EFFECT OF HYPOPHYSECTOMY ON INDUCTION OF MAMMARY CANCER AND CYP1 ENZYMES IN SPRAGUE-DAWLEY RATS

by

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ABSTRACT

Breast cancer is the most prevalent cancer among Canadian women and is the second leading cause of cancer-related deaths in North America. Hormonal influences including early menstruation, reproductive history, and hormone replacement therapy usage strongly affect breast cancer risk and development. In addition, hormone deprivation such as ovariectomy, hypophysectomy, and anti-estrogen therapy have been used as treatments to slow breast cancer growth.

In the present study, we determined the effect of hypophysectomy on mammary carcinogenesis. Twenty intact and hypophysectomized (at 7 to 7.5 weeks) virgin Sprague-Dawley rats were treated with a single intragastric administration of 20 mg of 7,12-dimethylbenz[*a*]anthracene (DMBA) or an equivalent volume of corn oil between 50 to 60 days of age. None of the hypophysectomized rats developed mammary tumors at 120 days post-treatment, whereas 55% of intact rats treated with DMBA developed mammary tumors. DMBA is a polyaromatic hydrocarbon procarcinogen that requires metabolic activation by the cytochrome P450 (CYP) system and microsomal epoxide hydrolase (mEH) prior to becoming carcinogenic. To determine if CYP and mEH enzymes needed for DMBA activation are down-regulated in hypophysectomized rats, CYP1A1, CYP1A2, CYP1B1, and mEH protein levels and CYP1-mediated enzyme activities were measured in liver and mammary tissue. Immunoblot analysis showed that there were no differences in hepatic CYP1A1 and CYP1A2 levels between DMBA- or corn oil-treated intact and hypophysectomized rats. The results also showed that mammary CYP1A1, CYP1A2, and CYP1B1 from hypophysectomized and intact rats were induced by DMBA. Microsomal EH levels in the liver and mammary gland were increased in hypophysectomized rats when

compared to the intact animals and DMBA treatment did not further affect mEH expression. MROD and BaP hydroxylase activities were similar in corn oil-treated hypophysectomized and intact rats and DMBA treatment increased both activities in hypophysectomized and intact rats to a similar extent. Based on the results of this study, the lack of mammary tumorigenesis in DMBA-treated hypophysectomized rats cannot be ascribed to the inability of either hepatic or mammary tissue to bioactivate DMBA.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AhR	aromatic hydrocarbon receptor
BaP	benzo[*a*]pyrene
BCIP	5-bromo-4-chloro-3-indoyl phosphate, *p*-toluidine salt
cDNA	complementary deoxyribonucleic acid
CO	corn oil
CYP	cytochrome P450
DMBA	7,12-dimethylbenz[*a*]anthracene
DNA	deoxyribonucleic acid
DRE	dioxin response element
E_1	estrone
E_2	estradiol
mEH	microsomal epoxide hydrolase
FSH	ollicule-stimulating hormone
GH	growth hormone
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
Hypox	hypophysectomized
LH	luteinizing hormone
i.g.	intragastric
IgG	immunoglobulin G
3-MC	3-methylcholanthrene
mA	milliamp
mg	milligram
ml	milliliter
MNU	*N*-methyl-*N*-nitrosourea
mRNA	messenger ribonucleic acid
MROD	methoxyresorufin *O*-demethylase
NADPH	nicotindiamide adenine dinucleotide phosphate tetrasodium salt
NBT	nitro blue tetrazolium
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PCDD	polychlorinated dibenzo-*p*-dioxin
pmol	picomole
PRL	prolactin
RT-PCR	reverse transcription polymerase chain reaction
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEMED	N,N,N′N′-tetramethylenediamine
mg	milligram
µl	microliter
XRE	xenobiotic response element

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1. INTRODUCTION

1.1 Incidence and Types of Human Breast Cancer

Breast cancer is the most common cancer among women and is the second leading cause of cancer-related death in North America. According to Canadian Cancer Statistics, it is estimated that 21,600 women were newly diagnosed with breast cancer in 2005 and there were 5,300 deaths from the disease (Canadian Cancer Statistics, 2005). On average, one in 9 women is expected to develop breast cancer during her lifetime. Breast cancer can also affect men. An estimated 150 men were newly diagnosed and 45 deaths were associated with breast cancer in 2005 (Canadian Cancer Statistics, 2005). Although breast cancer mortality rates among women over the past three decades have declined slightly, breast cancer continues to be the leading type of cancer among Canadian women.

Adenocarcinoma is a type of carcinoma that arises from epithelial gland cells. Most tumors occurring in breast tissue are adenocarcinomas where the cancer originates in the luminal mammary epithelial cells that make up the inner lining of ducts and alveoli involved in milk synthesis and transport (Nandi *et al.*, 1995). The most common type of breast carcinoma in women, between 40 to 89 years of age, is invasive ductal carcinoma (81%). The less prevalent type of breast cancer is ductal carcinoma *in situ,* also known as non-invasive ductal carcinoma (Weaver *et al.*, 2006). The most frequent site of origin of malignant ductal carcinoma is the terminal ductal lobular unit or type 1 lobule, where the unit exhibits high proliferative activity and less differentiation such as branching and clusters (Russo and Russo, 1998). Type 2 and type 3 lobules are more complex in morphology, with more branching and a higher number of ductules per lobule (see Figure 1.1).

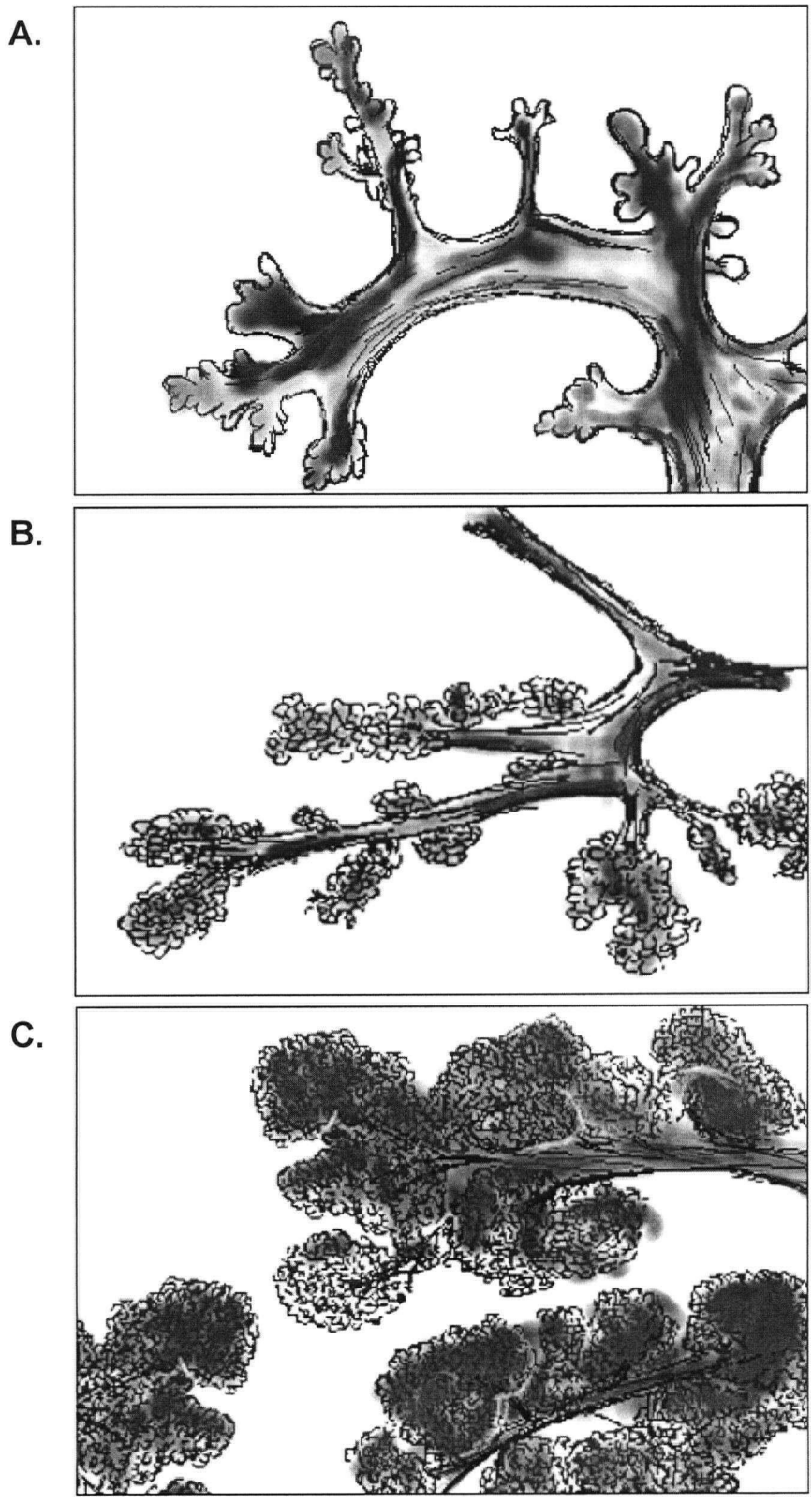


Figure 1.1 Types of lobule in human mammary gland. A) Type 1 lobule of nulliparous woman; B) Type 2 lobule of nulliparous woman; C) Type 3 lobule from parous woman.

Type 1 and type 2 lobules are structures most frequently found in the breast of nulliparous women of all ages, whereas the more differentiated type 3 lobule predominates in the breast of parous women. After menopause, the mammary parenchyma regresses to structures similar to type 1 lobule (see Figure 1.2). It is postulated that the level of differentiation of lobular structures may be associated with the development of neoplastic lesions with different malignant potential in the breast (Russo and Russo, 2004).

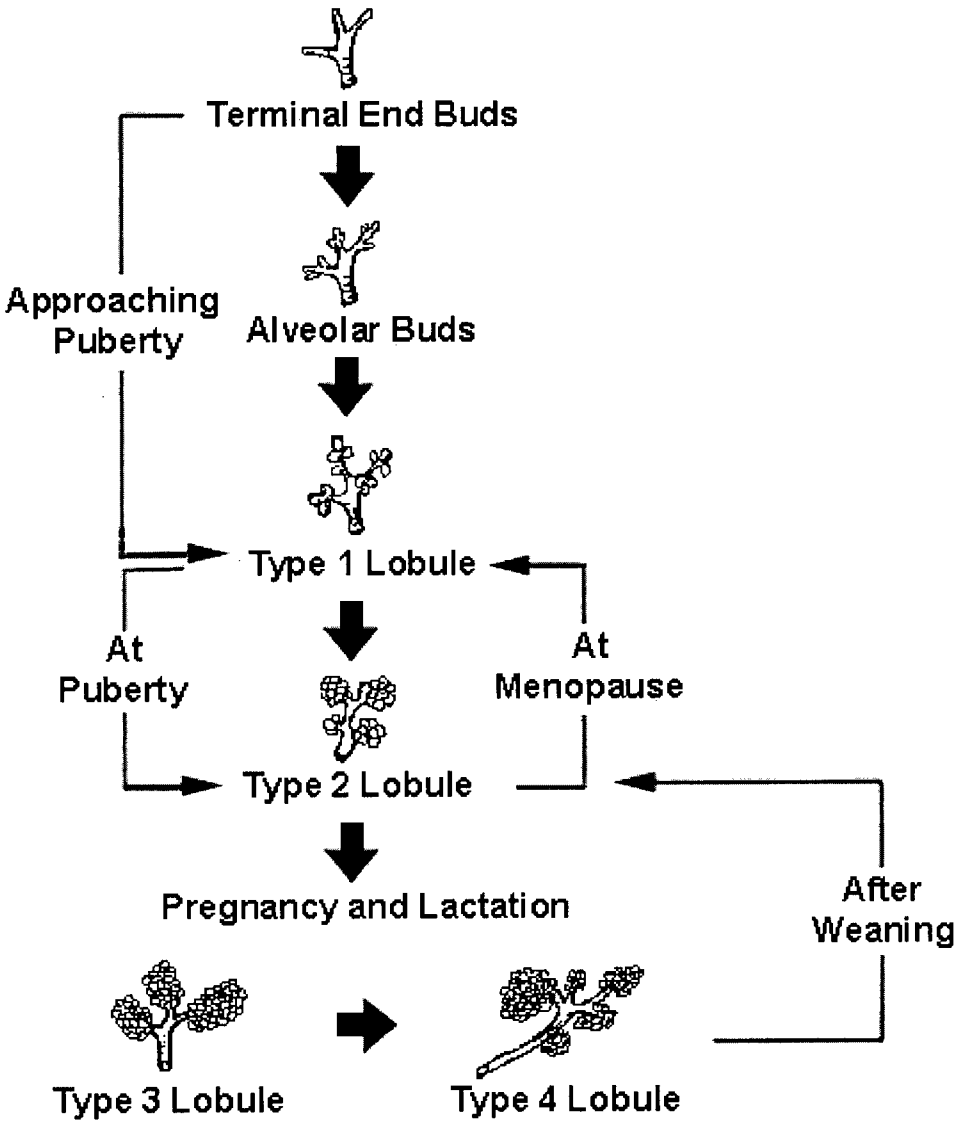


Figure 1.2 Development of the human mammary gland. Approaching puberty, terminal end buds begin to give rise to alveolar bud and to type 1 lobule. At puberty, type 1 lobule differentiates to type 2 lobule, which comprises of a higher number of ductules per lobule. At the last trimester of pregnancy and lactation period, the mammary gland developed into type 3 and type 4 lobules. After weaning, the structure regresses to type 2 lobule and back to type 1 lobule at menopause.

1.2 Etiology and Risk Factors of Human Breast Cancer

Breast cancer is a multifactorial disease. There is no single cause of breast cancer. Both endogenous and exogenous factors are involved in breast carcinogenesis. Researchers identified various risk factors associated with breast cancer including age, environment and lifestyle, genetics, and endocrinologic factors. Age is a well-recognized factor for breast cancer development. Breast cancer risk steadily increases with increasing age. The incidence of breast cancer is nearly nonexistent in women younger than 24 years of age and is maximal in post-menopausal women. Environmental and lifestyle factors are also of great importance in the pathogenesis of breast cancer. Unlike cigarette smoking and lung cancer, no causative relationship between environmental or lifestyle factors and breast cancer has been proven, but excessive alcohol consumption, a high-fat diet, increased body mass index in postmenopausal women, as well as excessive radiation exposure are recognized to play a role in the occurrence of breast cancer. Furthermore, familial background or genetics can also influence the risk of developing breast cancer. It is estimated that approximately 5 to 10% of breast cancers are due to a specific inherited mutation in breast cancer susceptibility genes. There are two major hereditary breast-ovarian cancer susceptibility genes, BRCA1 and BRCA2, both of which are tumor suppressor genes (Sakorafas and Pavlakis, 2004, Ganz, 2005). Epidemiological studies strongly support endocrinologic and reproductive factors associated with increased breast cancer risk. These factors include early menstruation, late menopause, nulliparity or late first full-term pregnancy, extended use of oral contraceptives, and prolonged use of hormone replacement therapy (Anderson, 2002, Lewis *et al.*, 2004). A vast majority of women (about 75%), who develop breast cancer have no familial, hereditary, or genetic risk for breast cancer, but these women may have an alteration in tissue response to

hormones or a variation in estrogen metabolism (Ganz, 2005). However, the mechanisms whereby estrogens are carcinogenic to the human breast are not completely understood. A better understanding of how estrogens and hormones initiate and regulate breast cancer growth and development is needed to prevent and treat breast cancer.

