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# Developmental and Endocrine Regulation of P450 Isoforms in Rat Breast

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**SUMMARY**

Cytochrome P450 was partially purified from rat breast tissue from 1-, 2-, 3-, 6-, 9-, and 15-week-old pregnant, lactating, or 3-week postlactation rats. The detergent-solubilized P450 was spectrally quantified, and the P450 isozyme pattern in the different samples was characterized by Western blot analysis with antibodies against cytochromes P450 1A1, 1A2, 2A, 2B, 2D4, 3A, 4A, 2E, and 19. The yield of P450 was 5–60 pmol/g wet weight tissue, with the highest yields in 1- and 2-week-old pups and lactating rats. The cytochromes P450 expressed in the breast can be divided into six main groups on the basis of their pattern of regulation: (a) those present in all samples (4A, 2E1, and 2D4), (b) those highly expressed in 1- to 3-week-old rats (2D4 and 3A), (c) those expressed only after 9 weeks of age [P450 19 (aromatase)], (d) those induced in pregnancy and maintained during lactation (1A1), (e) those induced in pregnancy and not maintained during lactation (2A), and (f) those induced 3 weeks after lactation (2B, 2A, and 3A). Reverse transcription-polymerase reaction amplification was used to confirm the presence of P450 isoforms in the breast. The mRNAs of cytochromes P450 1A1, 2A1, 2B1–3, 2D1, 2D3, 2D4, 2E1, and 4A3 were detected on analysis of total breast RNA. The mRNA of CYP 3A1 was not convincingly detected in untreated rat breast but was inducible by treatment with pregnenolone-16-α-carbonitrile. The presence of these various forms of P450 in the breast and their regulation by age and endocrine status may have implications for *in situ* metabolism of steroids and steroid antagonists and for activation of procarcinogens.

The cytochrome P450 supergene family is divided into families and subfamilies based on amino acid sequence similarity (1). In mammals, 10 families have been described that can be functionally subdivided into those involved in the synthesis of steroids and bile acids and those that metabolize exogenous compounds. The xenobiotic-metabolizing subfamilies (mainly P450s 1A, 2A, 3A, 2B, 2C, 2D, and 2E) contain a large number of isoforms with different substrate specificities. In the course of biotransformation of various drugs, reactive intermediates may be produced that can bind covalently to nucleic acids or otherwise interfere with cell functions. The role of cytochrome P450 enzymes in the initiation of carcinogenesis is believed to be mediated by the capacity of specific isozymes to metabolically activate procarcinogens. The tissue-specific expression of P450 isozymes may thus influence the relationship between bioactivation and detoxification of procarcinogens.

Breast cancer is the most common cancer among women in Europe and North America. The vast majority of all cases of breast cancer appear to be sporadic; only approximately 5% can be attributed to inherited predisposition conferred by the *BRCA1* locus and other genes (2, 3). Population studies in which second-generation migrants from low-risk countries assume the high incidence of breast cancer of their host countries indicate that environmental factors may be responsible for the geographical differences in breast cancer rates (4). It has been suggested that the capacity of the breast to metabolize carcinogens may be important in cancer susceptibility (5). Metabolism and metabolic activation of mammary procarcinogens, such as 7,12-dimethylbenz-(a)-anthracene, 2-aminoanthracene, *N*-2-fluorenylacetamide, and heterocyclic amines, have been shown *in vitro* with isolated rat and human mammary cells (6–9) and with microsomes from the mammary gland (10, 11). These reactions are known to be catalyzed by cytochrome P450 enzymes. Catalytic activities characteristic of P450s 1A and 2B have been detected in microsomes from human, mouse, and rat normal breast tissue as well as from mammary tumors (12–18). These catalytic activities have been low, and the contribution of these forms to the overall content of P450 in the breast has not been evaluated.

The level of P450 in breast microsomes from lactating rats

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**ABBREVIATIONS:**: TCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin; PCN, pregnenolone-16-α-carbonitrile; BNF, β-naphthoflavone; PB, phenobarbital; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; PCR, polymerase chain reaction; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; RT, reverse transcription; DMBA, 7,12-dimethylbenz(a)anthracene; AHH, aryl-hydrocarbon hydroxylase.

has been reported to be 500-fold lower than in liver microsomes (19). Because of the very low levels of P450 in the rat breast, it is difficult to accurately quantify the enzyme. This is one reason why the constitutive isozyme profile of P450s in the breast has not been successfully characterized. At 1–5 pmol P450/mg microsomal protein in the rat breast (19), it would be necessary to load 1 mg of microsomal protein in each lane to detect signals by Western blot analysis. In one study of human breast in which 50 μg of microsomal protein from tumor and peritumoral tissue were loaded in each lane, no P450 1A1/2, 2B1/2, 2C8–10, 3A4, or 2E1 could be detected (20). Surprisingly, Forrester *et al.* (21) could detect P450 2C on Western blots with microsomes from normal human breast and breast tumors when 7.5 μg was loaded in each lane. The P450 2C band from these human samples comigrated with the rat P450 2C6 but not with any 2C in the human liver. No P450 1A, 2A, 2B, 2C, 2D, or 3A was detectable. In the same study, the P450 3A antibody, which did not recognize a protein by Western blot analysis of breast microsomes, gave widespread immunohistochemical staining in the breast. The significance of this staining remains to be clarified. An interesting model system was used by Smith *et al.* (22) to study the constitutive and inducible forms of P450 in human breast tumors. Human breast tumors were grown as xenografts in immune-deficient mice, and xenograft microsomes from untreated and xenobiotic-treated mice were examined by Western blot analysis for various forms of P450 (22). In untreated mice, no P450 1A, 2B, or 4A was detectable when 15 μg of xenograft microsomal protein was loaded in each lane. A band that comigrated with rat P450 2A1 but not with human P450 2A6 was detected. Another study on human breast tumors has shown positive immunohistochemical staining with a P450 1A antibody in tumor cells but not in the surrounding normal epithelium (23). In the MCF-7 breast cancer cell line, P450 1A1 is inducible by TCDD, whereas P450 1B1 is constitutive (24, 25).

