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Role of mammary epithelial and stromal P450 enzymes in the clearance and metabolic activation of 7,12-dimethylbenz(a)anthracene in mice

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Abstract

Microsomal cytochrome P450 (P450) enzymes, which are important in the metabolism of carcinogens, are expressed in both epithelial and stromal cells in the mammary gland. The aim of this study was to investigate the roles of mammary epithelial P450 enzymes in the bioactivation and disposition of 7,12-dimethylbenz(a)anthracene (DMBA), a breast carcinogen, in the mammary gland. A new mouse model (named MEpi-Cpr-null) was produced, wherein P450 activities in the mammary epithelial cells are suppressed through tissue-specific deletion of the gene for P450 reductase (*Cpr*), an enzyme required for the activities of all microsomal P450 enzymes. Comparisons between wild-type and MEpi-Cpr-null mice showed that the tissue-specific deletion of *Cpr* in the mammary epithelial cells was accompanied by significant increases in the levels of DMBA and DMBA-DNA adduct in the mammary gland following a single intraperitoneal injection of DMBA at 50 mg/kg. Immunohistochemical and immunoblot analysis further revealed greater induction of CYP1B1 expression by the DMBA treatment in the mammary stroma of the MEpi-Cpr-null mice than in that of the WT mice. These findings not only demonstrate that the epithelial P450 enzymes play important roles in the clearance of DMBA, but also suggest that P450 enzymes in both mammary epithelial and stromal cells contribute to carcinogen-mediated DNA damage.

Keywords

Cytochrome P450; cytochrome P450 reductase; gene knockout; mammary gland; 7,12-dimethylbenz[a]anthracene; DNA adduct; mice

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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1. Introduction

The etiology of breast cancer is largely unknown. Exposure to environmental carcinogens has been proposed as a possible contributing factor, although experimental proof or epidemiological evidence for associating breast cancer with exposures to a particular environmental compound has yet to be obtained. Most chemicals require metabolic activation to become ultimate carcinogens. Microsomal cytochrome P450 (P450 or CYP) monooxygenases play essential roles in the metabolism of chemical carcinogens (Guengerich et al., 1988).

Although the liver is the major site for metabolic disposition and activation of chemical carcinogens, some fatty extrahepatic tissues, including the mammary gland, can accumulate hydrophobic compounds, such as polycyclic aromatic hydrocarbons (PAH), thus increasing the potential importance of local metabolic activation in PAH-induced mammary carcinogenesis. The ability of the mammary gland to metabolically activate procarcinogens has been demonstrated by studies in which the chemicals were directly injected into mammary gland of rodents, resulting in localized DNA adduct formation (Arif et al., 1997; Todorovic et al., 1997). Aromatic DNA adducts have been detected in human breast tissue (Perera et al., 1995; Li et al., 1996) and confirmed to be related to PAH exposure; their presence was also associated with the genetic polymorphisms of CYP1A1 (Li et al., 2002), a major P450 isoform for the metabolism of PAH carcinogens. Many P450 isoforms have been detected in rat breast tissue through immunoblot analysis, including CYP1A1, 1A2, 2A, 2B, 2D4, 3A, 4A, 2E1, and 19 (Hellmold et al., 1995; 1998). The expression of CYP1A1, 1B1, 2C, 2D6, 2E1, and 3A4/5 mRNAs was also detected in the human breast tissue (Williams et al., 2000). The expression of CYP2A5, CYP2B, CYP3A, and CYP19 has also been detected in the mouse mammary gland by immunoblot analysis (Gu et al., unpublished results).

DMBA (7,12-dimethylbenz[a]anthracene), is one of the most potent mammary carcinogens in animals, including mice (Median et al., 1982). DMBA requires multiple steps of metabolic activation; the resulting ultimate carcinogens are unstable and short lived. Stable DNA adducts are detectable in rodent mammary tissue following oral treatment with DMBA (Izzotti et al., 1999; Kleiner et al., 2001). Both CYP1A1 and CYP1B1 are major P450 isoforms for metabolic activation of PAH carcinogens, including DMBA, based on in vitro and in vivo metabolism studies (Parkinson and Ogilvie, 2008; Kleiner et al., 2004). CYP1A1 and CYP1B1 display stereospecific metabolism of DMBA, with CYP1A1 producing the anti-diolepoxides and CYP1B1 producing the syn- isomer.

