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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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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.

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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.

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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, 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

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.

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(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 and Phillips, 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, 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 and Werb, 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/−) 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 μm) of mammary gland were processed according to a published protocol (Fang et al., 2008a,b). 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 goat anti-rabbit 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 CO_2 overdose at 24 h 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-μl portion of the tissue homogenate (or blood serum) was spiked with 10 μl benzo(a)pyrene (1 μg/ml) as internal control. The samples were then mixed with 100 μl of formic acid (50%), followed by extraction with 2 ml of hexane. After centrifugation at 3000 × g, for 10 min, the hexane phase was collected, and dried under nitrogen. The residue was dissolved in 100 μl of hexane; aliquots (2 μl) 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 × 0.25 mm, 0.25 μm in film