single-stranded cDNA was denatured at 94° for 2 min. The amplification reactions were performed for 30 or 40 cycles, each consisting of 1 min at 94° and 90 sec at 65°. At the end of the last cycle, a final extension step was performed for 10 min at 72°. Amplification products were analyzed on a 1.5% agarose gel. The expectant DNA fragments amplified from mRNA were 0.43 kbp for CYP4A5, 0.30 kbp for CYP4A6, and 0.52 kbp for CYP4A7. As a positive control, the cDNA (0.45 kbp) for glyceraldehyde 3-phosphate dehydrogenase was amplified using the primer pair 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′, using the same methods and an annealing temperature of 60°.

2.5. Immunohistochemical staining for CYP4A ω-hydroxylase in rabbit parotid glands

For immunofluorescence techniques, sections (10 μm) of rabbit parotid gland, fixed in 2% paraformaldehyde, were prepared using a Cryostat (Bright, 5030 microtome). The sections were placed on gelatin-coated slides and incubated with 0.1 M phosphate buffer (pH 7.5) containing 1% normal goat serum, 0.3% Triton X-100, and 0.2% anti-CYP4A5 serum for 24 hr at room temperature. After the slides were washed several times with 0.1 M phosphate buffer (pH 7.5)–saline (PBS), they were further incubated with a second antibody, fluorescent isothiocyanate (FITC)-conjugated IgG fraction of goat anti-guinea pig IgG (1:1000 dilution) for 24 hr. After washing several times with PBS, the slides were mounted with glycerol and observed under a fluorescent microscope (AH2-HL, Olympus). Negative control sections were prepared using nonimmune guinea pig serum as the primary antibody.

Table 1
Effects of DEHP, phenobarbital, and 3-methylcholanthrene on the P450 monooxidation system in rabbit parotid gland microsomes

Treatment	Cytochrome P450 contents (pmol/mg protein)	Laurate ω-hydroxylase activity (pmol/min/mg problem)	Prostaglandin A_1 ω-hydroxylase activity (pmol/min/mg protein)	7-Ethoxycoumarin *O*-deethylase activity (pmol/min/mg protein)
Untreated (N = 4)	42.3 (6.5)	11.9 (3.9)	ND[a]	0.93 (0.64)
DEHP (N = 4)	37.9 (7.1)	83.5* (38.0)	2.3* (0.8)	1.13 (0.39)
Phenobarbital (N = 4)	58.0* (1.0)	10.5 (2.7)	ND	3.55* (0.61)
3-Methylcholanthrene (N = 5)	78.7* (19.6)	8.3 (5.0)	ND	4.82* (3.44)

Treatments of rabbits and analyses were carried out as described in "Materials and methods." Values are means, with the standard deviations in parentheses.
[a] ND = not detected.
*,** Significant difference between drug-treated and untreated groups: $*P < 0.01$, and $**P < 0.05$.

3. Results

Microsomes from parotid glands of untreated rabbits were found to contain 42.3 pmol of P450/mg protein. This value was about 2.8 times higher than the value for microsomes from rabbit colon mucosa, whereas it was about 2.5 times lower than those for rabbit kidney cortex, lung, or small intestine mucosa [21]. Table 1 shows that the parotid gland microsomes catalyzed ω-hydroxylation of laurate with a specific activity of 11.9 pmol/min/mg protein in the presence of NADPH. Administration of DEHP for 14 days resulted in a 7-fold increase in laurate ω-hydroxylase activity, while P450 content was little influenced. Furthermore, laurate ω-hydroxylation by the DEHP-treated rabbit microsomes was greatly inhibited by pretreatment with guinea pig antiserum against CYP4A5, whereas no inhibition was observed with the nonimmune serum or the antiserum against CYP2B4, suggesting that CYP4A5 or immunochemically related P450s such as CYP4A6 and CYP4A7 are involved in the reaction (Fig. 1). PGA_1 ω-hydroxylase activity was also induced by treatment with DEHP, although no activity was detected in microsomes from the control parotid glands (Table 1). On the other hand, no PGE_1 ω-hydroxylase activity was detected in parotid gland microsomes of either control or DEHP-treated rabbits (data not shown). Since among four rabbit CYP4A enzymes only CYP4A4 has been shown to be capable of ω-hydroxylating PGE_1 [5], the possibility of the presence of CYP4A4 was ruled out.