1.3 Carcinogenicity of Estrogens

Epidemiological evidence indicates that early menarche and late menopause are associated with increased breast cancer incidence. Results of the Women Health Initiatives Study (Rossouw *et al.*, 2002) and the Million Women Study (Beral *et al.*, 2003) suggest an overall increase in breast cancer risk from the use of hormone replacement therapy in post-menopausal women. In all of these cases, the level or duration of exposure to estradiol is enhanced. In addition, animal studies provide direct evidence for the role of estrogens in tumorigenesis. Colerangle and Roy (1995) demonstrated that exposure of female Noble rats to diethylstilbestrol and estrone produced a rapid acceleration in cell proliferation and progressively more differentiated epithelial structures in the mammary gland, when compared against control rats. Moreover, prolonged exposure to estrogens was carcinogenic in the liver, kidney, pituitary, and various organs of rats and mice (Weisz *et al.*, 1998, Spady *et al.*, 1999).

There are several proposed mechanisms to explain how estrogens are carcinogenic. One mechanism is based on the mitogenic properties of estrogen. Consequently, an increase in cell proliferation enhances the chance for genetic errors during DNA replication, resulting in an increased probability of mutations. Another proposed mechanism suggests that estrogen mediates carcinogenicity through its metabolites, 16α-hydroxy-, 2-hydroxy- and 4-hydroxy-estradiol (Rudali *et al.*, 1975, Liehr *et al.*, 1986, Li and Li, 1987, Lippert *et al.*,

2003, Lewis *et al.*, 2005). Accumulating evidence implicates covalent binding of estrogen metabolites to DNA causing mutations leading to cancer (see Figure 1.3, Cavalieri and Rogan, 2004). Furthermore, reactive oxygen species, generated by redox cycling of estrogen metabolites can be genotoxic indirectly (Jefcoate *et al.*, 2000, Yager, 2000). It remains unclear as to which proposed hypothesis contributes to the carcinogenic property of estrogens.

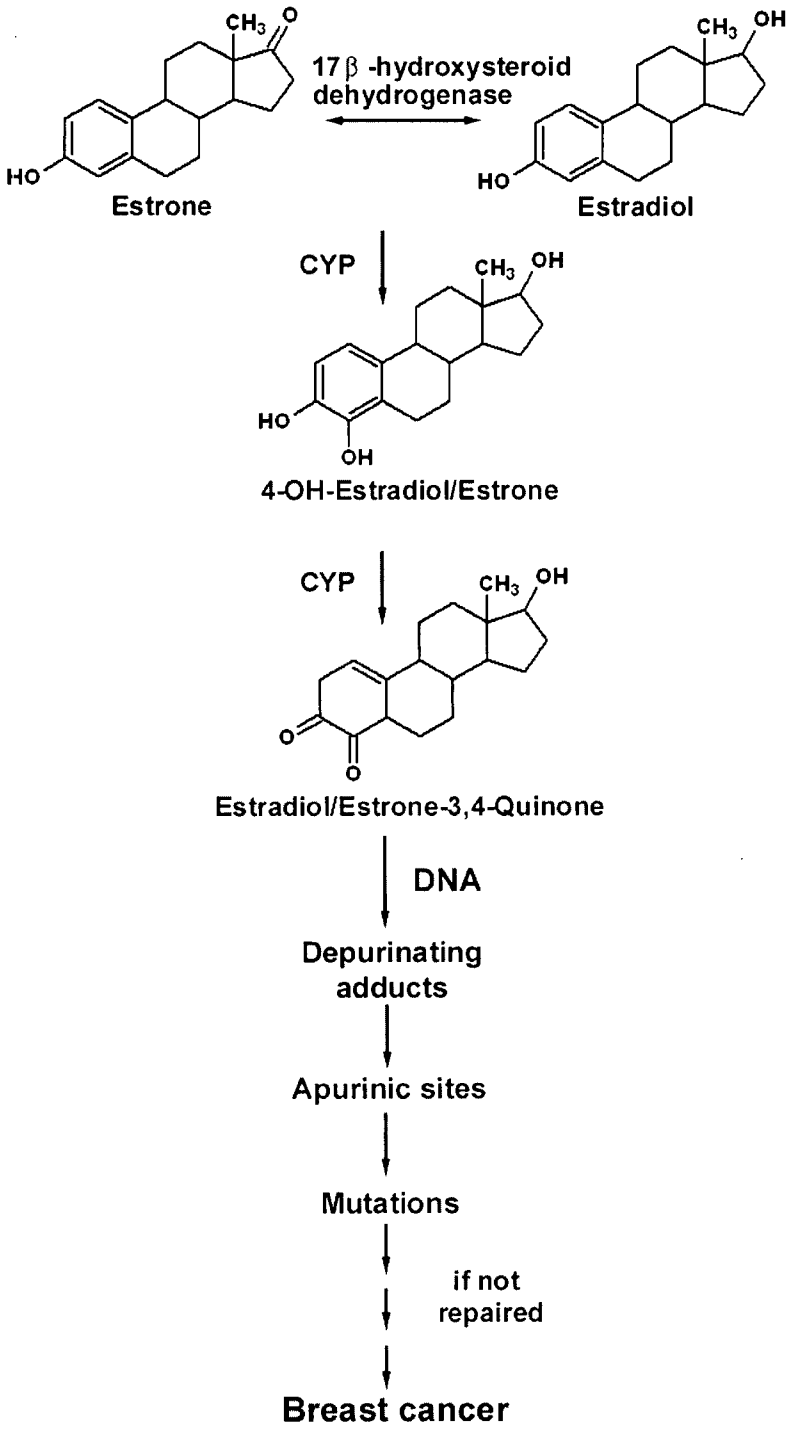


Figure 1.3 The bioactivation of estrone and estradiol leading to breast cancer. Estradiol is freely converted to estrone in the body by 17β-hydroxysteroid dehydrogenase in the body. Estradiol undergoes bioactivation by CYP enzymes to 4-hydroxyestradiol (4-OH-Estradiol) and Estradiol-3-4-quinones. The reaction of quinone estrogen with DNA to generate the series of events leading to breast cancer.

1.4 Hormonal Regulation of Breast Cancer

The etiology of breast cancer has a strong hormonal component. Most notably, menstrual status, reproductive history, and estrogen supplementation are risk determinants of breast cancer (Boyle and Leake, 1988). These observations suggest that ovarian and pituitary hormones play an important role not only in the normal breast development, but also in the development of breast cancer and its progression. Treating breast cancer has always been a challenge as there are multiple pathways regulating breast cancer growth. With hormone-dependent breast cancers, hormone deprivation is the principal mean to slow cancer growth.

1.4.1 Ovarian Hormones and Mammary Cancer

Estrogen and progesterone are two key hormones produced by the ovary. Estrogen, in particular, is an important hormone in mammary tumorigenesis. The concept of hormone dependency began when physicians observed remission and regression of breast tumors in patients who underwent ovariectomy (Davis, 1958). Later, the discovery of estrogen receptors (ER) in breast tumors confirmed an estrogen signal-transduction pathway in breast tumor growth (Jensen and Jacobson, 1962).

These results were further supported by animal studies that showed ovarian hormones stimulated mammary epithelial cell proliferation. Daily 17β-estradiol administration to a particular strain of rat, the ACI rat, caused mammary tumor development (Turan *et al.*, 2004). Dao (1962) demonstrated ovariectomy either prior to or within 7 days of 3-methylcholanthrene (3-MC) or 7,12-dimethylbenz[*a*]anthracene (DMBA) administration suppressed rat mammary tumor incidence. A similar observation is seen in humans where

the hormonal status of the individual such as pre- or post- menstrual, pregnancy or lactation can significantly affect mammary cancer incidence and multiplicity.

Although there is much evidence showing ovarian hormones act as a stimulator or promoter of mammary tumorigenesis, Huggins *et al.* (1962) demonstrated that the administration of large amounts of 17β-estradiol (20 μg) together with progesterone (4 mg) reduced DMBA-induced mammary tumor incidence from 100% in the control group to 52% in the estradiol plus progesterone-treated group. In addition, daily administration of high doses of estradiol, ranging from 20 μg to 30 mg per rat have been shown to have a protective effect on mammary cancer development and lead to tumor regression in *N*-methyl-*N*-nitrosourea (MNU)-induced intact rats (Rajkumar *et al.*, 2001). Cancer can also be controlled by supplying large amounts of estradiol in some breast cancer cases in post-menopausal women (Kennedy, 1962). Estradiol-induced apoptosis was also demonstrated at the cellular level. When MCF-7 cells are grown in estrogen-depleted medium to mimic the hormonal environment of breast tumors of post-menopausal women, treatment with a high concentration of estradiol ($\geq$ 0.1 nM) promotes a 60% reduction in cell growth (Song *et al.*, 2001). Furthermore, the response of human breast tumors to estrogen changes from stimulatory to inhibitory after prolonged estrogen deprivation according to the new theory of antihormonal resistance evolution (Lewis *et al.*, 2004). Hormone-induced cell apoptosis is a novel concept in breast cancer therapeutic intervention (Lewis *et al.*, 2004). Taken together, these lines of evidence support the dual role of estradiol in mammary cancer growth and development.

1.4.2 Pituitary Factors and Mammary Cancer

Ovarian hormones are not the only hormonal factor that regulates mammary tumor

growth and development. The use of estrogen deprivation therapies including anti-estrogenic agents such as tamoxifen, aromatase inhibitors, and ovariectomy lead to regression of some but not all breast tumor cases (Lewis *et al.*, 2004). Moreover, patients with advanced breast cancer, who underwent hypophysectomy (surgical removal of the pituitary glands) experienced ovary-independent beneficial effects (Wennbo and Tornell, 2000, Gebre-Medhin *et al.*, 2001). These observations suggest that one or more pituitary factors play a role in breast cancer growth. Animal studies performed by Huggins *et al.* (1958) also demonstrated a significant regression in tumor size after hypophysectomy in rats with 3-MC-induced mammary cancer, whereas ovariectomy reduced tumor size in most but not all rats. In addition, Sterental *et al.* (1962) showed that ovariectomy and adrenalectomy resulted in mammary tumor regression in DMBA-induced rats and that administration of estrogen reactivated tumor growth. In contrast, hypophysectomy led to mammary tumor regression but the administration of estrogen failed to reactivate tumor growth.

Among pituitary hormones, prolactin (PRL) and growth hormone (GH) are identified as pituitary factors that are primarily responsible for breast cancer growth and development in humans. High circulating levels of PRL and GH have been associated with breast tumor development. It has been shown that human breast cancer and mammary tumor cell lines express growth hormone receptor (Decouvelaere *et al.*, 1995) and prolactin receptor (Murphy *et al.*, 1984) and blocking PRL receptors results in inhibition of growth in cultured mammary tumor cells (Fuh and Wells, 1995). More recently, it was shown that human mammary tumors and normal mammary tissue produce both PRL and GH locally, suggesting a possible autocrine function of the hormones in tumor development and growth. The role of PRL and GH in experimental animal mammary tumors has also been investigated. The importance of GH in mammary tumorigenesis is demonstrated with GH-deficient Spontaneous Dwarf rats.

These rats failed to develop mammary tumors when treated with chemical carcinogens such as DMBA and MNU (Swanson and Unterman, 2002). Moreover, exogenous GH or PRL administered to hypophysectomized rats bearing mammary tumors promoted significant tumor growth compared to hypophysectomized rats injected with vehicle (Li and Yang, 1974). However, there are few studies that investigated the effect of individual pituitary hormone in the initiation process of chemical carcinogenesis. Thus, it is undetermined as to whether PRL, GH, or other factors are responsible for the growth of mammary tumors.

1.5 Cytochrome P450 Enzymes Overview

The cytochrome P450 (CYP) enzyme system is a superfamily of monooxygenases capable of catalyzing oxidative biotransformation of endogenous compounds and xenobiotics to more water-soluble products. CYP enzymes are classified into families and subfamilies according to their similarity in amino acid sequence (Nebert *et al.*, 1989). The expression of individual CYP enzyme is tissue and species specific. The CYP enzyme system catalyzes oxidations of a wide range of substrates. These substrates include endogenous compounds such as fatty acids and steroids, and xenobiotics such as drugs and toxins. The biotransformation of these compounds takes place in two phases. During Phase I, enzyme-catalyzed modification adds or exposes a functional group to the parent compound that can be used to attach a conjugate. CYP enzymes are part of the Phase I pathway and catalyze reactions such as hydroxylation, epoxidation, and dealkylation. In CYP-mediated hydroxylation, the added hydroxyl group then serves as the site for further modifications in Phase II metabolism. As mentioned in section 1.3, a proposed mechanism for estrogens to exert its carcinogenicity is via CYP-mediated biotransformation to its carcinogenic estrogen metabolites. Circulating estrogens are metabolized by CYP-mediated hydroxylation (Phase I metabolism) or by direct

conjugation to sulfate or glucuronide (Phase II metabolism). A majority of estrogens undergo conjugation, resulting in hormonally inactivated estrogens, whereas a relatively small amount of estrogens are converted to reactive catechol estrogens by CYP enzymes (Cavalieri *et al.*, 1997).

Mammalian species have similar but distinct sets of CYP enzymes. Between humans, rats, and mice, there are specific CYP enzymes that share similar function and regulation among species (e.g. CYP1A1, CYP1A2, CYP1B1). However, some CYP enzymes are unique within a particular species (e.g. CYP2C7, CYP2B2, CYP2C13 in rats). Mammalian CYPs are membrane bound and can be isolated by breaking open the cells and isolating the microsomal membrane (endoplasmic reticulum) fraction. In mammals, the main site of biotransformation takes place in the liver, where CYP enzymes are the most abundant. Extrahepatic tissues such as lungs, kidneys, brain, and mammary gland also express CYP enzymes, but the expression of CYP enzymes in these tissues is relatively low compared to the liver and not all hepatic forms are found in extra-hepatic tissues.

1.5.1 CYP1 Enzymes Overview

In humans and rats, the CYP1 family contains two subfamilies, CYP1A and CYP1B. The CYP1A subfamily contains two members, CYP1A1 and CYP1A2. In rats, constitutive expression of CYP1A enzymes is low in tissues such as small intestine, skin, lung, and liver. In the liver microsomes prepared from uninduced adult male rat, CYP1A enzymes account for approximately 1 to 3% of total CYP (Ryan and Levin, 1990). More recently, CYP1B1, a single member of the CYP1B subfamily, had been identified in humans, mice, and rats. CYP1B1 protein is difficult to detect in non-steroidogenic tissues of uninduced animals and its mRNA is present at a very low level in human liver, lymphocytes, endometrium, breast,

and lung epithelial cells (Murray, 2001). In the rats, CYP1B1 protein is expressed at a relatively high level in adrenal and testes (Bhattacharyya *et al.*, 1995, Leung *et al.*, 2005). Moreover, tumors of the kidney, prostate, and breast in humans were associated with an increased expression of CYP1B1 proteins (Murray, 2001).