Because based on these studies it is not possible to state with certainty which forms of P450 are expressed constitutively in the breast, the role of these enzymes in *in situ* metabolism of procarcinogens, steroids, and steroid antagonists cannot be evaluated.

In the present study, we devised a protocol for reliable measurement of the P450 content of the breast, which permitted characterization of the expression pattern of P450 enzymes in breast tissue as a function of age, pregnancy, and lactation.

## Materials and Methods

**Chemicals.** *Thermus aquaticus* polymerase, avian myeloblastosis virus reverse transcriptase, RNasin, Sephadex G-50 nick columns, deoxynucleotide triphosphates, and oligo(dT) were purchased from Promega (Madison, WI). [$^{32}$P]ATP and enhanced chemiluminiscence detection reagents were purchased from Amersham (Buckinghamshire, England). GeneScreen Plus membranes were obtained from DuPont-NEN (Boston, MA). All other chemicals were of analytical grade and obtained from Kebo Lab AB (Stockholm, Sweden), Sigma Chemical Co. (St. Louis, MO), or Merck AG (Darmstadt, Germany).

**Animals.** Sprague-Dawley rats were obtained from ALAB (Stockholm, Sweden). Pups were separated from their mothers at the age of 7, 14, or 21 days. Females and males were separated in the 3-week-old group but not in the 1- or 2-week-old groups. Virgin females were examined at 6, 9, and 15 weeks of age, and males were examined at 6 weeks of age. Pregnant rats were 9 weeks old. Three sets of pregnant rats were timed and used at days 7, 14, and 20 of pregnancy. Lactating rats were used 2–3 weeks postpartum. Pups were weaned at day 21, and postlactation rats were used 2–3 weeks after removal of the pups. The animals were allowed to acclimatize for a few days after arrival and were kept on hardwood bedding under standardized conditions of light (6:00 a.m. to 8:00 p.m.), temperature (21 ± 1°), and humidity. Food and water were available *ad libitum*. The animals were killed by decapitation after $CO_2$ anesthesia. PCN (25 mg/kg) and BNF (40 mg/kg) were administered as corn oil suspensions. PB (80 mg/kg) was dissolved in saline. All agents were given to 6-week-old female rats as single intraperitoneal injections on three consecutive days.

**Preparation of microsomes or total membrane fractions.** For microsomal and total membrane preparations, breast tissue from 6–30 rats was pooled to obtain sufficient material. The abdominal mammary glands were excised and immersed in cold 0.25 M sucrose. The tissue was weighed and transferred to homogenization buffer composed of 100 mM Tris·HCl, pH 7.4, 20% glycerol, 1.15% KCl, 0.2 μM dithiotreitol, and 1 mM EDTA. The tissue was minced with scissors and subsequently homogenized with a Polytron homogenizer (PT 3000, Kinematica, Lucerne, Switzerland). The homogenate was filtered through a piece of gauze. PMSF (0.2 mM) was added before homogenization. To prepare microsomes, the homogenate was first centrifuged at 9000 × *g* for 30 min, and the microsomes were isolated from the supernatant by additional centrifugation at 105,000 × *g* for 1 hr. The microsomes were resuspended in 50 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% glycerol. The total membrane fractions were obtained by direct centrifugation of the homogenate at 105,000 × *g* for 1 hr. All centrifugation was performed at 4°.

**Purification of P450 by hydrophobic chromatography.** The total membrane fraction was resuspended in solubilization buffer composed of 50 mM potassium phosphate buffer, pH 7.5, 20% glycerol, 0.5% (w/v) sodium cholate, 0.2% Emulgen 911, and 0.2 mM EDTA. PMSF (0.2 mM) was added before the solubilization. After 16 hr, insoluble material was sedimented by centrifugation at 105,000 × *g* for 1 hr. The solubilized material was diluted 4-fold with 50 mM potassium phosphate buffer containing 20% glycerol and chromatographed on a column (5 × 2.5 cm) of *p*-chloroamphetamine-coupled Sepharose as described previously (26). The column was washed with 100 ml 1:4 diluted solubilization buffer, and P450 was eluted with solubilization buffer. P450 was quantified according to Omura and Sato (27) with a Hitachi U-3200 spectrophotometer, and the fractions containing P450 were pooled and, in some cases, concentrated by dialysis against Aquacide II (Calbiochem-Novabiochem Corporation, La Jolla, CA).

**Purification of P450 by anion exchange chromatography.** Selected samples were chromatographed on a DE-52 cellulose column (2 × 2.5 cm) equilibrated in 50 mM potassium phosphate buffer containing 0.2% Emulgen 911 and 20% glycerol. The P450 was eluted with 200 mM potassium chloride.

**Western blotting analysis.** Proteins were precipitated with chloroform/methanol and separated by gel electrophoresis according to Laemmli (28) with a 9% separating gel. Transfer of the proteins to a nitrocellulose filter was performed as described by Towbin *et al.* (29). In some experiments, the proteins were loaded in a single well of 7.5 cm, and after electroblotting the filter was cut into 0.5-cm strips. This permitted probing of the same blot with all antibodies. As an alternative, the samples were loaded in small wells on the same gel. In both cases, 10 pmol P450 was loaded per 0.5-cm well. The filter was blocked in TBS (20 mM Tris·HCl, pH 7.5, and 150 mM NaCl) containing 0.2% Nonidet P-40 and 10% fat-free milk, rinsed in TBS, and incubated with primary antibodies against rat liver P450s and a secondary anti-rabbit, anti-sheep, or anti-mouse IgG coupled to horseradish peroxidase. The protein/antibody complex was visualized by enhanced chemiluminiscence. We obtained the following generous gifts: rabbit IgGs against rat P450s 1A1 and 1A2 (Dr. R. J.