The importance of potential interplays between the epithelial and stromal cell populations in breast development and carcinogenesis has been increasingly recognized in recent years (Wiseman et al., 2002). However, although metabolic activation in the mammary gland is believed to play a major role in chemical carcinogenesis in the breast tissue, the respective roles of the epithelial and stromal P450s in chemical carcinogenesis are largely unknown. In that connection, we have been developing *in vivo* models for determining tissue-specific contributions to chemical toxicity, including the relative contributions of the epithelial and stromal P450 enzymes to chemical carcinogenesis in the breast tissue.

The NADPH-P450 reductase (CPR) is the obligate redox partner for microsomal P450 enzymes (Black and Coon, 1987); deletion of the *Cpr* gene results in the inactivation of all microsomal P450 enzymes in targeted cells or tissues (Gu et al., 2003). Germline disruption of the mouse *Cpr* gene led to a spectrum of embryonic defects and mid-gestational lethality, indicating that CPR is essential for early embryonic development (Shen et al, 2002). Through crossbreeding between the *Cpr-lox* mouse (Wu et al., 2003) and various Cre

transgenic mice, several tissue-specific Cpr-null mouse models have been produced, including the liver-specific Cpr-null mouse (Gu et al., 2003), the lung-specific Cpr-null mouse (Weng et al., 2007), the cardiomyocyte-specific Cpr-null mouse (Fang et al., 2008a), the intestinal epithelium-specific Cpr-null mouse (Zhang et al., 2009), and the brain neuron-specific Cpr-null mouse (Conroy et al., 2010). Studies on these tissue-specific Cpr-null models have yielded direct evidence for the roles of P450 enzymes in the metabolic activation or disposition of various drugs and toxicants in the targeted tissues and organs.

The aim of this study was to develop a mammary epithelium-specific Cpr-null mouse, and to apply this model to determine the role of mammary P450 enzymes in the metabolic activation of PAH carcinogens (such as DMBA). Here, we report the successful generation of a mammary epithelium-specific Cpr-null (MEpi-Cpr-null) mouse model, produced through crossbreeding between the Cpr-lox mouse and the MMTV-Cre mouse; the latter is a well-characterized Cre transgenic mouse, widely used in many studies for mammary epithelium-specific gene deletion (Wagner et al., 2001, 2003; Cui et al., 2002; Loladze et al., 2006; Feng et al., 2007). We confirmed specific deletion of the Cpr gene in mammary epithelial cells, through immunohistochemical analysis. We then compared tissue levels of DMBA and DMBA-DNA adducts in DMBA-treated WT and MEpi-Cpr-null mice. We further examined expression of CYP1A1 and CYP1B1, two P450 enzymes possibly involved in DMBA metabolism in the mammary gland, through immunohistochemical and immunoblot analyses. We believed that our studies on the MEpi-Cpr-null mouse have yielded the first direct evidence for the specific role of mammary epithelial (vs stromal) P450 enzymes in the metabolic disposition and activation of a PAH carcinogen.

2. Materials and Methods

2.1. Generation of the MEpi-Cpr-null mice

The MMTV-Cre transgenic mouse (on a mixed B6/129 background) was obtained from Jackson Laboratory (Bar Harbor, ME) (Wagner et al., 2001). The Cpr-lox mouse [Cpr(lox/lox)]; congenic on B6 background) (Wu et al., 2003), was available at the Wadsworth Center. MMTV-Cre hemizygous transgenic mice were first crossed with Cpr(lox/lox) mice to generate MMTV-Cre(+/-)/Cpr(lox/lox) mice, which were crossed again with Cpr(lox/lox) mice, producing MMTV-Cre(+/-)/Cpr(lox/lox) mice (designated MEpi-Cpr-null) and MMTV-Cre(-/-)/Cpr(lox/lox) littermates (WT control). Genotype analyses for the Cre transgene and the Cpr allele were performed as described previously (Gu et al., 2003; Wu et al., 2003). All animal studies were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center.