The CYP1 enzymes are highly inducible. Induction of CYP1A enzymes is regulated via the aryl hydrocarbon receptor (AhR) (Li *et al.*, 1998). The unliganded AhR exists in the cytosol in a multiprotein complex. Upon binding of the inducing agent (AhR agonist), the multiprotein system dissociates and AhR translocates to the nucleus where it forms a heterodimer with aryl hydrocarbon nucleus translocator. This heterodimer binds to the specific DNA region termed dioxin or xenobiotic response element (DRE or XRE) of the CYP1A gene, thus enhancing its rate of transcription (Vrzal *et al.*, 2004). Chemical inducers of CYP1A enzymes include PAHs (its own substrate), β-naphthoflavone, polychlorinated dibenzo-*p*-dioxin (PCDD) and other environmental contaminants (Ryan *et al.*, 1982). The magnitude of CYP1A induction can differ depending on the dose of inducer, the structure of the inducer, as well as the target tissue. Humans, rodents, and other vertebrates appear to induce CYP1A through the same induction mechanism. Therefore, CYP1A enzyme induction has become a widely used determinant for exposure to environmental contaminants.

The role of CYP1 enzymes in the bioactivation of PAHs, nitrosamines, and aryl amines to their carcinogenic forms has been well documented (Guengerich, 1990). CYP1A induction had been associated with an increased risk in cancer development as an increased level of the enzyme could lead to an enhanced bioactivation of procarcinogens. Similar to the CYP1A enzymes, CYP1B1 is induced by PCDDs and other AhR agonists. Several regulatory elements within the promoter region in the CYP1B1 gene have been identified to be structurally similar to the regulatory elements within the CYP1A genes (Murray *et al.*,

2001). Although the induction of CYP1B1 expression by AhR agonists has been documented, there are other non-AhR-mediated mechanisms that could also contribute to the regulation of CYP1B1. For example, rat CYP1B1 is inducible by AhR agonists in liver, kidneys, and lungs, and is also inducible by ACTH (adrenocorticotrophic hormone) in the adrenal glands (Bhattacharyya *et al.*, 1995). Although basal expression of CYP1B1 is low relative to CYP1A1 and CYP1A2 in non-steroidogenic tissues; CYP1B1 protein expression was enhanced in a variety of human cancers including prostate (Tokizane *et al.*, 2005) and colon (Gibson *et al.*, 2003) . Along with emerging evidence supporting the role of CYP1B1 in procarcinogen bioactivation, CYP1B1 expression has become an important determinant of tumorigenesis.

1.6 Epoxide Hydrolase Overview

Another Phase I reaction, hydrolysis, is carried out by the enzyme, epoxide hydrolase (EH). Mammalian EH has three forms, cholesterol EH, soluble EH, and microsomal EH. Microsomal EH (mEH) is membrane bound and found in nearly all tissues. However, relative levels vary with tissue, species, sex, and age. Microsomal EH catalyzes the addition of water to epoxides or arene oxides to produce trans-hydroxy(diol) products (Morisseau and Hammock, 2005). The primary role of mEH is to convert foreign metabolically-derived epoxides to diols and in the process converts reactive metabolic intermediates to less mutagenic or carcinogenic products (Hassett *et al.*, 1998). However, mEH also play a role in the activation of PAHs to DNA-damaging diol-epoxides.

The inducibility of mEH in mammals is low relative to CYP1 enzymes. Exposure of primary human hepatocytes to compounds such as β-naphthoflavone and phenobarbital caused moderate increases of less than 3-fold in mEH mRNA expression, whereas CYP1A2

and CYP3A mRNA levels were induced more than 10-fold by these compounds (Hassett *et al.*, 1998). In addition, *in vivo* studies that measured hepatic mEH protein level and enzymatic activity in rats exposed to *trans*-stilbene oxide, phenobarbital, Aroclor 1254 or 3-MC also demonstrated modest inductions of mEH (less than 3-fold relative to the control values) (Thomas *et al.*, 1981). Despite the low expression of mEH protein, the presence of mEH is important in the formation of carcinogenic DMBA diol epoxide in the bioactivation of DMBA.

1.7 Animal Model of Human Breast Cancer

Animal models are useful experimental systems for the study of mammary cancer. A unique feature of mammary tumors in rats is that the induced tumors are 80 to 90% hormone dependent, whereas spontaneous mammary cancers in dogs and mice are primarily hormone-independent. In humans, approximately one-third of all breast cancer cases are hormone-dependent (Nandi *et al.*, 1995). Mammary cancers can be induced in rats by physical and chemical means including exposing the rats to 1) estrogens, 2) ionizing radiation, and 3) carcinogen chemicals (Huggins, 1965). Chemically induced mammary tumors in rats are, in general, papillary adenocarcinomas (Russo and Russo, 2000). Many are histologically similar to human ductal carcinoma *in situ* or early invasive adenocarcinomas (Clark, 2002).

1.8 Chemical Carcinogen Induction of Mammary Cancer in Rodents

Among different induction methods, the administration of a chemical carcinogen is the most efficient in inducing mammary carcinomas in a large percentage of rats. The most commonly used chemical carcinogens for studying the biology and therapeutic strategies of

mammary cancer are MNU and DMBA (Macejova and Brtko, 2001). MNU has been used more recently for the induction of mammary tumors whereas DMBA has been widely used since the 1950's. Critical parameters that affect induction of mammary tumors by chemical carcinogens include the nature and dose of the compound, and the species, strain, age, and hormonal status of the animal (Huggins, 1965). Many research groups have been successful at inducing mammary tumors in virgin adult female Sprague-Dawley rats with a single intragastric administration of 20 mg of DMBA between 50 to 60 days of age. Sprague-Dawley rats are widely used for chemically-induced mammary carcinoma studies because of their high susceptibility to chemical carcinogens. The first palpable mammary tumor usually appears around 8 to 10 weeks post DMBA treatment, and tumor number and size increase with time. Spontaneous mammary neoplastic lesions including fibroadenoma and adenocarcinoma also occur in Sprague-Dawley rats; however, these spontaneous mammary tumors do not appear before 50 weeks of age (Son and Gopinath, 2004). Based on previous experiments, the DMBA-induced Sprague-Dawley rat is a widely accepted animal model with which to study hormonal regulation of human breast cancer.

In our laboratory, a single intragastric dose of 20 mg of DMBA had been widely used for the induction of mammary cancer in performing breast cancer studies. By using the same induction method, data such as mammary tumor incidence, multiplicity, latency could be compared between former and future experiments. Therefore, DMBA was chosen to induce mammary tumors in my study. Another chemical carcinogen, MNU, is a direct acting alkylating agent that causes genetic damage or mutations to *ras* genes, which plays a significant role in signal transduction and regulation of cellular proliferation. The administration of DMBA can be achieved by intragastric and intravenous routes, whereas MNU can be administered by subcutaneous, intravenous, or intraperitoneal injections.

(Macejova and Brtko, 2001).

1.9 DMBA Overview

Many aromatic organic chemicals are capable of inducing mammary cancer in the rat after a single dose. Aromatic compounds with two or more rings are referred to as polycyclic aromatic hydrocarbons (PAHs). DMBA, which consists of 4 rings, is a type of PAHs (see Figure 1.4) and is relatively potent in inducing skin and mammary tumors in rodents (Cavalieri and Rogan, 2002). Environmental PAHs are generally found in by-products of combustion, in tobacco smoke and in cooked foods. Other examples of PAHs include naphthalene, anthracene, and benzo[*a*]pyrene (BaP). The carcinogenic activities of PAHs are not associated with the parent hydrocarbon but are a result of the biotransformation of the parent PAH into ultimate carcinogenic metabolites, which covalently bind to DNA causing mutation.

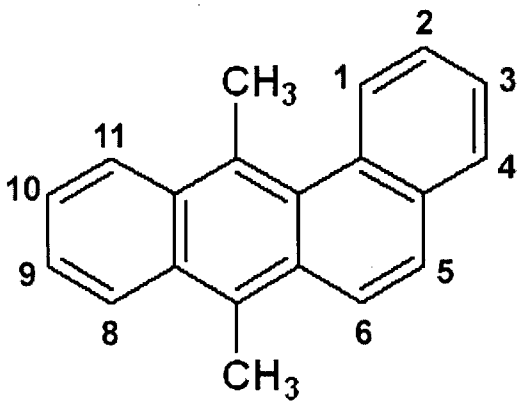


Figure 1.4 The chemical structure of DMBA.

1.9.1 Bioactivation of DMBA

The metabolic activation of DMBA has been studied intensively. There are approximately 30 DMBA metabolites formed by rat liver microsomes (Yang and Dower, 1975). DMBA is a procarcinogen that requires metabolic activation by CYP and mEH enzymes prior to becoming carcinogenic. CYP and mEH enzymes are located primarily in the liver and are also found in extrahepatic tissues including mammary tissue. Mammary epithelial cells have been shown to be capable of metabolizing DMBA (Christou *et al.*, 1995); therefore, the importance of mammary tissue in DMBA activation should not be neglected.

Several investigators observed that the initiation of DMBA-induced mammary carcinogenesis in rodents was altered by pretreating the animals with compounds that affect CYP1 enzyme levels and activities (MacDonald *et al.*, 2001, Chan and Leung, 2003). These studies demonstrated the importance of CYP1 enzymes in the initiation of mammary carcinogenesis by DMBA. In an *in vitro* study investigating the effect of different recombinant human and rodent CYP enzymes in the oxidative metabolism of DMBA, both human and rodent forms of recombinant CYP1A1 were found to be the most active in exhibiting metabolic activity, followed by CYP2C9, CYP2B6, and CYP1A2 (Shou *et al.*, 1996). More recently, several investigators showed that CYP1B1 is required for DMBA-induced cancer using the CYP1B1-null murine model (Gonzalez, 2001). Furthermore, using human recombinant CYP enzymes, it was demonstrated that CYP1B1 had higher activity than either CYP1A1 or CYP1A2 for the activation of various procarcinogens such as BaP and DMBA (Shimada *et al.*, 2004). Thus, the presence of CYP enzymes, specifically CYP1, is critical for the bioactivation of DMBA to its carcinogenic metabolites.

Previous studies proposed two major pathways of DMBA activation: 1) one-electron

oxidation by CYP or peroxidase enzymes to form intermediate radical cations (RamaKrishna *et al.*, 1992) and 2) metabolic activation to electrophilically-reactive bay-region diol epoxides (Melendez-Colon *et al.*, 2000) (see Figure 1.5). One of these major DMBA activation pathways involves a one-electron oxidation catalyzed by CYP or peroxidase enzymes to form the reactive carbenium ion at the 12-methyl group. The radical cation intermediate then binds to DNA to form depurinating adduct and ultimately apurinic site. Another major DMBA activation pathway is the bay-region diol-epoxide formation. According to the bay-region theory, formation of the diol-epoxide of DMBA can lead to reactive intermediates that bind to DNA (Shou *et al.*, 1996). DMBA-trans-3,4-diol-1,2- epoxide is formed by sequential reactions in which the first step is CYP-dependent oxidation of the 3,4-position of the DMBA molecule. The oxidation of DMBA at the 3,4-position is mediated most actively by purified CYP enzymes such as CYP1A1 and CYP1B1 (Shou *et al.*, 1996, Buters *et al.*, 2003). The resulting DMBA-3,4-oxide is then hydrolyzed by mEH to the corresponding trans-dihydrodiol or broken down non-enzymatically to monohydroxyl groups. A second CYP-mediated epoxidation of the 1,2-positions of DMBA-3,4-diol yields the reactive bay region diol epoxide metabolite (see Figure 1.6).

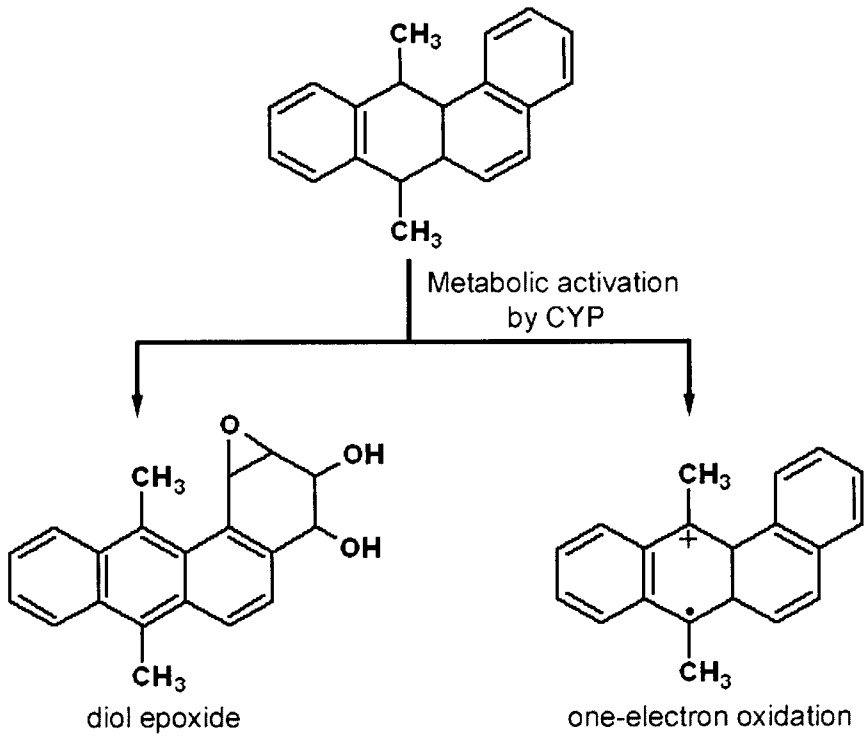


Figure 1.5 Metabolic activation of DMBA by one-electron oxidation and diol epoxide pathways.