Edwards, Department of Clinical Pharmacology, Royal Postgraduate Medical School, London, UK) (30, 31); rabbit IgG against rat P450 2A1, also recognizing 2A2 and 2A3 (Dr. F. J. Gonzalez, National Cancer Institute, National Institutes of Health, Bethesda, MD); sheep IgG against rat P450 4A isozymes (Dr. G. Gibson, University of Surrey, Guildford) (32); and mouse IgG against human P450 19 (aromatase) (Dr. E. R. Simpson, University of Texas at Dallas) (33). Rabbit IgG specific to rat P450 2D4 was prepared by Dr. A. Wyss (Karolinska Institute, Stockholm, Sweden) (34). Rabbit IgG against rat P450 2E1 was obtained from Oxygene Dallas (Dallas, TX) (35), and rabbit IgGs against rat P450s 2B1/2 and 3A isozymes were obtained from Human Biologics, Inc. (Phoenix, AZ).

**Isolation of total RNA.** Rat tissues for RNA preparation were quickly excised, immediately frozen in liquid nitrogen, and stored at −70° until use. Total RNA was isolated according to the guanidinium thiocyanate single-step method described by Chromczynski *et al.* (36).

**Oligonucleotide primers and probes.** All oligonucleotides were synthesized with an Applied Biosystems 380B DNA Synthesizer. Table 1 shows primers and probes used for PCR and Southern blot analysis. Conserved oligonucleotide primers were designed for the P450 subfamilies 2A, 2B, 3A, and 2D, and specific oligonucleotide probes were designed for the individual P450 isozymes. Specific primers were designed for P450s 1A1, 1A2, 2E1, 4A1, 4A2, 4A3, and 4A8. Oligonucleotide probes were end-labeled with $^{32}P$ to a specific activity of $1 \times 10^7$ cpm/$\mu$g.

**RT-PCR.** Five micrograms of total RNA were added to 10 mM Tris·HCl, pH 9, 50 mM KCl, 7.5 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM each deoxynucleotide triphosphate, 6 $\mu$M oligo(dT) primer, and 16 units avian myeloblastosis virus reverse transcriptase in a final volume of 50 $\mu$l. The reaction was allowed to proceed for 1 hr at 42°, after which the enzyme was inactivated by heating to 95°. RNA was omitted from the negative controls. A 2-$\mu$l aliquot of each reverse transcription reaction was used for the PCR reactions. The reaction was carried out in 50 mM KCl, 10 mM Tris·HCl, pH 9, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 1 $\mu$M sense and antisense primers, and 2.5 units *Thermus aquaticus* polymerase in 100 $\mu$l. The conditions used for amplification were 30 sec at 94°, 30 sec at 50°, and 1 min at 72° for 30 cycles.

**Southern blot analysis of PCR products.** Products obtained by PCR were separated on 2% agarose gels. Where indicated in the figure, the samples were precipitated to load the total PCR reaction on the gels. DNA was transferred to GeneScreen Plus membranes by capillary force in 0.4 M NaOH for 16 hr. Membranes were dried at room temperature and baked for 1 hr at 80°. The membranes were prehybridized in 50% formamide 5× SSC, 0.1 mg/ml salmon sperm DNA, 100 $\mu$g/ml yeast tRNA, 50 mM Tris·HCl, pH 7.5, 1 mg/ml sodium pyrophosphate, 1% SDS, 2 mg/ml polyvinylpyrrolidone, 2 mg/ml Ficoll, 5 mM EDTA, and 2 $\mu$g/ml bovine serum albumin (fraction V) for 3 hr at 42°. Hybridization was carried out under the same conditions with $^{32}P$-end-labeled oligonucleotides for 14–16 hr. Before autoradiography, the membranes were washed for 1–2 hr in 6× SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at 50°.

## Results

**Spectral quantification of P450 in rat breast.** The presence of interfering chromophores made it difficult to measure P450 in microsomal and total membrane fractions from breast tissue of pregnant and lactating rats. The P450 in these samples could be quantified only after chromatography of the solubilized membranes over a hydrophobic column. Only in the case of 1-week-old pups was the P450 measurable in the total membrane fraction. The standard method for purification of liver P450, anion exchange chromatography of microsomes, was also tried but found to be an unreliable method for isolation of breast P450. It was there-

TABLE 1
**Oligonucleotides used as primers for PCR amplification and probes for Southern blot analysis**

| P450 | Sense primer | Antisense primer | Internal probe |
|---|---|---|---|
| 1A1 | ATTGTCAGGACAGGAGGCTGG | TCAGACTTGTATCTCTTATGGTGC | CTTATCATCTGAGAGCTGGACATTGGCATT |
| 1A2 | GAAGAACTTCCCCAGGAAGAG | CACCTTGGGCTCTGTCACAAGTAG | CTTCTCCTGAGGGATGAGACCACCGTTGTC |
| 2A-conserved | GAGGCTTTGGTGGACCAAGCTGAGGA | GAACATGTCATAGAGGCTGCCC | |
| 2A1 | | | TGGGGAGCGGGCAAAACAAC |
| 2A2 | | | TGTGGAACAGGCCAAGCGTA |
| 2A3 | | | CGAGTCATTTCGAAAGACGA |
| 2B-conserved | CCTTCTGCGCATGGAGAAGGAGAA | CTGTGGGTCATGGAGAGCTG | |
| 2B1 | | | TTCAGATCTTGTCCCTATTGGAG |
| 2B2 | | | TGCAGATCTTGCCCCAATTGGTT |
| 2B3 | | | TTCTGATGTTTCTCCAATGGGTT |
| 2B12 | AGGGCAACCTTGGACCCCAG | TGGGTCATGGAGAGCTGAAC | GCAGATCTGACCCCGATTGG |
| 2D-conserved | GCAGCGAAGCTTACCAATGCTGTCATCCATGAGGT | ATCTATGGATCCGGCATGAAGGCCTCATGCTTCAC | |
| 2D1 | | | GCCACGATTCACGAGTTGT |
| 2D3 | | | GTCCCAATGAATTTGCCA |
| 2D4 | | | GCCTCACAAGACTTCTCGT |
| 2E1 | CTGATTGGCTGCGCACCCTGC | GAACAGGTCGGCCACAGTCAC | CACCTTCAGTCACTGGACATC |
| 3A-conserved | ACCTGCTTTCAGCTCTCACACT | AATCCCGCCGGTTTGTGAAGAC | |
| 3A1 | | | GGCACTCCACATCGAATTTCCA |
| 3A2 | | | AGCACTCCATGTCAAATCTCCC |
| 4A1 | AAGGCCAATGGCGTCTACAGATTG | ATTCAAGTTCCCAATGGCCTGGAT | AAAGATTTCTATAGAGGAGTCTTGACCTGCCAGCTGT |
| 4A2 | AAGCCTTATCAATCCCTTGCTCCC | GGCACTCCTCACACGAAAGAAAAT | GAAGATCTCTAGAGGGTGATCCTGGTCATCAAGCTTC |
| 4A3 | AAGGCTTCTGGAATTTATCAATTC | GGCACTCCTCACACGAAAGAAAGT | GAAGATCTCTAGAGGGTGATCCTGGTCATCAAGCTTC |
| 4A8 | AAAGCTCATGGTTCCTACAGATTT | CATGTTCTGTACACGGAAAAAAAA | GAAGATCTCCAGGGTGGAATCCTGTCCAACAATCTG |
| β-Actin | ACTGACTGGGCCCATTGAACACGGCATTGT | ACTGACTGGTACCGCAGCTCATAGCTCTTCTCC | GGCATTGTGATGGACTCCGGA |