2.2. Histopathology and immunohistochemical analysis of CPR and P450 expression

Mammary glands and other organs (liver and kidney) were dissected from 2-month-old female virgin MEpi-Cpr-null mice and wild-type littermates. The tissues were fixed in 10% neutral buffered formalin for histological examination, as described previously (Gu et al., 2003). For immunohistochemical detection of the expression of CPR, CYP1A1, and CYP1B1 in the mammary glands, paraffin sections (4 μ m) of mammary gland were processed according to a published protocol (Fang et al., 2008). The sections were analyzed using the following polyclonal antibodies: rabbit anti-rat CPR (Chemicon, 1:1000), rabbit anti-rat CYP1A1 (Chemicon, 1:500), and rabbit anti-human CYP1B1 (Santa Cruz, 1:500). Alexa Fluor 594 Tyramide Signal Amplification Kit (Molecular Probes, Eugene, OR) was used for visualization of the expression sites (Red) of CPR, CYP1A1 or CYP1B1, and the nucleus was stained with DAPI (Blue). The negative control sections were incubated with normal rabbit serum (Biogenex, San Ramon, CA) in place of the primary antibody.

2.3. Immunoblot analysis of CPR and P450 expression

For immunoblot analysis of the protein expression of CPR, CYP1A1, and CYP1B1 in the mammary glands and other tissues (liver and kidney), microsomal samples from the tissues were fractionated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Polyclonal antibodies to rat CPR (Stressgene, 1:2000), rat CYP1A1 (Chemicon, 1:1000), and human CYP1B1 (Chemicon, 1:1000), were used in the analyses. Peroxidase-labeled rabbit anti-goat IgG (Sigma- Aldrich, St. Louis, MO) was detected with an enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ) and the signal intensity of the detected bands was quantified using a densitometer.

2.4. Animal treatments with DMBA

DMBA and other chemicals were purchased from Sigma-Aldrich unless stated otherwise. Two-month-old female MEpi-Cpr-null mice and WT littermates (n=5-10, per strain and treatment group) were treated with a single i.p. dose of DMBA at 50 mg/kg (DMBA in olive oil, 10 mg/ml), or with vehicle only. Mice were sacrificed by CO₂ overdose at 24 hr after dosing. Mammary glands, blood serum, and liver were immediately frozen on dry ice and then stored at -80°C until used for determination of DMBA concentrations or DMBA-DNA adduct levels, or for immunoblot analysis. The mammary glands for immunohistochemical analysis of CYP1A1 and CYP1B1 expression were fixed in neutral buffered formalin as described above.

2.5. Determination of DMBA concentration in the tissues

The mammary gland and other tissue (blood serum and liver) samples were homogenized in phosphate-buffered saline (at 0.2 g/ml). A 50- μ l portion of the tissue homogenate (or blood serum) was spiked with 10 μ l benzo(a)pyrene (1 μ g/ml) as internal control. The samples were then mixed with 100 μ l of formic acid (50%), followed by extraction with 2 ml of hexane. After centrifugation at 3,000 g, for 10 min, the hexane phase was collected, and dried under nitrogen. The residue was dissolved in 100 μ l of hexane; aliquots (2 μ l) of the extracted samples were analyzed on an Agilent model 6890 GC interfaced to an Agilent model 5973 mass spectrometer (EI mode, at 70 eV). An Rxi column (30 m $\times$ 0.25 mm, 0.25 μ m in film thickness) was used in the GC system. The GC temperature was programmed as the following: initially set at 70°C for 2 min, then increased to 200°C at a gradient of 10°C/min, and maintained for 5 min, followed by an increase to 280°C at a gradient of 5°C/min, stayed for 5 min, finally increased to 300°C at a gradient of 2°C/min. The mass spectrometer was operated in the SIM (Selected Ion Monitoring) mode at a helium gas flow rate of 1.0 ml/min, with m/z values of 256 and 252 for DMBA and benzo(a)pyrene, respectively. Authentic DMBA standard was used for quantification. The recovery of DMBA from tissue homogenates was >80%.