To identify which members in the CYP4A subfamily are expressed in the parotid gland, western blot analysis using antiserum against CYP4A5 and RT–PCR analysis were performed. No significant signal was detected by western blot analysis, indicating a very low expression level of the CYP4A subfamily in rabbit parotid glands (data not shown). Amplification by RT–PCR (40 cycles) using specific primers showed that CYP4A5, CYP4A6, and CYP4A7 mRNAs were expressed at very low levels in the parotid glands of untreated rabbits (Fig. 2A), but treatment with DEHP resulted in a marked elevation in the CYP4A7 mRNA level (Fig. 2B). When mRNAs from untreated or DEHP-treated rabbit kidney were used as templates for RT–PCR (30 cycles), similar results were obtained (Fig. 2, C and D).

The cellular distribution of the CYP4A enzymes in rabbit parotid glands was also investigated by immunohistochemical staining using antiserum against CYP4A5 (Fig. 3). Intense immunoreactivity was observed in the epithelial cells of the intercalated, striated, and interlobular ducts, whereas no immunoreactivity was detected in the acini. Treatment with DEHP did not affect the immunoreactivity. Since the secretory cells, major components of the total gland volume, were not immunoreactive, the expression level of the CYP4A enzymes in the whole gland was likely to be extremely low. This finding indicates that the CYP4A

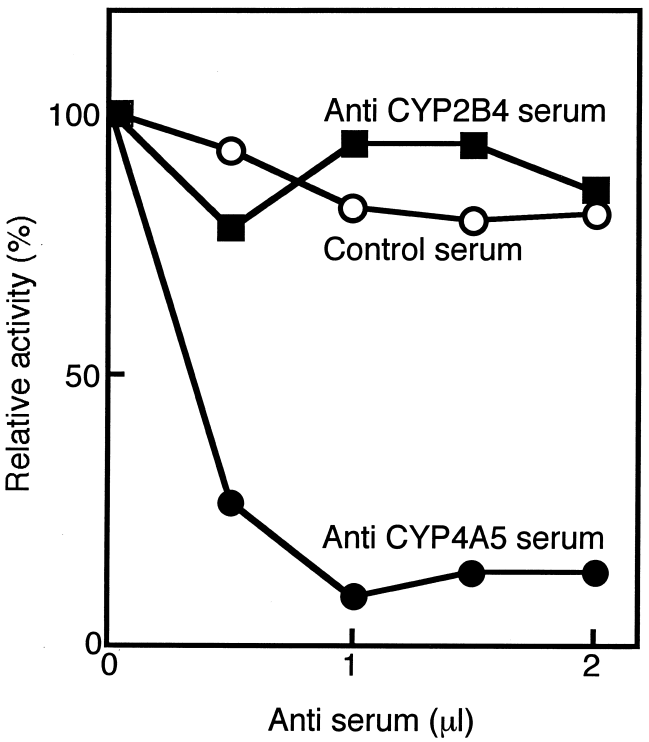

Fig. 1. Inhibition of laurate ω-hydroxylase activity in rabbit parotid gland microsomes by antisera against cytochrome P450s. Microsomes (200 μg) isolated from parotid glands of DEHP-treated rabbits were preincubated for 30 min at 25° with the indicated amounts of nonimmune serum (○), or antiserum against CYP4A5 (●) or CYP2B4 (■) before the addition of 1 mM NADPH and 20 mM laurate. The ω-hydroxylation products were extracted and identified as described in "Materials and methods." Representative graph of experiments were performed two times.