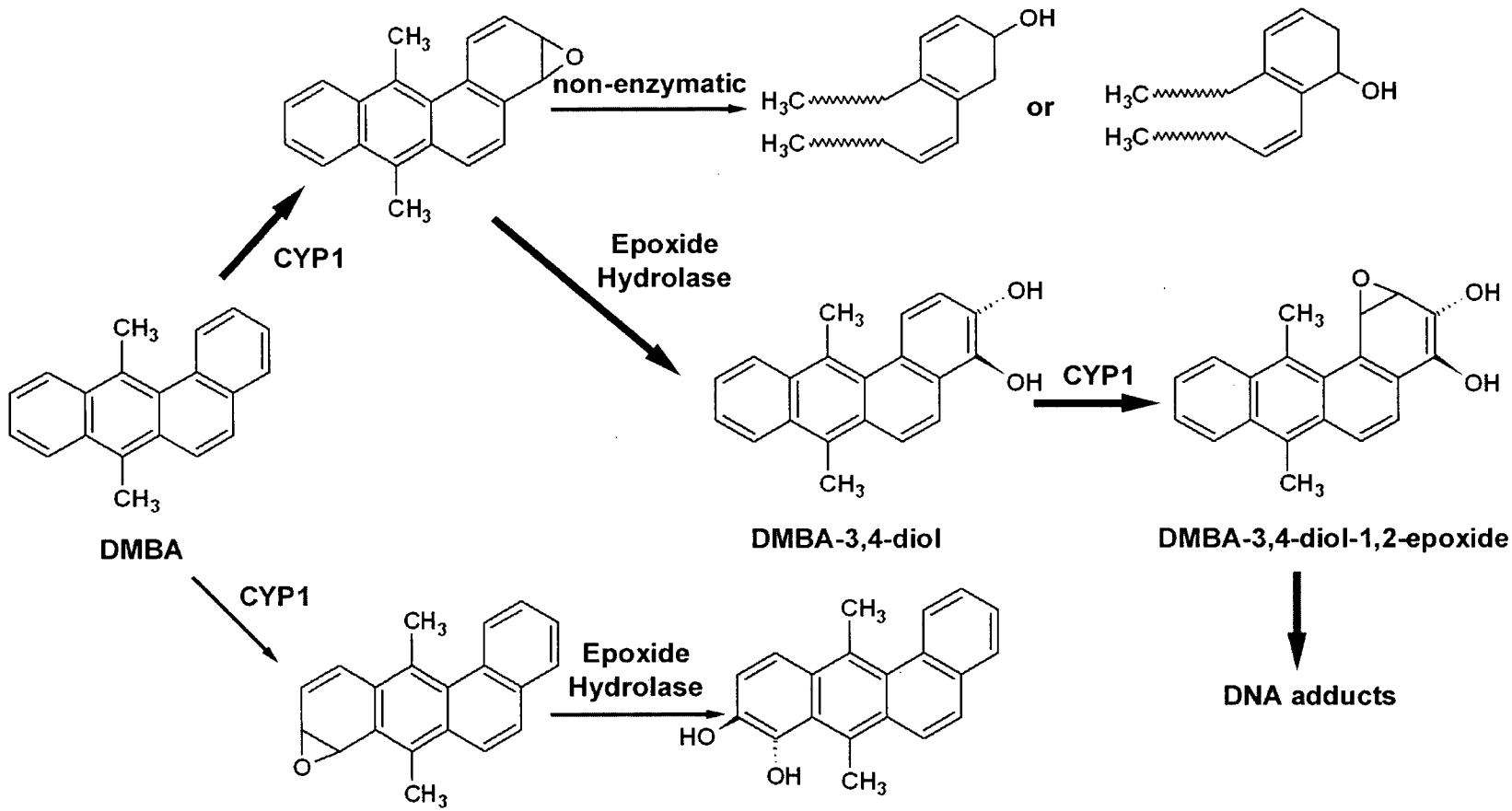


Figure 1.6 The bioactivation pathways of DMBA to its carcinogenic metabolite.

1.9.2 Carcinogenic Metabolites of DMBA

DNA adduct formation is a critical step in the process by which reactive PAH metabolites cause cancer (see Figure 1.7). Reactive DMBA metabolites covalently bind to DNA to form adducts. The DMBA metabolite-DNA adducts either remain intact in the DNA known as stable DNA adduct or release from DNA by cleavage of the glycoside bond forming depurinating adduct (see Figure 1.8). The unrepaired apurinic site is associated with mutation in genes leading to initiation of cancer. Earlier studies suggested that the 3,4-diol-1,2-epoxide of DMBA is the most active metabolite in binding to DNA in hamster V79 cells (Huberman *et al.*, 1979) and mouse embryo cells (Dipple and Nebzydoski, 1978). However, a limited number of *in vivo* studies investigated the carcinogenicity of individual DMBA metabolites. An *in vivo* study involving the topical application of DMBA and its metabolite derivatives on mouse epidermis demonstrated that DMBA-3,4-dihydrodiol derivatives formed the highest number of DNA adducts which further supports the diol epoxide pathway (Schoepe *et al.*, 1986). A more recent study by Melendez-Colon *et al.* (2000) supports stable DNA-adduct formation by diol epoxides as the primary carcinogenic pathway of PAHs. The study demonstrated that apurinic sites were primarily formed after short periods of exposure of MCF-7 cells to DMBA and BaP. However, when DMBA and BaP were incubated for a longer time (24 hours), stable DNA adducts formed by diol epoxides represented the majority of all DNA lesions, presumably due to an induction of CYP enzyme expression. Most published reports also support the theory that carcinogenic PAHs require CYP and mEH enzyme activity to elicit the formation of mutagenic and carcinogenic diol epoxide metabolites (Chou and Yang, 1978, Huberman *et al.*, 1979)

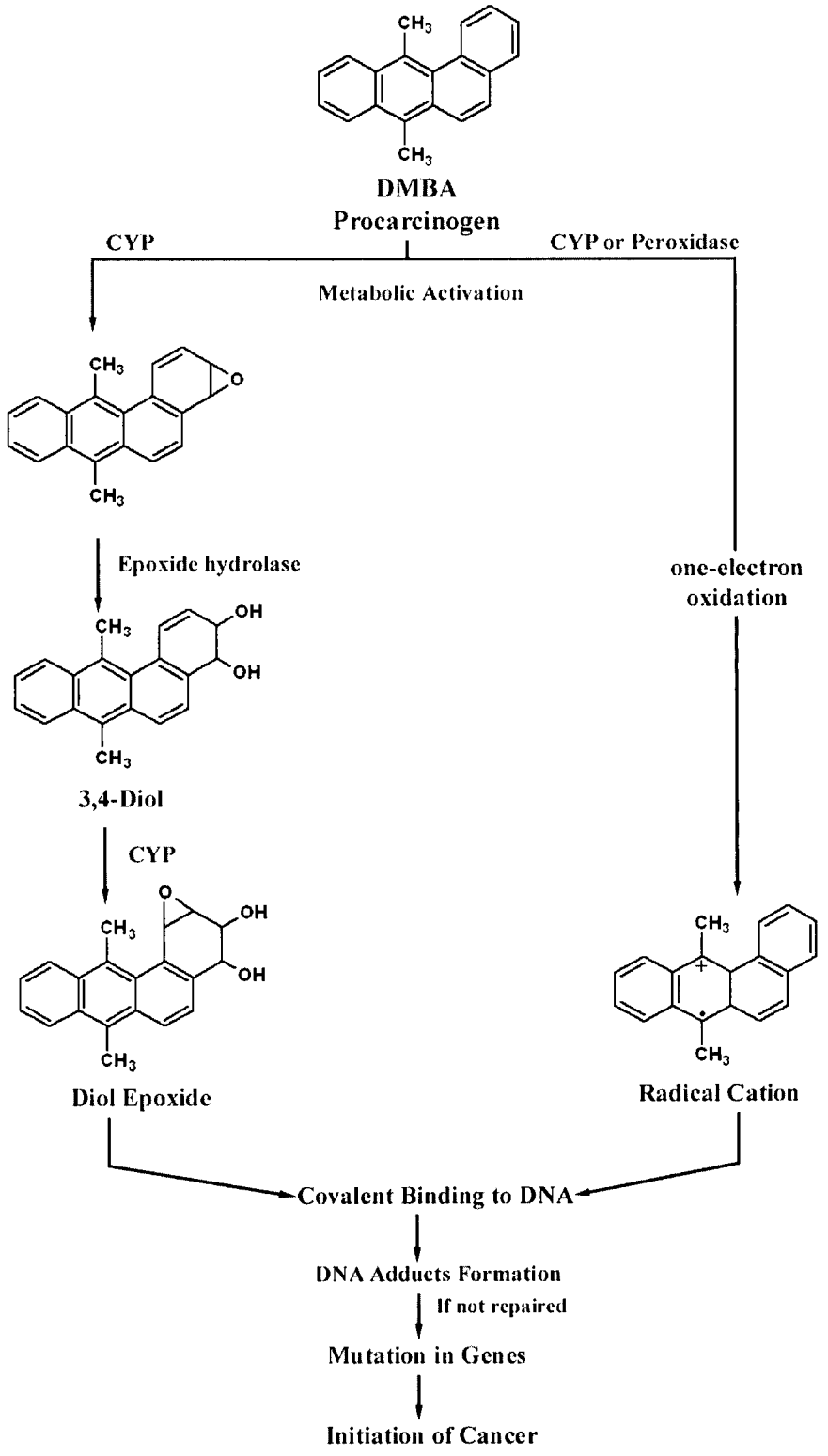


Figure 1.7 Overview of metabolic activation of DMBA leading to cancer

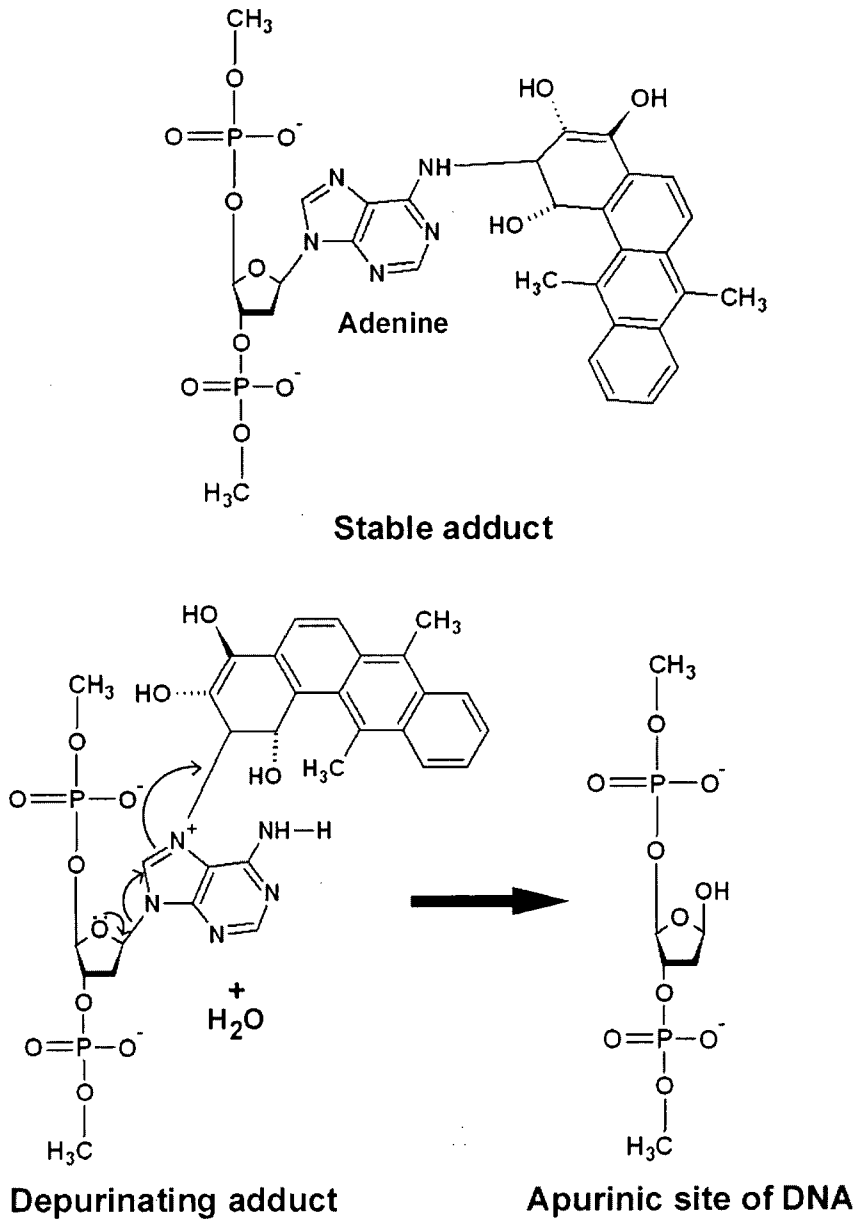


Figure 1.8 Formation of stable and depurinating DNA adducts and the generation of apurinic sites.

1.10 Rationale and Goals of the Present Study

The overall goal of the study is to determine the role of hormones in mammary tumorigenesis using the hypophysectomized rat model. A better understanding of the role of hormones in breast cancer growth and development can provide insight into methods of prevention as well as novel treatment therapies. This study was performed to illustrate the importance of hormones in mammary tumorigenesis as well as to validate the use of hypophysectomized rat as *in vivo* model to study DMBA-induced mammary tumorigenesis. Our study results will provide information on mammary tumorigenesis and expression of DMBA-bioactivating enzymes in intact and hypophysectomized rats. These results will benefit future hormonal studies perform using the hypophysectomized rat model.

The goal of this study is:

- To determine the effect of pituitary ablation on mammary tumor incidence, tumor burden, tumor multiplicity, and tumor latency.

My study will use intact and hypophysectomized female Sprague-Dawley rats as the experimental animal model and DMBA as the chemical carcinogen to induce mammary cancer. Mammary tumorigenesis in the hypophysectomized and intact rats will be monitored and analyzed.

1.11 Experimental Hypotheses

1. Hypophysectomy prevents mammary tumor development in DMBA-treated rats.
2. Expression of CYP and mEH enzymes involved in the bioactivation of DMBA is similar between hypophysectomized and intact rats.

1.12 Specific Objectives

- To assess mammary tumor incidence, tumor burden, tumor multiplicity, and tumor latency in DMBA-treated hypophysectomized and intact adult female virgin Sprague-Dawley rats
- To measure CYP1A1, CYP1A2, CYP1B1, and mEH enzyme levels by immunoblot analysis with specific antibodies in liver and mammary tissues of hypophysectomized and intact rats.
- To determine CYP1-mediated enzyme activities in liver microsomes of hypophysectomized and intact rats using the benzo[*a*]pyrene hydroxylase assay and methoxyresorufin *O*-demethylase (MROD) assay.