2.6. ³²P-postlabeling assay for analysis of DMBA-DNA adducts

DNA was isolated from mammary glands as reported previously (Gupta et al., 1984). The nuclease-P1-enhanced version of the ³²P-postlabeling assay for DMBA-DNA adducts was performed as reported previously (Reddy et al., 1986; Galvan et al., 2005). Briefly, DNA (10 μ g) was digested with micrococcal nuclease (0.04 U/ml) and spleen phosphodiesterase (0.4 μ g/ml) at pH 6.0 and 37° C, for 3.5 h. The DNA was then treated with nuclease P1 (0.6 μ g/ml) for 40 min, followed by labeling with [γ -³²P]ATP (4000 Ci/mmol, MP Biochemical) and T4 polynucleotide kinase (0.5 U/ml, US Biochemical Corporation) at pH 9.5 and 37°C for 30 min. The labeled products were separated by two-dimensional polyethylenimine (PEI)-cellulose thin-layer chromatography (2D-PEI-TLC) (Galvan et al., 2005). The solvent system for the first dimension was 3.8 M lithium formate and 6.8 M Urea, pH 3.4, and for the second dimension, it was 0.72 M NaH₂PO₄, 0.45 M Tris, and 0.76 M Urea, pH 8.2. The

2D sheets were exposed to autoradiographic films, with use of intensifying screens and a typical exposure time of 16 h at -80° C. The radioactivity of the adduct spots was also quantified via the use of a Packard Instant Imager, and data were expressed as relative adduct-labeling (RAL) values, which were calculated by using the formula: $RAL = \text{c.p.m. in adduct(s)} / [\text{specific activity (ATP)} \times \text{pmol DNA-P labeled (1 } \mu\text{g DNA} = 3240 \text{ pmol DNA-P)}]$ (Reddy et al., 1986).

2.7. Statistical Analysis

All data are expressed as mean $\pm$ S.D. Statistical differences between two groups were examined using Student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. General characterization of the MEpi-Cpr-null mice

The MEpi-Cpr-null mice were viable, fertile, and normal in size and body weight, and exhibit no obvious physical or behavioral abnormalities, compared to WT littermates. There was no embryonic lethality in the MEpi-Cpr-null mice, based on analyses of genotype distribution in pups derived from crossbreeding between MMTV-Cre(+/-)/Cpr(lox/+) and MMTV-Cre(-/-)/Cpr(lox/lox) mice (data not shown). Histological examination of the mammary glands of the MEpi-Cpr-null mice did not reveal any structural abnormalities (data not shown).

3.2. Mammary epithelium-specific loss of CPR in the MEpi-Cpr-null mice

The epithelium-specific *Cpr* gene deletion in the mammary gland was revealed through immunohistochemical analysis of 2-month old female WT and MEpi-Cpr-null mice. CPR protein was detected in both mammary epithelial cells and stromal cells in WT mice (Fig. 1A). In MEpi-Cpr-null mice, CPR was no longer detected in the epithelial cells of the mammary duct (in ~80% of all mammary ducts viewed), whereas CPR expression in the stromal cells appeared to be unaffected (Fig. 1A). However, there was no significant decrease in total CPR protein level in microsomes prepared from whole mammary tissue of the MEpi-Cpr-null mice, compared to that of WT mice, as determined by immunoblot analysis (Fig. 1B and 1C). The expression of CPR was not affected in the liver or kidney of the MEpi-Cpr-null mice, as analyzed by immunohistochemistry (data not shown).