fore not routinely used. In the one case where the different methods of P450 isolation, hydrophobic or anion exchange chromatography were compared, i.e., in 1-week-old rat breast, approximately the same yield of P450 was obtained. More important, the different methods resulted in the same P450 profile by Western blot analysis (data not shown).

Fig. 1 shows the yield of breast P450 from pups, female and male rats of different ages, and pregnant, lactating, and postlactation rats. The highest yield of P450, ~60 pmol/g wet weight tissue, was obtained from the breasts of 1- and 2-week-old pups. By 3 weeks of age, the level of P450 had decreased to 12 ± 3 pmol/g wet weight in both males and females. There was a further decrease to 6 pmol/g wet weight at 6 and 9 weeks in the male. In female rats, there was a transient increase to 18 ± 5 pmol/g at 6 weeks, but by 9 weeks the level was not different than that in males. There was no further change at 15 weeks. In pregnant rats, the yield of breast P450 increased to approximately the level of 6-week-old females, 19 ± 4 pmol/g wet weight. The highest levels of P450 in adult rats were measured in breast P450 preparations from lactating rats (34 ± 12 pmol/g wet weight), whereas the P450 yield from postlactation rat breast was very low (~4 pmol/g wet weight). Representative P450 spectra from 6-week-old female rats and lactating rats are shown in Fig. 2.

**Western blot analysis of P450 forms in rat breast.** The relative amounts of different P450 forms in 3-, 6-, and 9-week-old female and pregnant rats are shown in Fig. 3. In 3–9-week-old rat breast, no P450 1A1 and very little P450 2A could be detected by Western blot analysis, but P450s 3A, 4A, and 2E antibodies produced immunoreactive bands at all three ages. P450 19 and 2B antibodies showed weak immunoreactive bands in 3- and 6-week-old breast and increased signals in the 9-week-old breast. P450 2D4 was present in the breast of 1–9-week-old rats. The P450 2D4 signals were stronger than those in the liver when similar amounts of P450 were loaded onto the gel. During pregnancy, there was a striking induction of P450s 1A1 and 2A and an increase in the intensity of the P450 4A band. The P450s 3A, 2E, and 2D4 antibodies gave weak signals in the pregnant breast. The antibodies against P450s 2B and 3A produced multiple bands, and therefore the results were somewhat difficult to interpret. The blurred band above the band in the liver controls that was present on the blots probed with these antibodies was most likely an artifact caused by mercaptoethanol (37). Both antibodies recognized bands in the breast P450 that comigrated with bands in the liver microsomes. In addition, the P450 2B antibody reacted with a third band of lower molecular weight, which is most likely due to cross-reactivity with P450 2A (38). To assist in the identification of P450 3A, rats were treated with PCN. There was an induction in the P450 3A signal in the breast of 6-week-old PCN-treated female rats, shown in Fig. 4.

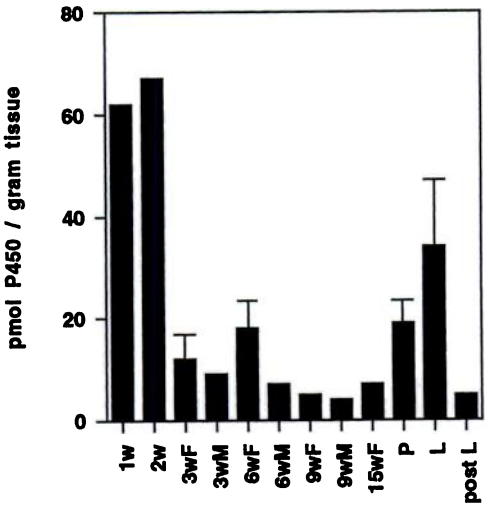


**Fig. 1.** Yield of P450 by hydrophobic or anion exchange chromatography of solubilized membranes of enriched P450 fractions prepared from breast tissue from 1- and 2-week (*w*)-old pups; 3-, 6-, and 9-week-old female (*F*) and male (*M*) rats; and 15-week-old virgin female rats and rats that were pregnant (*P*), lactating (*L*), and postlactation. Standard deviations are presented when three or more independent preparations were made.