2. MATERIALS AND METHODS

2.1 Chemicals

Chemicals and reagents were obtained from the following sources:

Aldrich Chemical Company Inc. (Milwaukee, WI, USA)

Resorufin

BD Gentest (Woburn, MA, USA)

Anti-rat CYP1B1 antibody, cDNA-expressed rat CYP1B1 protein

BDH Chemicals (Toronto, ON, Canada)

Magnesium chloride ($MgCl_2 \cdot 6H_2O$)

Bio-Rad (Richmond, CA, USA)

Bromphenol blue

BIOSOURCE International (Camarillo, CA, USA)

Alkaline phosphate conjugated, goat F(ab′)2 anti-rabbit IgG, gamma and light chain specific, affinity purified

Fisher Scientific (Fair Lawn, NJ, USA)

Acetone (pesticide grade), acrylamide; ammonium persulphate, N,N′-methylene-bis-acrylamide (BIS), 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), Folin and Ciocalteu phenol reagent, glycerin, glycine, hexane (optima grade), methanol (reagent grade), potassium phosphate (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4), sodium

carbonate (Na_2CO_3), sodium chloride, sodium dodecyl sulphate (SDS), sodium phosphate (Na_2HPO_4); sodium hydroxide, β-mercaptoethanol, N,N,N′,N′-tetramethylehylenediamine (TEMED), 4-nitro-blue tetrazolium chloride (NBT), tris (hydroxymethyl) aminomethane (Tris)

J.T. Baker Chemical Co (Phillipsburg, NJ, USA)

Sodium dithionite

Molecular Probes (Eugene, OR, USA)

Methoxyresorufin

NCI Chemical Carcinogen Repository Midwest Research Institute

3-hydroxy benzo[*a*]pyrene

Pacific Milk Division (Vancouver, BC, Canada)

Skim milk powder

Pall Corporation (Pensacola, FL, USA)

Nitrocellulose membrane

Praxair (Vancouver, BC, Canada)

Carbon monoxide gas (99.5% purity)

Sigma-Aldrich (St Louis, MO, USA)

Benzo[*a*]pyrene, bromphenol blue; corn oil, cupric sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$), 7,12-dimethylbenz[*a*]anthracene (DMBA), ethylenediaminetetraacetic acid (ETDA), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), β-nicotinamide adenine dicucleotide phosphate reduced formed (NADPH), polyoxyethylene sorbitan monolaurate

(Tween 20), potassium chloride

VWR Scientific Products (West Chester, PA, USA)

Blotting paper

Dr. S.M. Bandiera (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada)

Purified rat cytochrome P450 1A1, purified rat cytochrome P450 1A2, purified rat epoxide hydrolase, rabbit anti-rat cytochrome P450 1A2 serum, rabbit anti-rat epoxide hydrolase IgG

Table 2.1 Buffers and reagents used in this study

Buffers or reagent	Contents
Tris-KCl buffer	50 mM Tris; 1.15% KCl; pH 7.4 at 4°C
EDTA-KCl buffer	10 mM EDTA; 1.15% KCl, pH 7.4 at 4°C
0.1 M KPO_4 buffer (for Total CYP determination)	0.1 M KH_2PO_4/K_2HPO_4; 20% glycerol; 0.1 mM EDTA, pH 7.4
Lowry Reagent C	98% of a; 1% of b; 1% of c a) 2% $NaCO_3$ anhydrous in 0.1 M NaOH b) 2% Na/K tartrate tetrahydrate c) 1% $CuSO_4 \cdot 5H_2O$
Sample dilution buffer	62.5 mM Tris-HCl, pH 6.8 at room temperature; 10.8% glycerol; 0.001% bromphenol blue; 1% SDS; 5% β-mercaptoethanol
Separating gel	0.375 M Tris-HCl, pH 8.8 at room temperature; 0.1% SDS; 7.5% acrylamide bis; 0.042% ammonium persulphate; 0.03% TEMED
Stacking gel	0125 M Tris-HCl, pH 6.8 at room temperature; 0.1% SDS; 3% acrylamide bis; 0.08% ammonium persulphate; 0.05% TEMED
Electrophoresis buffer (4X)	0.1 M Tris; 0.767 M glycine; 0.4% SDS
Transfer buffer (10X)	0.25 M Tris; 1.92 M glycine; 0.1% SDS
Transfer buffer (1X)	10% Transfer buffer (10X) in 3.5 part distilled water and 1 part methanol
Modified Phosphate Buffered Saline (PBS) (10X)	1.37 M NaCl; 26 mM KCl, 81 mM Na_2HPO_4; 15 mM KH_2PO_4; 2 mM EDTA
Blocking buffer	1% BSA; 3% skim milk powder in modified PBS, pH 7.4
Antibody dilution buffer	1% BSA' 3% skim milk powder; 0.05% Tween 20 in modified PBS, pH 7.4
Wash buffer	0.05% Tween 20 in modified PBS
Substrate solution	0.1 M Tris-HCl, 0.5 mM $MgCl_2$, pH 9.5
0.1 M HEPES buffer	0.1 M HEPES; 5 mM $MgCl_2$, pH 7.8
100 mM KPO_4 buffer (for BaP hydroxylase assay)	100 mM KH_2PO_4/K_2HPO_4; 5 mM $MgCl_2$; 0.1 mM EDTA, pH 7.5

2.2 Animals

Forty-nine to fifty-three days old (7 to 7 ½ weeks old) intact and hypophysectomized female Sprague-Dawley rats (140 to 155 g) were purchased from Charles River (Montreal, Canada). Hypophysectomy was performed at 7 weeks of age by the supplier. The animals were acclimatized with monitoring for 3 days post-arrival. Hypophysectomized rats were supplemented with 5% sucrose in their drinking water as a supplement to their diet as recommended by Charles River. Hypophysectomized animals have reduced food consumption when compared against the intact animals; therefore, their drinking water was supplemented with 5% sucrose. The intact animals received regular drinking water. All animals in the study had access to food and water *ad libitum* and were housed in pairs or triplets on corn-cob bedding in polycarbonate cages. Animal quarters were maintained at a temperature of 20 to 23°C and had a 12-hour photoperiod. All treatment and experimental procedures were performed in accordance with the principles and policies of the Canadian Council on Animal Care.

2.3 Study Designs

2.3.1 Study 1: DMBA-Induced Mammary Tumorigenesis in Sprague-Dawley Rats

Study 1 investigated the effect of hypophysectomy on DMBA-induced mammary tumorigenesis. The treatment plan for study 1 is outlined in Figure 2.1. Intact and hypophysectomized female Sprague-Dawley rats were assigned to one of the two treatment groups, corn oil (CO) or DMBA. Each treatment group consisted of 20 animals with the exception of hypophysectomized animals where 27 animals were treated with DMBA. At approximately 60 days of age, intact and hypophysectomized rats were treated with either a single intragastric (i.g.) dose of 20 mg of DMBA (dissolved in 1 ml of corn oil) or an equivalent volume of corn oil vehicle. Body weights were monitored every two days throughout the study period. Mammary tumors development including number, size, and latency was also monitored by palpitation twice weekly starting 5 weeks post treatment. The animals were monitored for 120 to 127 days after treatment and were then terminated by decapitation. Mammary tumors were excised and tumor number, size, location, and weight were recorded. Liver, ovaries, and uterus were also excised and weighed. During the monitoring period, an animal would be scheduled for immediate termination when it 1) developed ulceration or infection of the tumor site, 2) developed tumor mass where it significantly interferes with normal bodily functions or cause pain and distress, or 3) experienced more than 20% loss in body weight of a similar normal animal (taking into account the tumor mass).

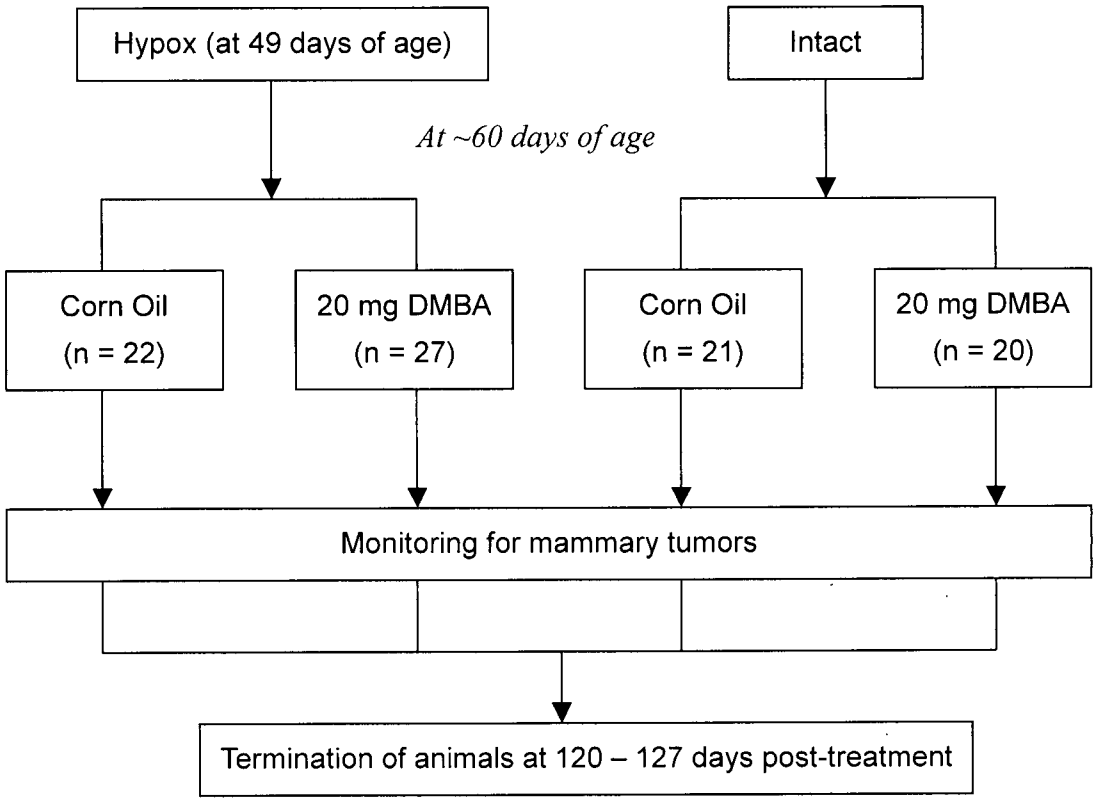


Figure 2.1 Treatment plan for study 1: DMBA-induced mammary tumorigenesis in Sprague-Dawley rats. Hypox: hypophysectomized rats; Intact: Intact rat.

2.3.2 *Study 2: Expression and Activity of DMBA-Bioactivating Enzymes in Intact and Hypophysectomized Rats*

Study 2 investigated the effect of hypophysectomy on CYP1 and mEH enzymes expression. Figure 2.3 summarizes the treatment plan for Study 2. Intact and hypophysectomized rats were assigned into one of the two treatment groups, corn oil or DMBA. Each treatment group consisted of 6 animals. At approximately 60 days of age, the animals received either a single intragastric dose of 20 mg DMBA or an equal volume of corn oil (vehicle). Twenty-four hours post dose, the animals were terminated by decapitation and liver and mammary tissue were harvested for microsome preparation and analysis.

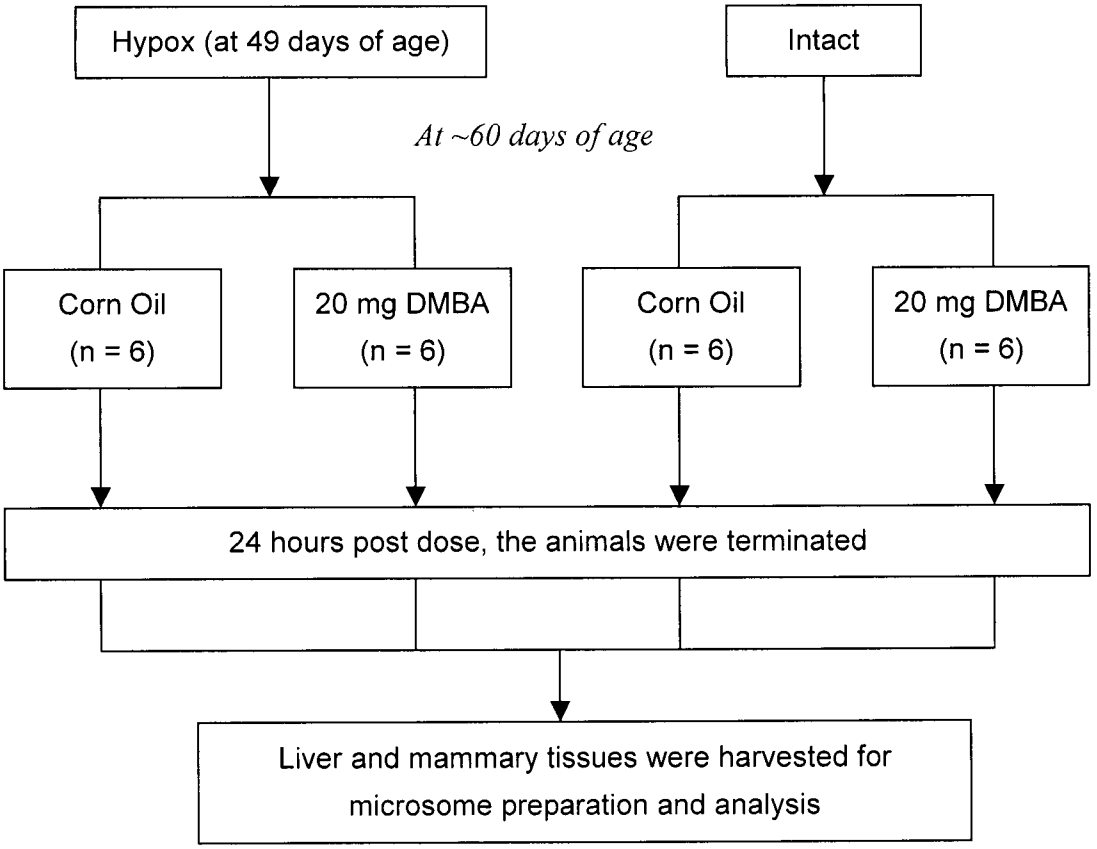


Figure 2.2 Treatment plan for study 2: Expression and activity of DMBA- bioactivating enzymes in intact and hypophysectomized rats.

2.4 Microsome Preparation

2.4.1 Liver

The whole liver was excised and homogenized in cold Tris-KCl buffer in a PotterElvehjem tissue grinder. The homogenate was centrifuged at 9,000 x g for 20 minutes at 4°C to generate the S9 fraction. The S9 fraction was then centrifuged further at 105,000 x g for 60 minutes at 4°C. The supernatant was then discarded and the remaining microsomal pellet was washed and resuspended in EDTA-KCl buffer using the homogenizer. The resuspended microsomal pellet was centrifuged again at 105,000 x g for 60 minutes at 4°C. After centrifugation, the final microsomal pellet was resuspended and homogenized in a minimal volume of 0.25 M sucrose. All samples and test tubes were kept on ice throughout the procedure. The final microsome preparations were stored in small aliquots at -76°C.

2.4.2 Mammary Tissue

Mammary tissue was processed similarly to liver as described in section 2.4.1 with the following modifications. The mammary S9 fraction underwent centrifugation at 105,000 x g for 60 minutes at 4°C only once and the pellet was not washed with EDTA-KCl buffer. The final microsome preparations were stored in small aliquots at -76°C.

2.5 Total CYP Determination

Total microsomal CYP content was determined by the method of Omura and Sato (1964). Microsome samples were diluted in 0.1 M KPO_4 buffer, pH 7.4 and divided into two matched spectrophotometer cuvettes (Hellma Canada Ltd). The reducing agent, sodium dithionite (~10 mg), was first added to both cuvettes and then carbon monoxide was gently bubbled into one of the cuvettes. The absorption difference between the two cuvettes was measured at absorption maxima at 450 nm with a SLM-Aminco DW-2C spectrophotometer (Urbana, IL). Total CYP concentration was calculated using the difference between the absorption maximum and the molar extinction coefficient of 91 cm^{-1} mM^{-1}.

2.6 Total Protein Determination

Total protein content was measured by the method of Lowry *et al.* (1951). Microsome samples were diluted in water with a dilution factor of 1:20 or 1:10 for liver microsomes and 1:5 for mammary tissue microsomes. Lowry Reagent C was first added to each diluted microsome sample with vigorous mixing. After 10 to 15 minutes, Folin and Ciocalteu's phenol reagent (diluted in 1:1 part with distilled water) was added to each sample. After incubation at room temperature for 30 minutes, absorbance was measured at 750 nm, using a microplate autoreader model EL309 (BIO-TEK Instruments Inc.). Different concentrations (0, 10, 20, 40, and 100 mg per ml) of bovine serum albumin (BSA) were processed by the same procedure and were used to generate a standard curve of absorbance versus protein concentration from which the concentrations of the microsome samples were extrapolated. All samples were analyzed in duplicate.