3.3. Expression of CYP1A1 and CYP1B1 in the mammary gland and their inducibility by DMBA treatment

We confirmed that both CYP1A1 and CYP1B1 proteins, two of the most important P450 enzymes for metabolic activation of DMBA and other PAH carcinogens, are constitutively expressed in mouse mammary gland at low levels (Fig. 2 and Fig. 3). CYP1A1 was primarily localized in the epithelial cells (Fig. 2A, left panel), whereas CYP1B1 was expressed mostly in the stromal cells (Fig. 3A, left panel). There was no obvious change in the basal levels and the distribution of mammary CYP1A1 and CYP1B1 in MEpi-Cpr-null mice, compared to WT mice. The inducibility of CYP1A1 and CYP1B1 in the mammary gland by DMBA was also studied in the two mouse strains. At 24 hr after a single i.p. injection of DMBA at 50 mg/kg, there was a marked increase in the levels of CYP1A1 and CYP1B1 (as indicated by the fluorescence intensity) in the mammary gland of both WT and MEpi-Cpr-null mice, compared to the level in vehicle-treated control mice (Fig. 2A and Fig. 3A). Although the induction of CYP1A1 occurred in both epithelial and stromal cells, it appears that the increase of CYP1A1 expression was more striking in the stromal cells than in the epithelial cells (Fig. 2A). In contrast, the induction of CYP1B1 appeared to be more remarkable in the epithelial cells than in the stromal cells (Fig. 3A). Interestingly, the overall extent of CYP1B1 induction by DMBA in the mammary gland of MEpi-Cpr-null mice (~6-

fold), as determined by immunoblot analysis (Fig. 3B and 3C), was substantially greater than that in WT mice (~2.5-fold), although there was no significant difference in the overall extent of CYP1A1 induction between MEpi-Cpr-null (~2.5-fold) and WT (~2.5-fold) mice following DMBA treatment (Fig. 2B and 2C).

3.4. Levels of DMBA in the mammary gland of MEpi-Cpr-null and WT mice after DMBA treatment

To examine the impact of epithelial CPR loss on P450 dependent metabolic clearance of DMBA from the mammary gland, we determined the levels of DMBA in the mammary gland, as well as liver and blood, of MEpi-Cpr-null and WT mice, at 24 hr following a single i.p. injection of DMBA at 50 mg/kg. Interestingly, the level of DMBA in the mammary gland of MEpi-Cpr-null mice was significantly (~50%) higher than the level in the mammary gland of WT mice (Fig. 4). As controls, no significant difference was found between the two mouse strains in DMBA levels in the liver and blood (Fig. 4).

3.5. DMBA-DNA adduct formation in mammary gland of MEpi-Cpr-null and WT mice following DMBA treatment

To further investigate the impact of epithelial CPR loss on P450 dependent metabolic activation of DMBA and the resultant DNA adduct formation in the mammary gland, we determined the levels of DMBA-DNA adduct, using the ³²P-postlabeling assay, in the mammary gland of MEpi-Cpr-null and WT mice, at 24 hr following a single i.p. dosing of DMBA (50 mg/kg). Surprisingly, the levels of DMBA-DNA adduct were significantly higher in the mammary gland of MEpi-Cpr-null mice, compared to that of WT mice (Fig. 5), although there was no obvious difference between the two strains in the patterns of the DNA adducts formed (labeled numerically from 1 to 7).

4. Discussion and Conclusion

In recent years, with the availability of tissue-selective Cpr-null mouse models, it has become possible to directly determine the *in vivo* contributions of P450 enzymes in a given organ or tissue to the disposition and toxicity of numerous xenobiotics. Evidence obtained so far indicated that extrahepatic P450 enzymes play important roles in the *in situ* metabolic activation and disposition of various drugs and toxicants (e.g., Weng et al, 2007; Fang et al, 2008b; Xiao et al., 2008; Zhang et al., 2009), and that the contribution of hepatic CPR/P450-dependent metabolism to drug toxicity in extrahepatic tissues is organ- and compound-dependent (e.g., Pass et al., 2005; Gu et al., 2007, Xiao et al., 2008; Zhang et al., 2007; 2009).