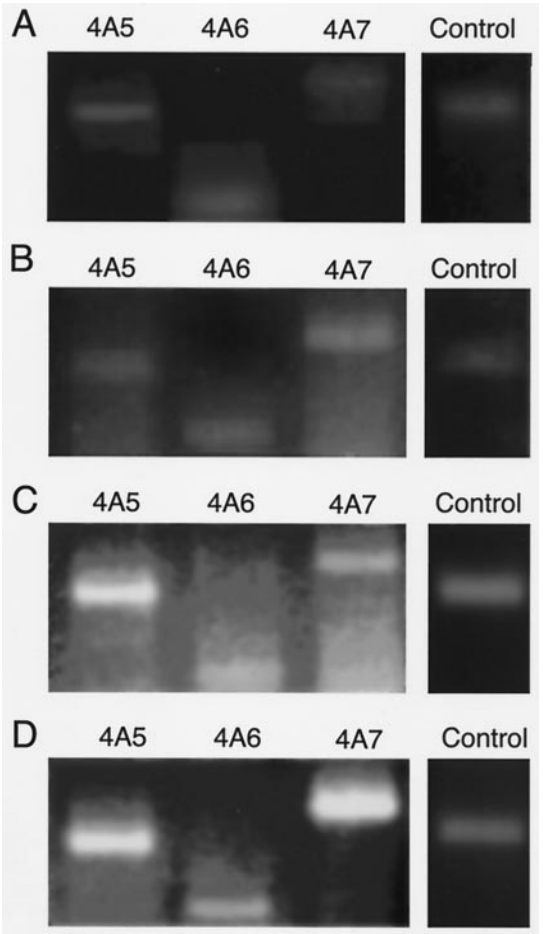

Fig. 2. RT–PCR analysis of RNA from rabbit parotid glands and kidneys. RT–PCR was performed using total RNA extracted from parotid glands of (A) untreated and (B) DEHP-treated rabbits, and from kidneys of (C) untreated and (D) DEHP-treated rabbits using templates and specific primers for the CYP4A5, CYP4A6, and CYP4A7 mRNA. PCR products were separated on a 10% agarose gel. RT–PCR products using a primer pair for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA are shown as controls. The sizes of the PCR products were: (CYP4A5) 0.43 kbp, (CYP4A6) 0.30 kbp, (CYP4A7) 0.52 kbp, and (G3PDH) 0.45 kbp. Representative experiments were performed three times.

enzymes are expressed exclusively in the ducts of rabbit parotid glands.

The expression of drug-metabolizing P450s in rabbit parotid glands was also examined (Table 1). Microsomal P450 content and 7-ethoxycoumarin *O*-deethylase activity were elevated 1.4- and 3.8-fold, respectively, after treatment with phenobarbital. On the other hand, treatment with 3-methylcholanthrene resulted in 1.9- and 5.2-fold increases in microsomal P450 content and 7-ethoxycoumarin *O*-deethylase activity, respectively (Table 1). Western blot analysis showed that the antiserum against CYP2B4 recognized a protein with an apparent molecular weight of 49 kDa, the molecular weight of CYP2B4 (Fig. 4A). Treatment with phenobarbital had no significant effect on the expression level of CYP2B4. In a similar experiment performed with antiserum against CYP1A1, no immunoreactive protein was detected in the microsomes of the control rabbits (Fig. 4B).

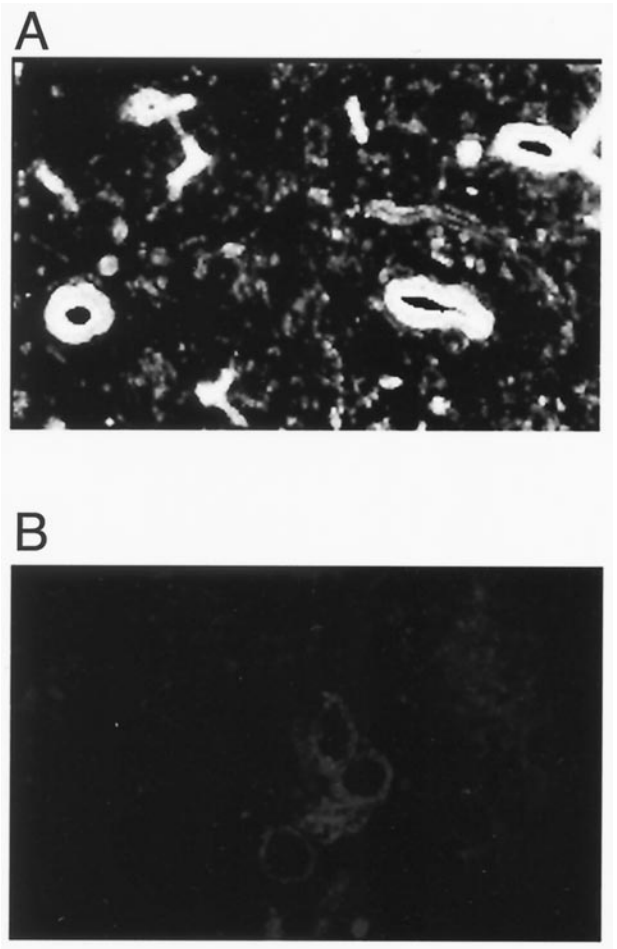

Fig. 3. Immunofluorescence micrograph of a rabbit parotid gland. (A) Epithelial cells of intercalated, striated, and interlobular ducts of untreated rabbit parotid glands were strongly immunostained by anti-CYP4A5 guinea pig serum and FITC-conjugated anti-guinea pig IgG. (B) Specific staining was not detected in the control experiment using nonimmune guinea pig serum. Original magnification: 80X.