2.7 SDS-PAGE and Immunoblot Assay

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using a Hoefer SE 600 vertical slab gel unit (San Francisco, CA). The discontinuous SDS-polyacrylamide gel consisted of a 3% acrylamide stacking gel and a 7.5% acrylamide separating gel. Microsomes were diluted in sample dilution buffer and boiled for 2 minutes. The denatured microsome samples were loaded on to the gel at 20 µl per well. Sample loading concentrations are summarized in Table 2.2. Electrophoresis was carried out at a constant current of 23 mA through the stacking gel (approximately an hour) and 46 mA through the separating gel (approximately 2 ½ to 3 hours) until the dye front reached the bottom of the gel. Proteins resolved by SDS-PAGE were transferred electrophoretically onto 0.2 micron pore size nitrocellulose membrane by the methods described by Towbin *et al.* (1979). The procedure was carried using Hoeffer TE 52 Transphor unit with Power Lid under constant current of 0.4 A for 2 hours in the cold cabinet. After the transfer, the membranes were left overnight in blocking buffer at 4°C. The membranes were incubated with polyclonal rabbit anti-rat CYP1A2 serum, anti-rat CYP1B1 antibody, or anti-rat EH IgG (see Table 2.3 for concentrations used) in antibody dilution buffer at 37°C for 2 hours with shaking, followed by three 5 to 10 minute incubations with wash buffer. The membranes were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000 dilution) for 2 hours at 37°C with shaking, followed again by three 5 to 10 minute incubations with wash buffer. Colorimetric detection of immunoreactive proteins was completed by reacting with 0.01% NBT and 0.005% BCIP in substrate solution at room temperature under subdued light. The reaction was stopped by discarding the substrate solution and washing the membrane with distilled

water when protein bands were sufficiently stained. The alkaline phosphatase reaction time varied between 1 to 8 minutes.

Table 2.2 Amount of microsomal protein loaded per lane for immunoblot assay

	CYP1A study	EH study	CYP1B1 study
Liver			
Hypox + CO	20	5	--
Hypox + DMBA	5	5	--
Intact + CO	20	5	--
Intact + DMBA	5	5	--
Mammary tissue †			
Hypox + CO	40	40	40
Hypox + DMBA	40	40	40
Intact + CO	40	40	40
Intact + DMBA	40	40	40

Values are presented as μg of total microsomal protein.

† Few mammary samples did not have sufficient total protein content to load 40 μg per lane. For these samples, the maximum amount of total protein was loaded by diluting microsomes with sample dilution buffer in a 1:1 ratio.

-- indicates immunoblot assay was not performed.

Table 2.3 Concentration of antibody used for immunoblot assay

	Liver	Mammary tissue
Anti-rat CYP1A2 serum	1:1000	1:500
Anti-rat CYP1B1	--	1:500
Anti-rat EH IgG	20 μg/ml	20 μg/ml

-- indicates immunoblot assay was not performed.

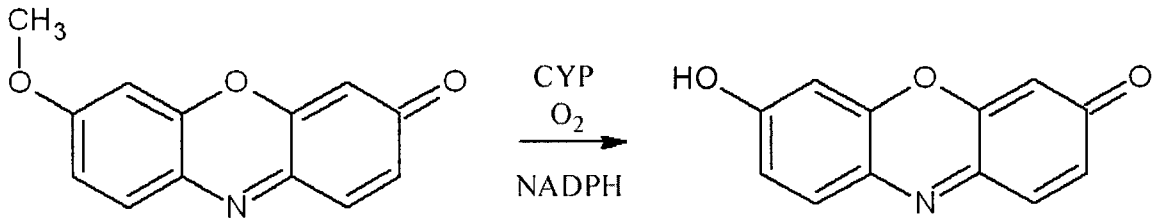
2.8 Quantitation of Immunoblot Staining

Staining intensities of protein bands on immunoblots were quantified with a pdi 320 oe scanning densitometer using Quanity One ® version 4.2.0 software (Bio-Rad Laboratories, Hercules, CA). Protein band intensity was measured as contour quantity (CQ), calculated by the software program as optical density x contour area (OD x mm^2). The CQ values were divided by the CQ value of a purified standard that was included in each gel. Final protein concentration was calculated using of calibration curve generated by loading various concentrations of purified standards on gels followed by immunoblotting and densitometric analysis as described above.

2.9 Enzyme Activity

2.9.1 Methoxyresorufin O-demethylase (MROD) Assay

Microsomal methoxyresorufin *O*-demethylase (MROD) activities were determined by a direct fluorometric method originally described by Burke and Mayer (1974) with modifications. The cytochrome P450-catalyzed demethylation of methoxyresorufin is illustrated in Figure 2.4. Assay mixtures contained 1.93 ml of 0.1 M HEPES buffer, 50 µl of microsomes diluted to 2 mg protein per ml in 0.25 mM sucrose, and 10 µl of 0.5 mM methoxyresorufin dissolved in DMSO (2.5 µM final concentration). After a preincubation of 5 minutes at 37°C, the reaction was initiated by the addition of 10 µl of NADPH dissolved in HEPES buffer (0.25 mM final concentration). The total volume of the reaction mixture was 2 ml and reactions were carried out at 37°C under subdued light. The formation of resorufin was measured as an increase in fluorescence using a Shimadzu RF-540 spectrofluorometer (Kyoto, Japan), with excitation and emission wavelength set at 530 and 582 nm, respectively. The slit width for both the excitation wavelength and emission wavelength were set at 2 nm. Fluorescence values were recorded at 5 minutes post initiation of the reaction. The amount of resorufin formed was determined from a standard curve of fluorescence versus resorufin concentration incubated in the same reaction mixture as the samples with distilled water replacing NADPH and sucrose replacing microsome. All measurements were performed in duplicate.



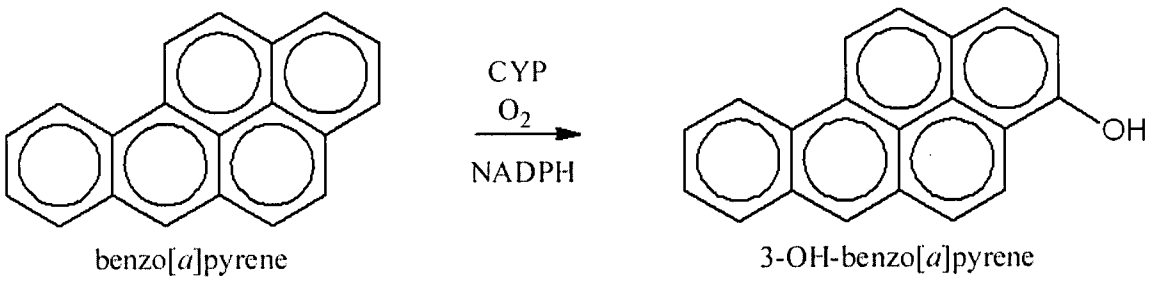
methoxyresorufin resorufin

Figure 2.3 The cytochrome P450-catalyzed demethylation of methoxyresorufin

2.9.2 Benzo[a]pyrene Hydroxylase Assay

Benzo[*a*]pyrene (BaP) hydroxylation activity was measured according to the method described by Nebert and Gelboin (1968). The biotransformation of benzo[*a*]pyrene to its metabolities is mediated by several CYP enzymes (see Figure 2.5). In this assay, rat hepatic microsomes were incubated with BaP and the formation of BaP metabolites was measured fluorimetrically. Reaction mixture contained 50 µl of hepatic microsomal protein diluted to 2 mg protein per ml in 0.25 mM sucrose, 500 µl of 100 mM KPO_4 buffer pH 7.5, 430 µl distilled water, and 10 µl of 8 mM benzo[*a*]pyrene (80 µM final concentration). After a 5-minute incubation at 37°C, the reaction was initiated by the addition of 10 µl of NADPH dissolved in distilled water (0.5 mM final concentration). The reaction was carried out in a shaking water bath at 37°C for 4 minutes and stopped with the addition of 1 ml cold acetone to the reaction mixture. Hexane (3.25 ml) was added to each tube for the first extraction. The tubes were capped and mixed vigorously for at least two minutes. The mixture was allowed to settle for 30 minutes prior to transferring 2 ml of the organic fraction to a new test tube. A second extraction with 4 ml of 1 M NaOH was added to the new tubes. The tubes were then shaken vigorously for at least two minutes and centrifuged at 3,000 x g for 10 minutes using a Beckman GP centrifuge (Palo Alto, CA). The upper organic phase was aspirated and

discarded. BaP metabolites were measured as the fluorescence of the remaining aqueous phase using a Shimadzu RF-540 spectrofluorophotometer interfaced with a Shimadzu DR-3 data recorder (Kyoto, Japan), with excitation and emission wavelength set at 396 and 522 nm, respectively. The slit width for both the excitation wavelength and emission wavelength were set at 2 nm. The amount of BaP metabolites formed was determined from a standard curve of fluorescence intensity versus concentration generated using known concentrations of a major BaP metabolite, 3-hydroxy-benzo[*a*]pyrene (3-OHBaP) incubated in the same reaction mixture as the sample with distilled water replacing NADPH and sucrose replacing microsome. All samples were performed in duplicate.



benzo[*a*]pyrene 3-OH-benzo[*a*]pyrene

Figure 2.4 The cytochrome P450-catalyzed hydroxylation of benzo[*a*]pyrene

2.10 Statistical Analysis

The differences in CYP and mEH enzymes protein levels and enzyme activities were analyzed by two-way ANOVA with Tukey-Kramer multiple comparison test (InStat version 3.00, GraphPad Software, Inc) with the exception in Study 1, where a one-way ANOVA with non-parametric (Dunn) post-hoc test was used to analyze body and organ weights due to non-uniform sample size. Differences with a p-value < 0.05 were considered to be statistically significant.

3. RESULTS

3.1 Study 1: DMBA-Induced Mammary Tumorigenesis in Sprague-Dawley Rats

3.1.1 Effects of Hypophysectomy and DMBA Treatment on Body Weight

The animals were weighed every two days throughout the study period. In the beginning of the experiment, intact animals had an average body weight of 170 ± 6 g and hypophysetomized animals had an average body weight of 143 ± 7 g. The average animal body weight of each treatment group is illustrated in Figure 3.1. Hypophysectomized animals had minimal weight gain of less than 33 ± 14 g from beginning to the end of the study. Intact animals, on the other hand, maintained steady weight gain with an average gain of 163 ± 29 g per rat over 120 days. At termination, the body weight of hypophysectomized rats was about 50% of that of the intact animals. There was a significant difference between body weights of intact and hypophysectomized rats.

DMBA treatment has a temporary effect on body weight. A single intragastric dose of 20 mg of DMBA or equivalent volume of corn oil was given on Day 0 to both intact and hypophysectomized rats. After treatment, DMBA-treated animals began to lose weight. The total decline in body weight was about 11 ± 7 g (5%) in the intact animals whereas hypophysectomized rats lost approximately 18 ± 6 g (9.5%) compared to the weight before treatment. The drop in body weight continued for 4 days after DMBA treatment and body weight gradually returned to the normal pattern. Statistical analysis showed no significant difference between the final body weight of DMBA and corn oil-treated intact rats or hypophysectomized rats (see Table 3.1).

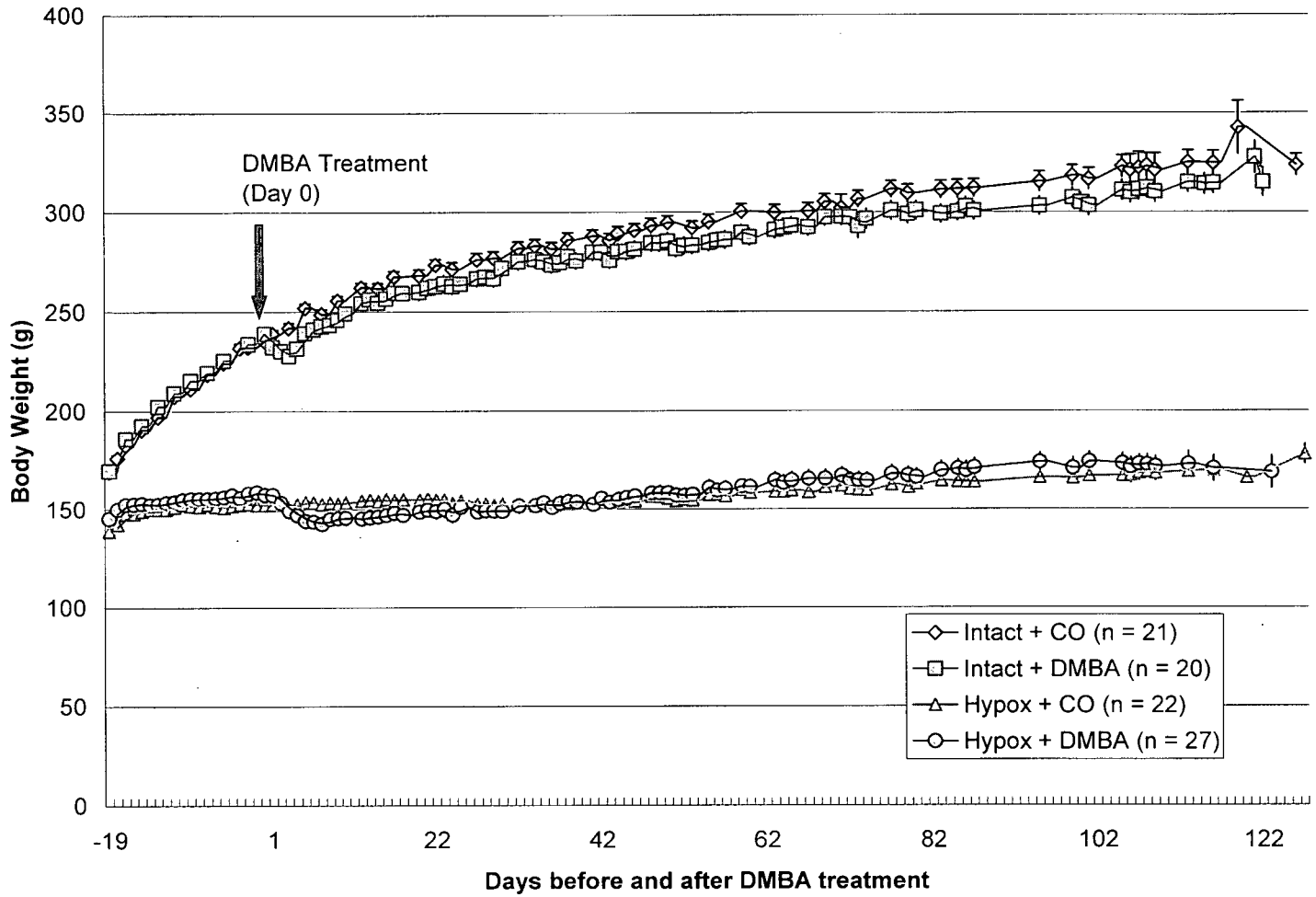


Figure 3.1 Daily mean body weight of rats in each treatment group ($n = 6$) during the experimental period of study 1. Values are expressed as mean ± SEM.