The *in vivo* role of mammary P450 enzymes in xenobiotic metabolism, or chemical carcinogenesis, is largely unexplored; appropriate animal models or research approaches that allow the alteration of the function of P450 enzymes in a mammary tissue-specific fashion was not available until now. The results from our studies on the MEpi-Cpr-null mouse provided the first direct evidence for a role of mammary epithelial P450 enzymes in the metabolism of a mammary carcinogen, DMBA. Our results also indirectly demonstrated the important role of mammary stromal P450 enzymes in the metabolic activation of DMBA.

CPR is expressed in both epithelial and stromal cells in the mammary gland, as indicated by our immunohistochemical data. CYP1A1 and CYP1B1, the two most important P450 enzymes for metabolic activation of PAH carcinogens, are expressed in the mammary gland at low levels in untreated mice, with CYP1A1 primarily localized in the epithelial cells, and CYP1B1 mainly detected in the stromal cells. Our observation of the selective expression of CYP1B1 in the stromal cells is consistent with the results of an earlier study, in which CYP1B1 was found to be selectively expressed in rat mammary stromal cells in culture

(Christou et al., 1995). Our study also confirmed that both CYP1A1 and CYP1B1 are highly inducible by DMBA in the mammary gland. Interestingly, the induction of CYP1A1 was more striking in the stromal cells, while the induction of CYP1B1 was more remarkable in the epithelial cells; as a result, both epithelial and stromal cells had increased capability for the metabolic activation of PAH carcinogens, such as DMBA, upon exposure to DMBA.

The importance of the interactions between the epithelial and stromal cells in mammary development and carcinogenesis has been increasingly recognized in recent years (Wiseman et al., 2002), but the role of the stromal cell P450s in chemical carcinogenesis has long been neglected, due partially to the fact that carcinogen-induced tumors are originated from the epithelial cells (Russo and Russo, 1996; Sharma et al., 2011), and in part to the lack of appropriate in vivo models that could distinguish between the relative contributions of the two mammary tissue compartments to metabolic activation of procarcinogen and consequent tumor formation. The results of our present study of the MEpi-Cpr-null mouse support a mechanism by which the P450 enzymes in mammary epithelial cells may modulate the bioavailability of potential P450 inducers or chemical carcinogens in all cells of the mammary gland, including the stromal cells. Thus, in DMBA-treated MEpi-Cpr-null mouse, compared to WT mouse, the inactivation of CPR in the mammary epithelial cells resulted in the loss of CPR/P450-dependent metabolic disposition of DMBA in the epithelial cells, and consequent increases in the levels of DMBA in both the epithelial and the adjacent stromal cells of the mammary gland. The increased levels of DMBA in the stromal cells, where the *Cpr* gene is intact and CYP1A1/CYP1B1 are functional, would lead to increased production of reactive DMBA metabolites and, consequently, increased formation of DMBA-DNA adducts, not only in the stromal cells, but likely also in adjacent epithelial cells to which the DMBA metabolites may be transported.

Notably, although we did not (and could not) measure the specific DMBA levels in the epithelial and stromal compartments, our finding of marked inductions of CYP1A1 and CYP1B1 in both cell types supports the notion that DMBA levels are increased in both compartments. In that connection, the greater induction by DMBA of CYP1B1 in the mammary gland of the MEpi-Cpr-null mice, than in that of the WT mice, is consistent with the observed, greater tissue levels of DMBA in the mammary glands of the null than in the WT mice.