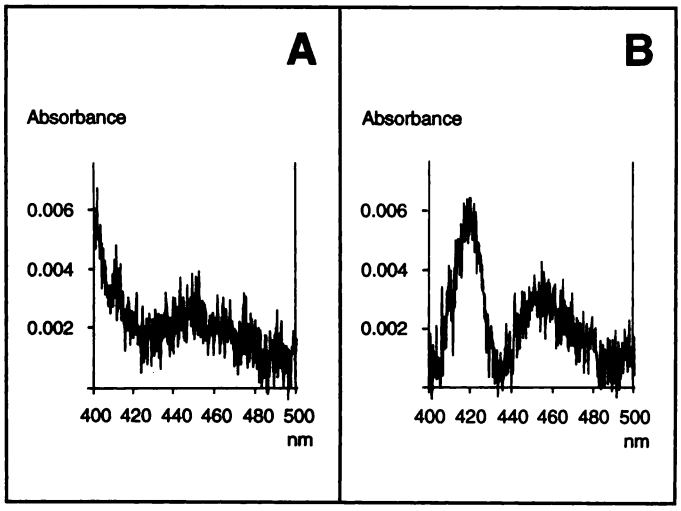


**Fig. 2.** Carbon monoxide difference spectra of breast P450 fractions prepared by hydrophobic chromatography of the solubilized total membrane fraction from female 6-week-old (A) and lactating (B) rats. P450 was measured in the eluate from the *p*-chloramphetamine-coupled Sepharose column.

Fig. 5 shows additional Western blot analysis of P450 fractions from pregnant, lactating, and postlactation rat breast as well as P450 from omental fat from pregnant rats. The increase of P450 1A1 in pregnancy was maintained during lactation but not at 3 weeks after lactation. P450 2A, 2B, 3A, 19, and 2D4 antibodies produced a similar pattern, with decreased intensity of the immunoreactive bands in the lactating breast, followed by an increase in the postlactation breast. Immunoreactive bands were observed in all breast samples with antibodies against P450s 4A and 2E. P450 1A2 was not detected in any of the the breast samples. Fig. 6 shows a Western blot that was probed with P450 1A1 antibody, stripped of antibodies, and reprobed with P450 1A2 antibody. A trace of P450 1A1 antibody could be observed in the BNF liver control after stripping, but it was completely absent in the breast samples. A strong P450 1A2 signal was observed in the BNF liver control, but there was no signal in the breast.

All samples were also analyzed by loading 150 pmol of P450 in a 7.5-cm well, cutting the filter into 0.5-cm-wide strips, and probing these with all available antibodies. This protocol does not permit qualitative comparison between P450 fractions but reveals the isozyme pattern in each prep-

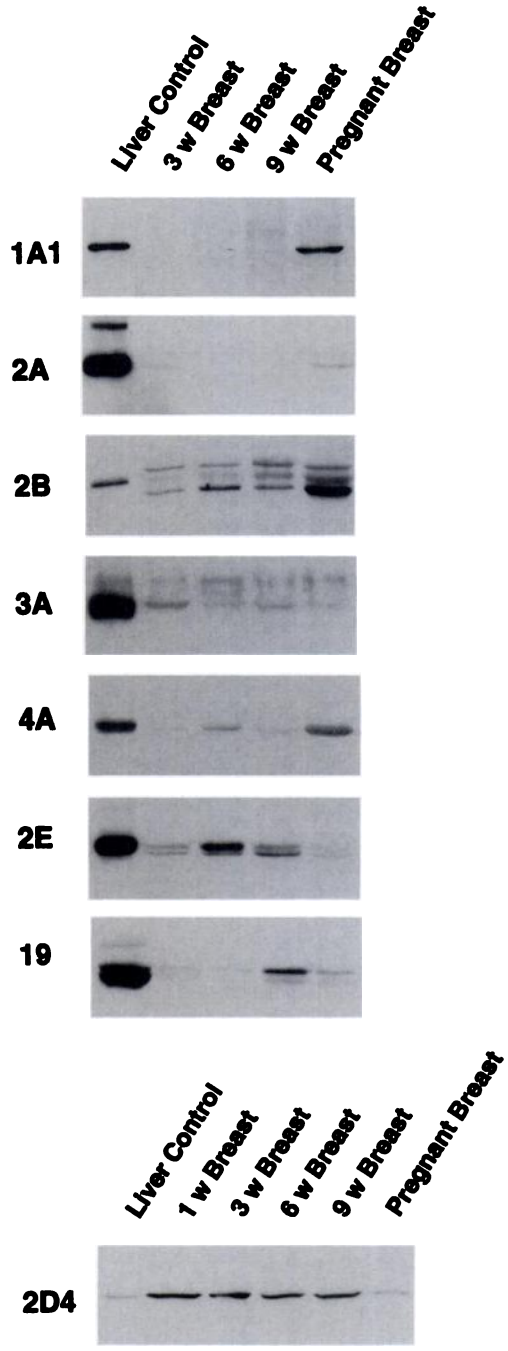


**Fig. 3.** Western blot analysis of rat breast P450. Comparison of breast P450 from 3-, 6-, and 9-week (*w*)-old virgin female rats and from pregnant rats. For P450 2D4, breast from 1-week-old pups was included. Ten picomoles of breast P450 were loaded per lane. Liver microsomes from 9-week-old female rats (10 pmol P450/lane) were used as a positive control in most cases, except for the analysis of P450 1A1, where liver microsomes from BNF-treated male rats (4 pmol/lane) were used, and for P450 19, where ovary microsomes (10 pmol/lane) from lactating rats were used.

aration. From these studies (data not shown), we were able to demonstrate that P450s 3A, 4A, 2E, and 2D4 were present in the breasts from 1- and 2-week-old rats. There was no qualitative difference in the P450 profile in the breasts of male and female 3- and 6-week-old rats and no differences in the P450 profile in the breast at days 7, 14, and 20 of pregnancy.