Table 3.1 Effects of hypophysectomy and DMBA treatment on final mean body weight

	n	Final body weight (g)
Intact + CO	21	332 ± 7
Intact + DMBA	20	321 ± 5
Hypox + CO	22	172 ± 3 †
Hypox + DMBA	27	169 ± 8 †

Values presented as mean ± SEM.
Final body weight was measured on the day of termination of rats.
† Signficantly different from their corresponding intact groups with a p-value < 0.05.

3.1.2 Effects of Hypophysectomy and DMBA Treatment on Organ Weight

At termination, liver, ovaries, and uterus were excised and weighed. The mean organ weights are presented as absolute weight and as a percentage of body weight in Table 3.2. Statistical analysis was performed using the relative (percentage) organ weights to determine the effect of hypophysectomy and DMBA treatment on organ weight. Comparing corn oil-treated intact and hypophysectomized animals, there was no significant difference in liver weight. Conversely, ovary and uterus weights of hypophysectomized animals were significantly lower than those of the intact animals. Liver, ovary and uterus weights of DMBA-treated hypophysectomized animals were not significantly different from their corresponding corn oil-treated groups. According to the results, hypophysectomy affected ovary and uterus weight whereas DMBA treatment had no effect on liver, ovary, or uterus weights.

Table 3.2 Effects of hypophysectomy and DMBA treatment on final mean organs weight

	n	Final liver weight (g)	Final ovary weight (g)	Final uterus weight (g)
Intact + CO	21	11.2 ± 0.4 (3.36)	0.12 ± 0.01 (0.036)	0.55 ± 0.02 (0.166)
Intact + DMBA	20	11.2 ± 0.2 (3.49)	0.16 ± 0.02 (0.050)	0.52 ± 0.03 (0.162)
Hypox + CO	22	5.5 ± 0.1 (3.22)	0.04 ± 0.01 (0.023) †	0.06 ± 0.01 (0.035) †
Hypox + DMBA	27	6.4 ± 0.5 (3.77)	0.03 ± 0.01 (0.018) †	0.06 ± 0.01 (0.035) †

Values presented as mean ± SEM.
Values in parentheses represent percentage of organ weight per body weight.
Final organ weights were measured on the day of termination of rats.
Ovary weight was measured from both ovaries.
† Signficantly different from Intact+CO and Intact+DMBA groups with a p-value < 0.05.

3.1.3 Effect of a Single Intragastric Dose of 20 mg of DMBA on Mortality

A single intragastric dose of 20 mg of DMBA was demonstrated to be very toxic to hypophysectomized rats. Twenty-seven hypophysectomized rats were treated with DMBA and only nine rats survived after the first week (see Table 3.3). Among the intact animals, there were no deaths associated with the same DMBA treatment. A report by Carter *et al.* (1988) noted DMBA can elicit acute symptoms of lethargy, decreased food consumption, diarrhea, and adrenal necrosis. The deaths seen in the hypophysectomized animals were associated with the acute toxicity of DMBA.

Table 3.3 Number of rats in each group before treatment and at termination

	n Before treatment	*n* At termination
Intact + CO	21	21
Intact + DMBA	20	20
Hypox + CO	22	22
Hypox + DMBA	27	9

3.1.4 Effect of Hypophysectomy on DMBA-Induced Mammary Tumor Incidence

Mammary tumor development was monitored for 120 to 127 days (17 to 18 weeks) post treatment. Mammary tumors occurred in 55% of the intact rats treated with DMBA (see Table 3.4). In addition, there was also one case of spontaneous mammary tumor formation in an intact rat in the corn oil group. Mammary tumor latency of the first palpable tumor was approximately 8 weeks after DMBA treatment and tumor number and tumor size (data not shown) increased with time (see Figure 3.2). The number of mammary tumors in the rats was estimated by palpitation and the exact tumor number, tumor size, and tumor location can only be obtained on the day of termination. On average, there were approximately 2.5 mammary tumors per tumor-bearing intact rat. Based on the results of previous studies, mammary tumor incidence will reach 100% in the intact rats treated with DMBA. However, all animals were scheduled for termination on 120 to 127 days post treatment because some tumors were beginning to ulcerate and tumor mass was becoming too large for the animal to bear in accordance to the guidelines recommended by the UBC Animal Care Committee.

Table 3.4 Mammary tumor incidence, weight, multiplicity, and latency in intact and hypophysectomized rats following treatment with DMBA and corn oil

	n	Tumor Incidence (%)	Average tumor weight (g)	Average number of tumor/animal	Tumor Latency
Intact + CO	21	1 (5)	6.18	1	12
Intact + DMBA	20	11 (55)	0.88 ± 0.49	2.5	8
Hypox + CO	22	0 (0)	--	--	--
Hypox + DMBA	9	0 (0)	--	--	--

Values presented as mean ± SEM ($n = 6$).
Values in parentheses represent percentage of tumor incidence in each group.
-- indicates data is not available.

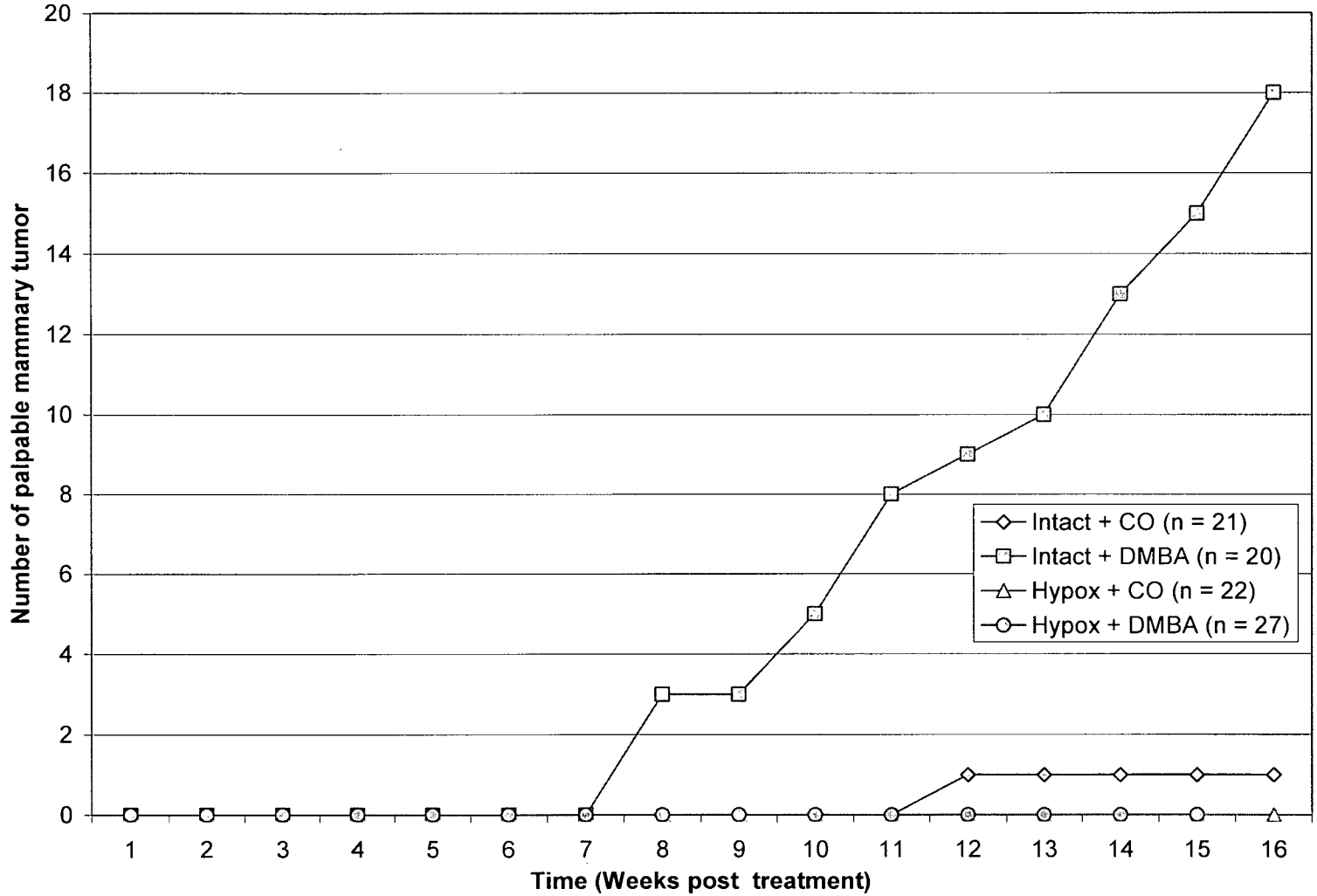


Figure 3.2 Total number of palpable mammary tumors in each group ($n = 6$) per week post DMBA or corn oil treatment.

3.2 Study 2: Expression and Activity of DMBA-Bioactiviating Enzymes in Intact and Hypophysectomized Rats

3.2.1 Effects of Hypophysectomy and DMBA Treatment on Body and Liver Weight

The animals were weighed daily and on the day of termination. Liver weight was collected 24 hours post DMBA treatment. Both intact and hypophysectomized rats were given either a single intragastric dose of 20 mg of DMBA or an equivalent volume of corn oil. Table 3.5 summarizes the effect of hypophysectomy and DMBA treatment on mean body and liver weights. When liver weights were expressed as a percentage of body weight, there was no difference in relative liver weight between the hypophysectomized and intact animals. The results demonstrated hypophysectomy alone did not affect relative liver weight. However, when the animals were treated with DMBA, the relative liver weight of the hypophysectomized animals was lower than that of the intact animals. Our results demonstrated that DMBA treatment along with hypophysectomy significantly reduced relative mean liver weight.

Table 3.5 Effects of hypophysectomy and DMBA treatment on body weight and liver weight in female rats terminated 24 hours post treatment.

Treatment	Body weight (g)	Liver weight (g)	Liver weight as a percentage of body weight (%)
Hypox + CO	172 ± 3	6.41 ± 0.26	3.73 ± 0.09
Hypox + DMBA	162 ± 1	4.91 ± 0.11	3.02 ± 0.05 †
Intact + CO	212 ± 5	8.14 ± 0.35	3.84 ± 0.08
Intact + DMBA	208 ± 4	7.89 ± 0.26	3.79 ± 0.05

Values presented as mean ± SEM (n = 6).
† Significantly different from all other treatment groups with a p-value <0.05.

3.2.2 *Effects of Hypophysectomy and DMBA Treatment on Total CYP Content*

Protein and total CYP concentrations were measured in hepatic microsomes to determine total CYP content. Total CYP content was expressed as nmol per mg of protein (see Table 3.6). The mean total CYP content was significantly higher in liver microsomes of intact rats treated with DMBA compared with intact rats treated with corn oil. Total CYP content was induced by DMBA treatment in the intact animals. In the hypophysectomized animals, however, there was no difference between corn oil-treated and DMBA-treated groups. The higher CYP content of the corn oil-treated hypophysectomized rats relative to corn oil-treated intact rats indicates that hypophysectomy increased total CYP content and that DMBA treatment of hypophysectomized rats did not further increase total CYP content.

Table 3.6 Effects of hypophysectomy and DMBA treatment on total hepatic CYP content.

Treatment	Total CYP content (nmol/mg protein)
Hypox + CO	0.97 ± 0.09 †
Hypox + DMBA	1.06 ± 0.03 †
Intact + CO	0.66 ± 0.02
Intact + DMBA	0.98 ± 0.05 †

Values presented as mean ± SEM (n = 6).
† Significantly different from Intact + CO group with a p-value <0.05.

3.2.3 *Effects of Hypophysectomy and DMBA Treatment on Hepatic CYP1A2 Protein Expression*

Hepatic microsomal proteins were resolved by SDS-PAGE. CYP1A1, CYP1A2, CYP1B1 and mEH enzymes in the microsomal samples were detected and quantified by immunoblot analysis as described in section 2.7. The immunoblot was probed with polyclonal rabbit anti-rat CYP1A2 serum, which recognizes both CYP1A1 and CYP1A2 proteins. Figure 3.3 is a scanned image of the immunoblot showing CYP1A1 and

CYP1A2 in hepatic microsomes from each treatment group as well as different concentrations of purified CYP1A1 and CYP1A2 standards. CYP1A2 was the lower band (lower molecular weight) and CYP1A1 was the upper band (higher molecular weight). A bar graph summarizing the mean hepatic CYP1A2 protein level for each treatment group is presented in Figure 3.4A. Hepatic CYP1A2 was significantly induced by DMBA in hypophysectomized and intact rats. Compared with the corn oil-treated control group, there was a 8-fold induction of CYP1A2 protein in the hypophysectomized rats and a 7-fold induction in the intact rat samples. The results demonstrated that CYP1A2 was present at similar levels in livers of both intact and hypophysectomized animals and the levels were induced to approximately the same extent by DMBA treatment.

3.2.4 *Effects of Hypophysectomy and DMBA Treatment on Hepatic CYP1A1 Protein Expression*

Hepatic CYP1A1 protein was undetectable in the liver microsomes from corn oil-treated animals whereas hepatic CYP1A1 protein was detected in DMBA-treated animals (Figure 3.3). The results suggested hepatic CYP1A1 protein was induced by DMBA. Protein expression of CYP1A1 in the livers of DMBA-treated intact and hypophysectomized rats was lower than protein expression of CYP1A2. Figure 3.4B illustrates hepatic CYP1A1 levels for hypophysectomized and intact rats. Statistical analysis showed there was no significant difference between hepatic CYP1A1 protein levels in the DMBA-treated hypophysectomized and intact rats.