However, treatment with 3-methylcholanthrene resulted in the induction of a protein with an apparent molecular weight of 58 kDa, corresponding to the molecular weight of CYP1A1. No induction was detected by treatment with phenobarbital.

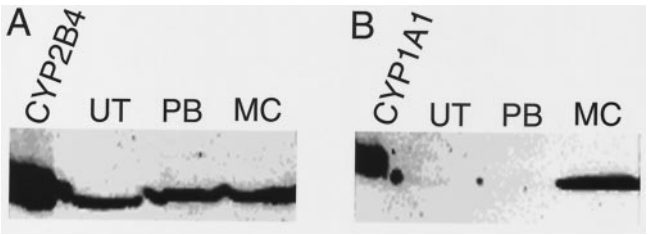

Fig. 4. Immunoblot analysis of rabbit parotid gland microsomes with antibodies against CYP2B4 and CYP1A1. Microsomes (50 μg) isolated from parotid glands of untreated (UT), phenobarbital-treated (PB), and 3-methylcholanthrene-treated (MC) rabbits were analyzed with antibodies against (A) CYP2B4, and (B) CYP1A1. Purified CYP2B4 and CYP1A1 were used as positive controls. Representative immunoblots of experiments were performed three times.

4. Discussion

More than 20 years have passed since morphological and functional similarities between salivary glands and kidneys were discovered [11]. Whereas renal P450s had been investigated extensively [3,4,9], nothing was known about the P450 monooxygenase system in salivary glands. The data presented in this paper provide evidence that at least five distinct forms of P450 (CYP1A1, CYP2B4, CYP4A5, CYP4A6, and CYP4A7) are expressed in rabbit parotid glands. Ogita *et al.* [3,12] were the first to purify three isoforms of P450 to electrophoretic homogeneity from rabbit kidney cortex microsomes, corresponding to CYP4A7, CYP1A1, and CYP2B4. Subsequent work from this laboratory separated and purified two additional fatty acid ω-hydroxylases from the microsomes of kidney cortex: CYP4A5 [14] and CYP4A6 [4]. Taken together, these results clearly indicate that rabbit parotid glands and kidneys are identical in P450 composition. It is of interest to determine how these findings are correlated to the functional resemblance between the two organs. The ω-hydroxylase P450s are also significantly expressed in the mucosal cells of the rabbit small intestine [6] and colon [7], which are implicated in the transport of water and ions. Schwartzman's group discovered a new way to clarify the physiological role of the CYP4A enzymes. A number of studies have revealed that 20-HETE, the ω-hydroxylation product of arachidonic acid, has potent effects on renal tubular and vascular function [9,10]. A recent report of Evans and Turne [22] indicating the involvement of a product of the P450 pathway of arachidonic acid in the up-regulation of the rat salivary gland $Na^{+,}$ $K^{+,}$ $2Cl^{-}$-cotransporter may be related to our results. However, we were unable to detect arachidonic acid ω-hydroxylase activity in parotid gland microsomes. The lack of arachidonic acid ω-hydroxylase activity may be due to its greater lability. On the other hand, the fact that the synthesis and physiological function of 20-HETE have been studied mainly using the rat CYP4A ω-hydroxylases (CYP4A1, CYP4A2, and CYP4A3) should also be considered. Alternatively, the possibility that the arachidonic acid-dependent regulation of ion transport and blood pressure is not the only function of the rabbit fatty acid ω-hydroxylases cannot be excluded. Hosny *et al.* [23] recently described the inability of recombinant CYP4A5 to catalyze arachidonic acid ω-hydroxylation. It seems that CYP4A7, which is highly inducible with peroxisome proliferators, plays an important role in the degradation of fatty acids.

Apart from the CYP4A enzymes, drug-metabolizing P450s are also expressed in the parotid glands. Taking into consideration that these P450s are localized in particular regions suggests that they are important for the detoxication of xenobiotics in this tissue. On the other hand, the induction of CYP1A1 by 3-methylcholanthrene in parotid glands could result in the metabolic activation of carcinogenic polycyclic aromatic hydrocarbons. Recently, an immunohistochemical study of Yokose *et al.* [24] indicated that CYP2C8 is expressed in human salivary glands. The relationship between parotid gland P450s and carcinogenesis remains to be elucidated in future studies.

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