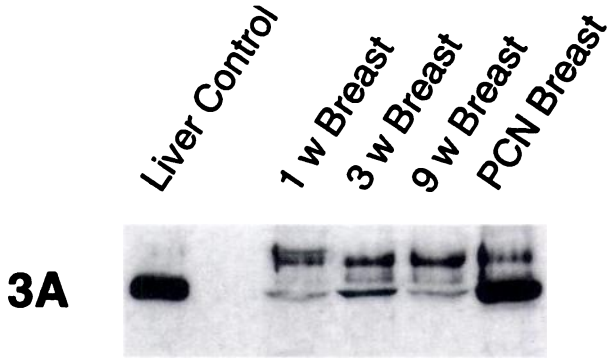


**Fig. 4.** Western blot analysis of breast P450 from PCN-treated 6-week-old female rats compared with breast P450 from 1-, 3-, and 9-week-old female rats. Ten picomoles of breast P450 were loaded in each tract. Liver microsomes from 9-week-old female rats (10 pmol P450/lane) were used as a positive control.

**Analysis of P450 from omental fat.** Omental fat was taken from pregnant, lactating, and postlactation rats, and P450 was prepared by chromatography of the solubilized total membrane fraction on a *p*-chloroamphetamine-coupled Sepharose column. Surprisingly, we were able to measure P450 in these samples. The yield of P450 in the fat was ~6 pmol/g from pregnant rats, ~4 pmol/g from lactating rats, and ~1 pmol/g from postlactation rats. As shown in Fig. 5, P450s 1A1, 4A, 2E, 19, and 2D4 were detectable in Western blots of omental fat P450 from pregnant rats. Omental fat from lactating and postlactation rats contained a similar pattern except that P450 1A1 was not detectable.

**Confirmation of P450 isozymes in rat breast by RT-PCR.** The oligonucleotides that were used for PCR and probing of Southern blots are listed in Table 1. In total RNA preparations from both pregnant and lactating rat breast, the mRNAs of P450s 1A1, 2E1, 2D1, 2D3, 2D4, and 4A3 were detected. The data for lactating rat breast are given in Fig. 7. In pregnant rat breast, the mRNAs of P450s 2B1, 2B2, and 2B3 were also detected (Fig. 7). A weak signal for P450 2A1 could be seen but was not successfully photographed. The P450 3A1 signal in pregnant breast was extremely low but was clearly visible in the breast of PCN-treated 6-week-old female rats (Fig. 7). The mRNAs of P450s 4A2 and 4A8 were detected only in lactating breast (data not shown), and the mRNAs of P450s 2A2 and 2A3 were not detected in lactating or pregnant breast.

## Discussion

In the present study, we quantified P450 in the breast from normal rat breast tissue and identified the P450 forms by Western blot and RT-PCR analysis. The protocol for partial P450 purification involves solubilization of the enzymes from microsomal and total membrane fractions followed by hydrophobic chromatography. It was therefore necessary to demonstrate that the recovery of P450 was efficient. Our value of 30 pmol/g wet weight breast tissue in lactating rats, corresponding to 3 pmol/mg microsomal protein, is similar to that observed by Ritter *et al.* (19), who measured P450 in microsomes from the breast of lactating rats. We could not measure P450 in lactating breast microsomes because of other interfering chromophores. It could, however, be measured in breast microsomes from 1-week-old rats, and with this P450

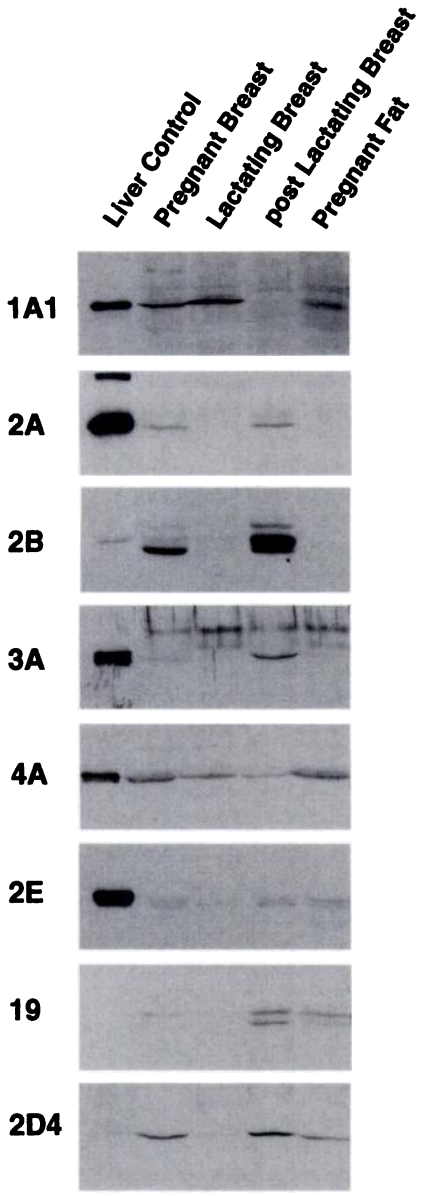


**Fig. 5.** Western blot analysis of rat breast P450 from pregnant, lactating, and postlactation rats compared with a P450 fraction from omental fat from pregnant rats. Ten picomoles of breast and fat P450 were loaded in each lane. Liver microsomes from 9-week-old female rats (10 pmol P450/lane) were used as a positive control in most cases, except for the analysis of P450 3A, where 5 pmol/lane was loaded; of P450 1A1/2, where liver microsomes from BNF-treated male rats (4 pmol/lane) were used; and of P450 2B, where liver microsomes from PB-treated rats (0.5 pmol/lane) were used.

preparation we could show almost 100% recovery of P450 after solubilization and hydrophobic chromatography.

The highest levels of P450 (60 pmol/g wet weight) were found in breast tissue from 1- and 2-week-old pups. Thereafter, we observed a decrease in the P450 content of the breast to 5 pmol/g wet weight when rats were 9 weeks old. In female rats, there was a transient increase at 6 weeks. During pregnancy and lactation, the P450 yield from the breast increased to 19 ± 4 and 34 ± 12 pmol/g, respectively, and after lactation, the level returned to normal for age-matched controls (5 pmol/g).