We also observed a greater increase in the MEpi-Cpr-null mouse, compared to WT mice, in DMBA-DNA adduct levels in the mammary gland, following DMBA treatment. This observation contrasted with the results of a previous study on lung DNA-adduct formation in the lung-Cpr-null mice (Weng et al., 2007). In the latter study, the levels of DNA adduct formed, as well as the number of lung tumors induced, by NNK (4-methylnitrosamino-1-3-pyridyl-1-butanone), a carcinogen from tobacco smoke, were significantly decreased in the lung-Cpr-null mice, compared to WT control mice. We believe that the greater increase in DMBA-DNA adduct formation, which is evident in all seven DMBA-DNA adduct spots, could be attributed to a greater increase in DMBA bioactivation in the stromal cells, rather than the epithelial cells. In the stromal cells, the CPR expression was intact, while CYP1A1 and CYP1B1 expression was greatly increased and the level of DBMA was also elevated. In contrast, in the majority of the mammary epithelial cells, where CPR expression was abrogated, DMBA bioactivation would be significantly decreased, despite the increase in CYP1A1 and CYP1B1 expression and the elevation in DMBA levels. Therefore, these results demonstrated indirectly that the mammary stromal cell P450 enzymes can make significant contributions to the metabolic activation of mammary carcinogens, such as DMBA, in the mammary gland.

An excessive burden of DNA adducts can overwhelm the repair system and cause mutations, which can lead to tumor initiation. Therefore, we expect that the MEpi-Cpr-null mice, which had higher tissue levels of DMBA-DNA adducts in the mammary gland than in WT mice, will also have greater sensitivity to DMBA-induced mammary tumorigenesis than will WT mice, assuming that the proximal or ultimate PAH carcinogens produced (such as DMBA diol epoxides) by the stromal cells can be transferred to adjacent epithelial cells to cause mutagenesis. Further studies are planned to compare DMBA-induced mammary tumor formation in the MEpi-Cpr-null and WT mice.

In conclusion, we have successfully generated MEpi-Cpr-null mouse, a new mouse model for determination of the in vivo roles of mammary epithelial and stromal P450 enzymes in chemical carcinogenesis. Our results demonstrated, for the first time, that suppression of P450 activities in the mammary epithelial cells results in significant increases in the levels of DMBA, as well as DMBA-DNA adducts, in the mammary gland. This finding supports our hypotheses that the epithelial P450 enzymes play an important role in the clearance of PAH carcinogens, and that both stromal and epithelial P450 enzymes contribute to the metabolic activation of PAH carcinogens, leading to carcinogen-mediated DNA damage in the mammary glands.

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Abbreviations

P450 or CYP	cytochrome P450
CPR	NADPH-cytochrome P450 reductase
DMBA	7,12-dimethylbenz[a]anthracene
PAH	polycyclic aromatic hydrocarbons
RAL	relative adduct labeling

Highlights

- A new mouse model having suppressed P450 activities in the mammary epithelial cells
- Increased DMBA level in the mammary gland following DMBA treatment
- Increased DMBA-DNA adduct level in the mammary gland following DMBA treatment

Fig. 1. Immunohistochemical and immunoblot analyses of CPR protein expression in the mammary gland of MEpi-Cpr-null and WT mice

A. Paraffin sections (4 μ m) of mammary gland from female virgin (2-month old) WT and MEpi-Cpr-null mice were analyzed by immunohistochemistry. The tissue sections were incubated with a polyclonal rabbit anti-rat CPR antibody. Alexa Fluor 594 Tyramide Signal Amplification Kit was used for visualization of CPR expression sites (Red). The nucleus was stained with DAPI (Blue). No signal was detected when the primary antibody was replaced by a normal rabbit serum (data not shown). The arrow indicates the epithelial cells. Results shown are typical of six mice per strain analyzed. Scale bar, 20 μ m. **B.** Microsomal proteins (10 μ g per lane) from pooled mammary glands of three WT or three MEpi-Cpr-null

mice were analyzed for CPR expression with a rabbit anti-rat CPR antibody. Three different microsomal samples were analyzed for each strain. **C.** Densitometry analysis of the immunoblot results. Values represent means $\pm$ S.D., n=3. There was no significant difference in CPR band intensity between MEpi-Cpr-null and WT mice.