With the partially purified breast P450, it was possible to load 10 pmol P450 in each lane and compare the P450 profiles in the breast under different conditions. In male and female rats at 3 and 6 weeks of age, signals of the correct size were observed on Western blots with antibodies against P450s 2B, 3A, 4A, 2E, and 2D4. It was not possible to detect P450 1A1, and we conclude that if this enzyme is present, the concentration is very low. In rats at 1 and 2 weeks of age, the P450 profile was slightly different from that found after 3 weeks of age. The predominant signals were those of P450s 2D4, 2E1, and 3A. During pregnancy, not only was there an increase in the P450 content of the breast, but also when the same amount of P450 was loaded in each lane, there was an increase in the intensity of P450 4A and 2A signals. In addition, there was a strong P450 1A1 signal, which was not detected before pregnancy. The P450 2D4, 2E, and 19 signals were decreased during pregnancy. During lactation, the P450 1A1 and 4A signals were similar to those in pregnancy, whereas the P450 2A, 2B, 3A, and 2D4 signals were decreased. After lactation, when the P450 content of the breast had returned to control values, there were strong signals with the P450 2B, 2A, and 3A antibodies.

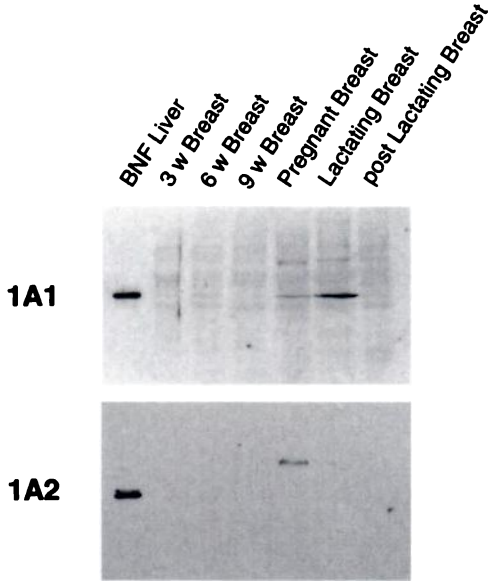


**Fig. 6.** Western blot analysis of P450 1A1 and 1A2 in the rat breast from 3-, 6-, and 9-week (*w*)-old female rats and pregnant, lactating, and postlactation rats. The same filter was probed with P450 1A1 antibody, stripped, and reprobed with P450 1A2 antibody.

RT-PCR analysis was used to confirm the presence of the mRNA for the cytochrome P450 forms detected by Western blot analysis and to identify specific isoforms. The mRNAs for P450 1A1, 2E1, 2D1, 2D3, 2D4, and 4A3 were detected in both pregnant and lactating rats. In addition, mRNAs of P450 2B1, 2B2, 2B3, and 2A1 were detected in the breast of pregnant rats. There was a weak P450 3A1 signal in untreated rat breast. In view of a previous report on the presence of P450 3A in human breast (21), the lack of P450 3A in rat breast was further investigated. After treatment with PCN, the mRNA for 3A1, as well as a strong P450 3A signal on Western blots, could be easily detected in the breast. We conclude that the constitutive level of P450 3A1 is low but that there is transcriptional regulation of this isoform by PCN in the breast. We have, so far, identified five forms of

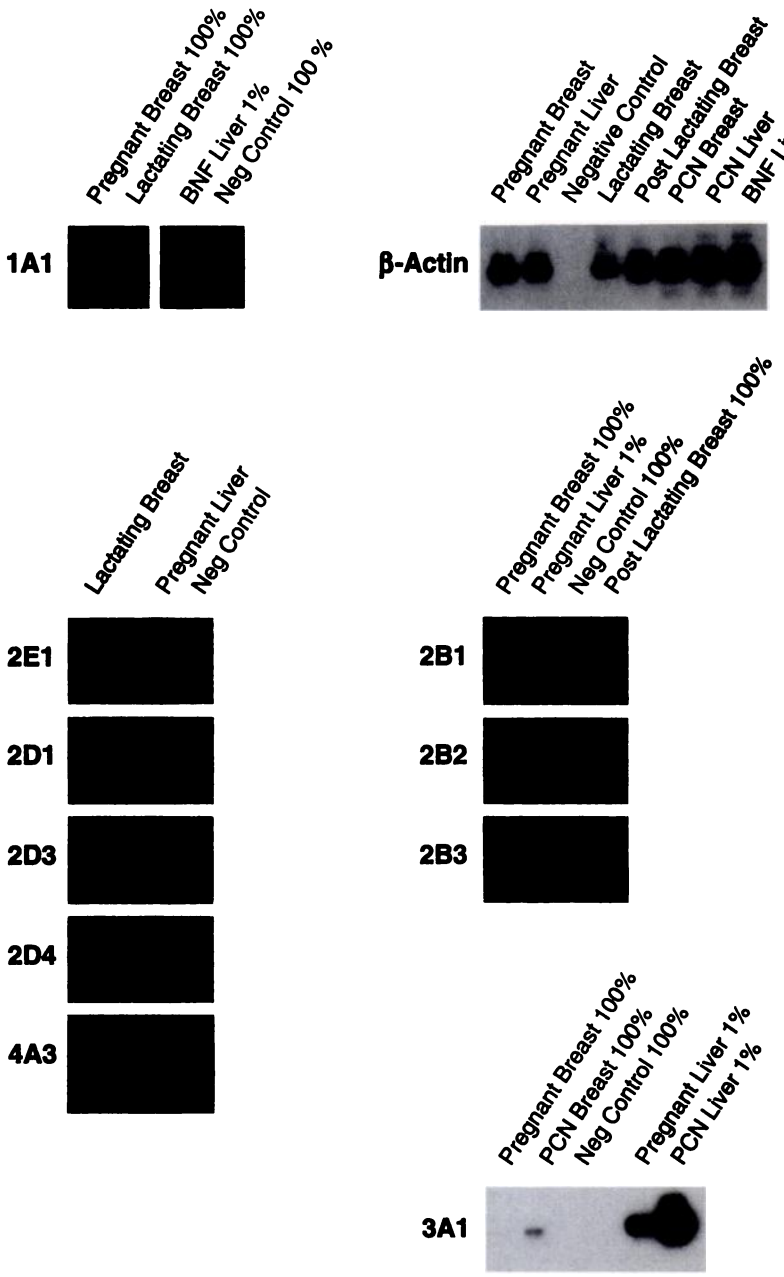


**Fig. 7.** RT-PCR and Southern blot analysis of total breast RNA from lactating, pregnant, postlactation, and PCN-treated rats. Liver total RNAs from pregnant rats and BNF- and PCN-treated rats were used as positive controls where indicated. RNA was omitted from the negative control. The amount of each PCR reaction loaded on the gel was 10% of the total volume unless otherwise indicated.

P450 (2D4, 2B1, 2B2, 2B3, and 2E1) that have not been previously reported in the breast.

P450 enzymes play an important role in both metabolic activation and detoxification of procarcinogens. Because environmental carcinogens may be important in the etiology of breast cancer, the role of breast P450 in the metabolism of procarcinogens must be evaluated. In this regard, P450s 1A1 and 2E1 are the most interesting forms. P450 1A1 is known to metabolize polycyclic aromatic hydrocarbons and, to some extent, heterocyclic amines (39), whereas P450 2E1 metabolizes ethanol, acetone, and a number of small organic molecules (40, 41). The metabolic fate of xenobiotics reaching the mammary gland does not depend only on the activating and deactivating reactions performed by P450 enzymes. Several phase II enzymes, which may be equally important, have been shown to be present in human breast samples (20, 21). P450 enzymes may have multiple roles. Metabolic activation may lead to the formation of DNA adducts or cytotoxins, whereas other reactions may have a protective effect. Our results revealed that the isozyme pattern of P450 changes during the development of the breast. This is true, for example, for P450 2D4, a major form of P450 in the breasts of 1–9-week-old rats. The function of P450 2D in the breast is not known, and the substrate specificity has not been characterized. Other members of the P450 2D subfamily metabolize numerous pharmaceutical agents and the tobacco smoke mutagen, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (42, 43).

The physiological and pharmacological roles of P450 in the breast also deserve consideration. Of the forms identified so far, the presence of P450 4A suggests metabolism of prostaglandins and fatty acids. We have confirmed the previous observation of others (14, 19) that breast P450 is inducible by xenobiotics. In the present study, PCN increased the levels of both the mRNA and the protein of P450 3A1. Because this inducer is dexamethasone-like in its action on P450 in the

liver, the role of glucocorticoids in the regulation of breast P450 must be examined. The inducibility in the breast of P450 3A, an enzyme that can metabolize tamoxifen (44, 45), raises the question of whether some of the resistance to tamoxifen, which is observed on long-term use of this antiestrogen, may be due to changes in its metabolism in the breast.

Several questions about the localization and function of these multiple forms of P450 in the breast remain to be answered. One of the most important issues is the identification of the cells that harbor the enzymes, i.e., whether they are located in epithelial, fat, or stromal cells and whether any forms are concentrated in undifferentiated epithelial cells. The susceptibility of the rat breast to DMBA-induced carcinogenesis has been shown to be higher in the undifferentiated breast of virgin rats, when the breast consists of an actively proliferating epithelium (5). Pregnancy, which causes differentiation of the mammary gland, has a protective effect against DMBA-induced carcinogenesis. Similar results have been found in epidemiological studies on human reproductive factors where a late (as opposed to an early) first full-term pregnancy confers a higher risk for breast cancer (4). An increase in AHH activity at puberty and an altered metabolism of DMBA between virgin and parous rats (14, 16, 46) are further indications of a relationship between metabolic activation in the breast and cancer susceptibility.

It is known that in human breast, aromatase (P450 19) is present in adipocytes. To our knowledge, the presence of aromatase in rat adipose tissue has not been reported (for a review, see Ref. 47). The increased intensity of aromatase bands in the 9-week-old breast may be a reflection of an increase in the fat content of the tissue. In an effort to evaluate the contribution of fat to the content of P450 in the breast, we examined the P450 profile in omental adipose tissue. We found that omental adipose tissue from pregnant, lactating, and postlactation rats contained measurable amounts of P450. Interestingly, as in the breast, P450 1A1 was detectable in the omental adipose tissue during pregnancy. The mechanism by which P450 1A1 is induced in the breast tissue from pregnant animals is not known. This induction was not accompanied by an increase of P450 1A1 in the liver (data not shown). P450 1A1 has been shown to be inducible in MCF-7 cells by ligands to the TCDD receptor; similarly, AHH activity has been shown to be induced in the breast by BNF and 3-methylcholanthrene (14, 25). The possibility of an active endogenous TCDD receptor ligand during pregnancy is intriguing and merits further investigation. The content of P450 in omental fat was no more than 20% of the P450 content of lactating breast, but omental fat may be different than fat in the breast. Whether the presence of 1A1 and other forms of P450 in the breast can be explained by the presence of the fat cells must be investigated with immunohistochemical techniques.

In summary, we used a panel of antibodies to characterize the P450 content of the rat breast. Some antibodies, such as the P450 2E, 3A, and 2B antibodies, produced multiple bands. RT-PCR was used to confirm the presence of P450 isoforms, and P450s 2E1, 2B1, 2B2, and 2B3 were successfully identified. mRNA of P450 3A could not be convincingly demonstrated in untreated breast, but it was detectable after PCN treatment. The quantitative importance of P450 3A in untreated breast remains to be evaluated.

In the breast from 3-week-old, pregnant, and postlactation rats, we could account for a large proportion of the total P450 content. In the prepubertal and lactating rats, the antibodies have not satisfactorily accounted for the P450 content of the breast. This indicates that other quantitatively important P450 forms may be present under those conditions.

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