Fig. 2. Immunohistochemical and immunoblot analysis of CYP1A1 protein expression in the mammary gland of vehicle-treated or DMBA-treated MEpi-Cpr-null and WT mice
 Female virgin MEpi-Cpr-null and WT mice (2-month-old) were treated with DMBA at 50 mg/kg (in olive oil) by i.p. injection, or with vehicle alone. The mammary glands were collected for analysis at 24 hr after the treatment. **A.** Paraffin sections (4 μ m) of mammary glands were analyzed with a rabbit anti-rat CYP1A1 antibody. Alexa Fluor 594 Tyramide Signal Amplification Kit was used for visualization of CYP1A1 expression (Red), and the nucleus was stained with DAPI (Blue). No signal was detected when the primary antibody was replaced by a normal rabbit serum (data not shown). Results shown are typical of six mice per strain analyzed. Scale bar, 20 μ m. **B.** Microsomal samples isolated from pooled mammary glands of three mice were used (samples loaded in duplicate, 10 μ g/lane), and CYP1A1 expression was detected with the same rabbit anti-rat CYP1A1 antibody as the one

used in immunohistochemistry. **C.** Densitometry analysis of the immunoblot results. Values represent means $\pm$ S.D., n=3. ** p<0.01, WT or MEpr-Cpr-null (Null) vs. control mice.

Fig. 3. Immunohistochemical and immunoblot analysis of CYP1B1 protein expression in the mammary gland of vehicle-treated or DMBA-treated MEpi-Cpr-null and WT mice
 Female virgin MEpi-Cpr-null and WT mice (2-month-old) were treated with DMBA at 50 mg/kg (in olive oil) by i.p. injection, or with vehicle only. The mammary gland was collected for analysis at 24 hr after the treatment. **A.** Paraffin sections (4 μ m) of mammary gland were analyzed with a rabbit anti-human CYP1B1 antibody. Alexa Fluor 594 Tyramide Signal Amplification Kit was used for visualization of CYP1B1 expression (Red), and the nucleus was stained with DAPI (Blue). No signal was detected when the primary antibody was replaced by a normal rabbit serum (data not shown). Results shown are typical of six mice per strain analyzed. Scale bar, 20 μ m. **B.** Microsomal samples isolated from pooled

mammary glands of three mice were used (samples loaded in duplicate, 10 μ g/lane), and CYP1B1 expression was detected with the same antibody to CYP1B1 as the one used in immunohistochemistry. **C.** Densitometry analysis of the immunoblot results. Values represent means $\pm$ S.D., n=3. ** p<0.01, WT or MEpr-Cpr-null (Null) vs. control mice; ## p<0.01, MEpi-Cpr-null (Null) vs. WT mice.

Fig. 4. Levels of DMBA in mammary gland of MEpi-Cpr-null and WT mice after DMBA treatment

Two-month-old female virgin MEpi-Cpr-null and WT mice were given a single i.p. dose of DMBA at 50 mg/kg. The mice were sacrificed at 24 hr after treatment with DMBA, and mammary glands, blood serum, and liver were collected and processed for determination of DMBA levels in the tissues using GC/MS. Values represent the means $\pm$ S.D., $n=5$. ** $p<0.01$, MEpi-Cpr-null vs. WT mice.

Fig. 5. Levels of DMBA-DNA adduct formation in mammary gland of MEpi-Cpr-null and WT mice after DMBA treatment

Two-month-old female virgin MEpi-Cpr-null and WT mice were treated with a single i.p. injection of DMBA, at 50 mg/kg in olive oil. WT control mice were treated with olive oil only. The mammary glands were obtained at 24 h after DMBA dosing for DNA adduct analysis using a ³²P-postlabeling method. **A.** Mammary DNA samples analyzed on the 2D TLC plates were exposed to autoradiographic films. The adduct spots (marked with circles) are labeled numerically from 1 to 7. The adduct spots were not detected in mammary glands of mice treated with vehicle. **B.** The radioactivity of the adduct spots was quantified and the data were expressed as relative adduct-labeling (RAL) values. Values represent means $\pm$ S.D., n=5 per strain. * p<0.05; MEpi-Cpr-null vs. WT.