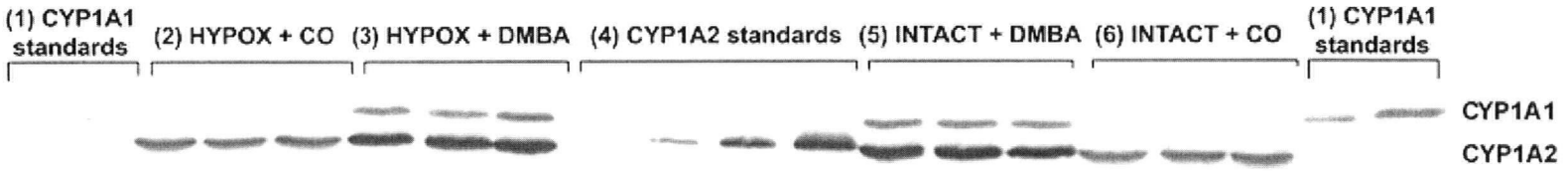
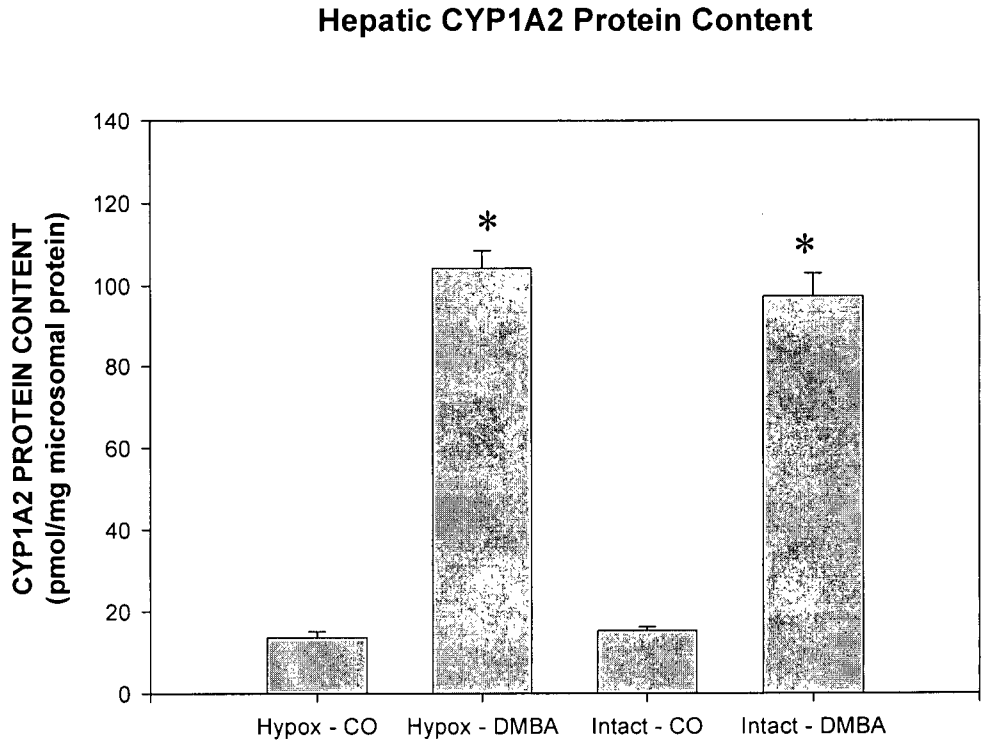


Figure 3.3 Immunoblot showing hepatic samples probed with rabbit anti-rat CYP1A2 polyclonal serum (1:1000 dilution). CYP1A1 is represented as the upper band and CYP1A2 as the lower band. From left to right, bands showing samples of (1) CYP1A1 standards of 0.05, 0.1, 0.25, and 0.5 pmols per lane, (2) Hypophysectomized rats treated with corn oil, loaded at 2.5 μg per lane, (3) Hypophysectomized rats treated with DMBA, loaded at 1 μg per lane, (4) CYP1A2 standards of 0.05, 0.1, 0.25, and 0.5 pmols per lane, (5) Intact rats treated with DMBA, loaded at 1 μg per lane, (6) Intact rats treated with corn oil, loaded at 2.5 μg per lane. The membrane was developed with substrate solution for 2 minutes.

A.



B.

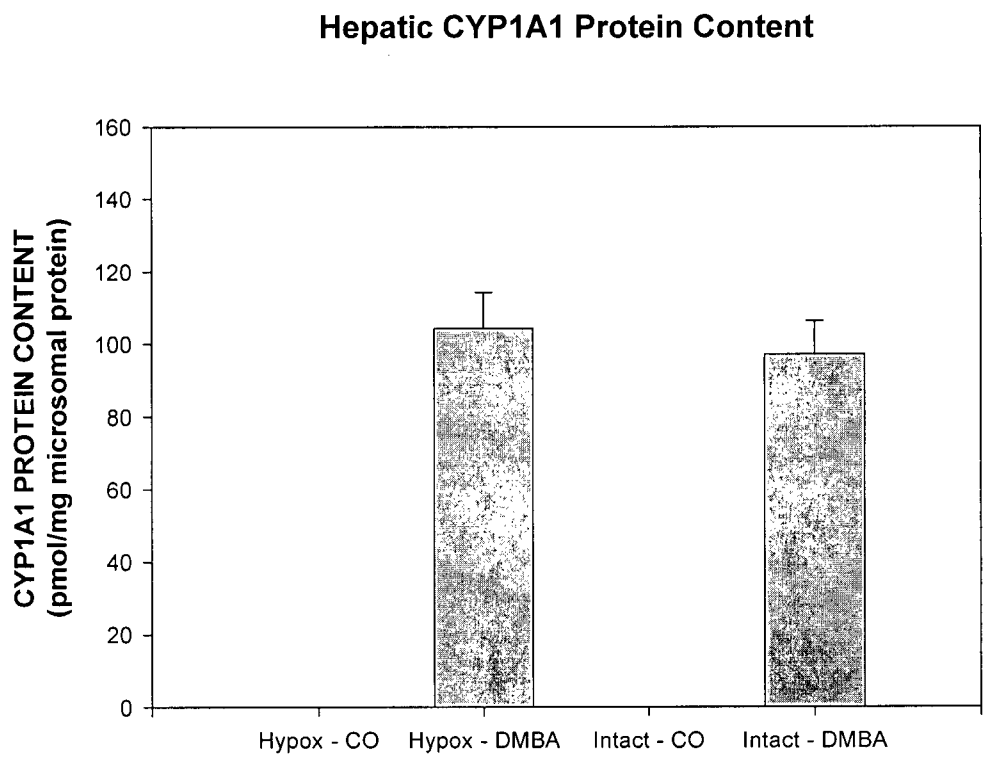


Figure 3.4 Summary of hepatic CYP1A1 (A) and CYP1A2 (B) protein content in corn oil- or DMBA-treated hypophysectomized and intact rats. Bars are shown as mean ± SEM ($n = 6$). * Significantly different from other treatment groups with a p-value <0.05.

3.2.5 *Effects of Hypophysectomy and DMBA Treatment on Hepatic CYP1B1 Protein Expression*

CYP1B1 was not examined in the hepatic microsomes because it was demonstrated previously in our laboratory that the protein level was too low to be detected using the immunoblot technique.

3.2.6 *Effects of Hypophysectomy and DMBA Treatment on Mammary CYP1A2 Protein Expression*

Mammary microsome samples were separated by SDS-PAGE and probed with anti-rat CYP1A2 serum to detect CYP1A2 and CYP1A1 enzymes. The amount of microsomal protein loaded per lane for the mammary tissue was 2 to 5 times greater than the liver in order to achieve similar protein band intensity. It suggests that mammary CYP1A2 protein content was much lower when compared with hepatic CYP1A2 protein content in rats. On the immunoblot, CYP1A2 protein bands of corn oil-treated mammary microsome of hypophysectomized and intact animals were poorly visible (see Figure 3.5). Upon treatment with DMBA, CYP1A2 protein was induced in the mammary tissue of both hypophysectomized and intact rats. No statistical analysis was performed because CYP1A2 protein bands were not detected consistently in the mammary microsome samples. Nevertheless, the results demonstrate that mammary tissue expresses CYP1A2 enzyme and that this enzyme was inducible by DMBA in hypophysectomized and intact rats.

3.2.7 *Effects of Hypophysectomy and DMBA Treatment on Mammary CYP1A1 Protein Expression*

On the immunoblot, CYP1A1 protein was undetectable in mammary microsomes from the corn oil-treated animals. Upon treatment with DMBA, mammary CYP1A1

protein level was induced in hypophysectomized and intact groups by visual examination (see Figure 3.5). However, mammary CYP1A1 and CYP1A2 protein contents were unable to be determined because the protein bands were not detected consistently in the mammary microsome samples.

3.2.8 Effect of Hypophysectomy and DMBA Treatment on Mammary CYP1B1 Protein Expression

CYP1B1 protein expression was investigated in the mammary microsome samples. Immunoblot analysis showed that CYP1B1 protein was present in the mammary tissue of DMBA-treated hypophysectomized and intact animals, but CYP1B1 protein was not detectable in the corn oil-treated animals. Mammary CYP1B1 protein levels of DMBA-treated intact animals were lower than DMBA-treated hypophysectomized animals according to our immunoblot analysis (see Figure 3.6). Referring to Figure 3.6, protein bands in the "Intact + DMBA" group were visible by eye on the actual blot. However, the band intensities were below the limit of quantitation of the scanner and the image did not appear on the scanned figure.

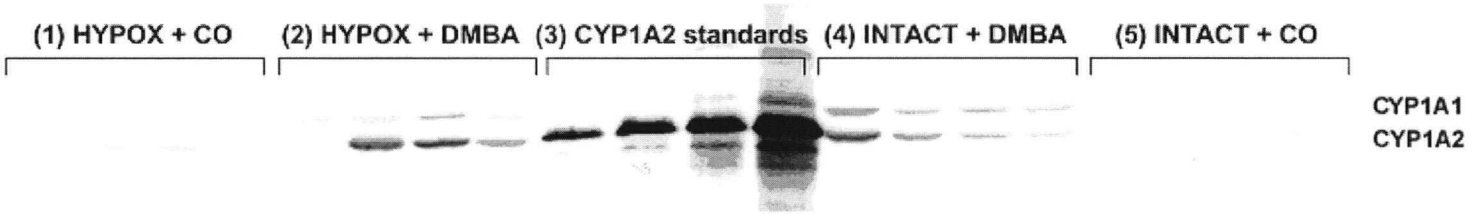


Figure 3.5 Immunoblot showing mammary samples probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution). CYP1A1 is represented as the upper band and CYP1A2 as the lower band. From left to right, bands showing samples of (1) Hypophysectomized rats treated with corn oil, (2) Hypophysectomized rats treated with DMBA, (3) CYP1A2 standards of 0.05, 0.1, 0.2, 0.5 pmol per lane, (4) Intact rats treated with DMBA, (5) Intact rats treated with corn oil. All microsomal samples were loaded at 40 μg of microsomal protein per lane. The membrane was developed with substrate solution for 3.5 minutes.

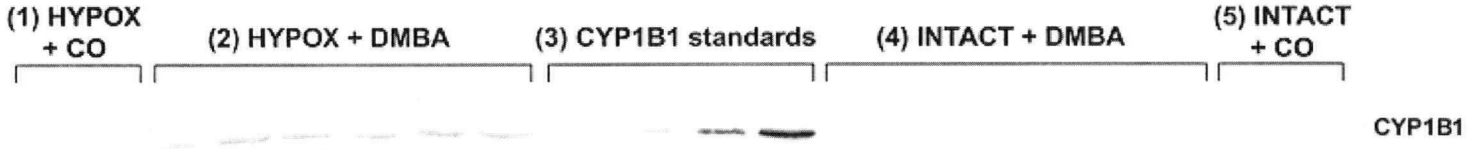


Figure 3.6 Immunoblot showing mammary samples probed with rabbit anti-rat CYP1B1 antibody (1:500 dilution). From left to right, bands showing sample of (1) Hypophysectomized rats treated with corn oil, (2) Hypophysectomized rats treated with DMBA, (3) CYP1B1 standards of 0.05, 0.1, 0.2, and 0.4 pmol per lane, (4) Intact rats treated with DMBA, (5) Intact rats treated with corn oil. All microsomal samples were loaded at 40 μg of microsomal protein per lane. The membrane was developed with substrate solution for 6 minutes.

3.2.9 *Effects of Hypophysectomy and DMBA Treatment on Hepatic mEH Protein Expression*

Microsomal epoxide hydrolase protein level was measured in rat hepatic microsome by probing the immunoblot with rabbit anti-rat EH IgG (Figure 3.7). The antibody detects mEH protein as a single band on the immunoblot. Immunoblot analysis showed that mEH protein was present in the hepatic microsomes prepared from intact and hypophysectomized animals. There was 15 ± 2 μg of hepatic mEH protein per mg of microsomal protein in control hypophysectomized rats versus 11 ± 3 μg of hepatic mEH protein per mg of microsomal protein in control intact rats (Figure 3.9A). Statistical analysis showed mEH protein in the livers of corn oil-treated hypophysectomized rats is significantly higher than that of in the livers of the corn oil-treated intact rats. Comparing hepatic mEH protein expression between corn oil- and DMBA-treated rats, DMBA treatment did not induce mEH in the livers of either intact or hypophysectomized animals. A bar graph in Figure 3.9A summarizes hepatic mEH protein expression in each treatment group.

3.2.10 Effects of Hypophysectomy and DMBA Treatment on Mammary mEH Protein Expression

Immunoblot analysis showed that mEH protein was present in the mammary tissue of both intact and hypophysectomized animals. Similar to the results of hepatic mEH, mammary mEH protein expression is significantly higher in control hypophysectomized rats than in intact rats. Futhermore, DMBA treatment did not induce mEH protein level in the mammary tissue of either intact or hypophysectomized animals (see Figure 3.8). There was approximately 0.5 ± 0.1 μg of mEH protein per mg of microsomal protein in control hypophysectomized rats versus 0.3 ± 0.1 μg of mEH protein per mg of microsomal protein in control intact rats. A bar graph in Figure 3.9B illustrates mammary mEH protein expression in each treatment group.

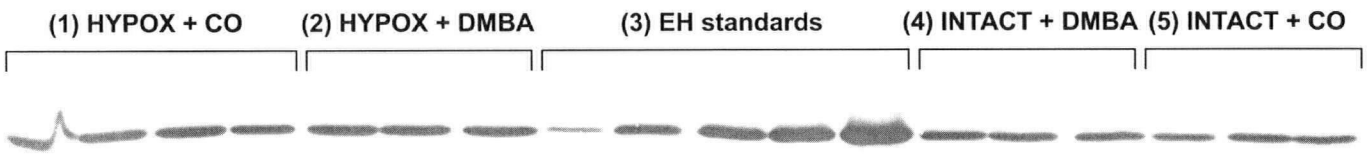


Figure 3.7 Immunoblot showing hepatic samples probed with rabbit anti-rat mEH IgG (20 μg/ml). From left to right, bands showing samples of (1) Hypophysectomized rats treated with corn oil, (2) Hypophysectomized rats treated with DMBA, (3) mEH standards of 0.01, 0.05, 0.1, 0.25, 0.5 μg per lane, (4) Intact rats treated with DMBA, (5) Intact rats treated with corn oil. All microsomal samples were loaded at 5 μg of microsomal protein per lane. The membrane was developed with substrate solution for 2 minutes.

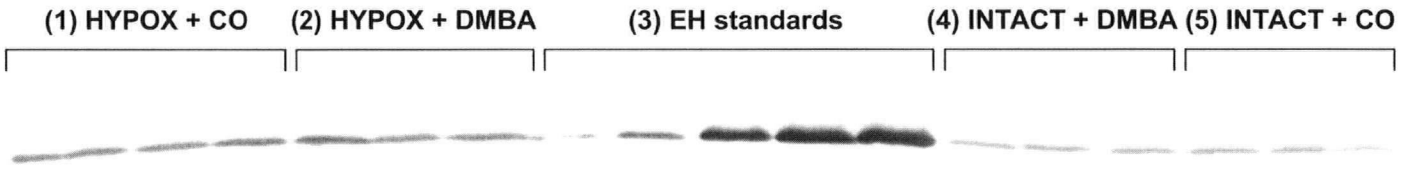
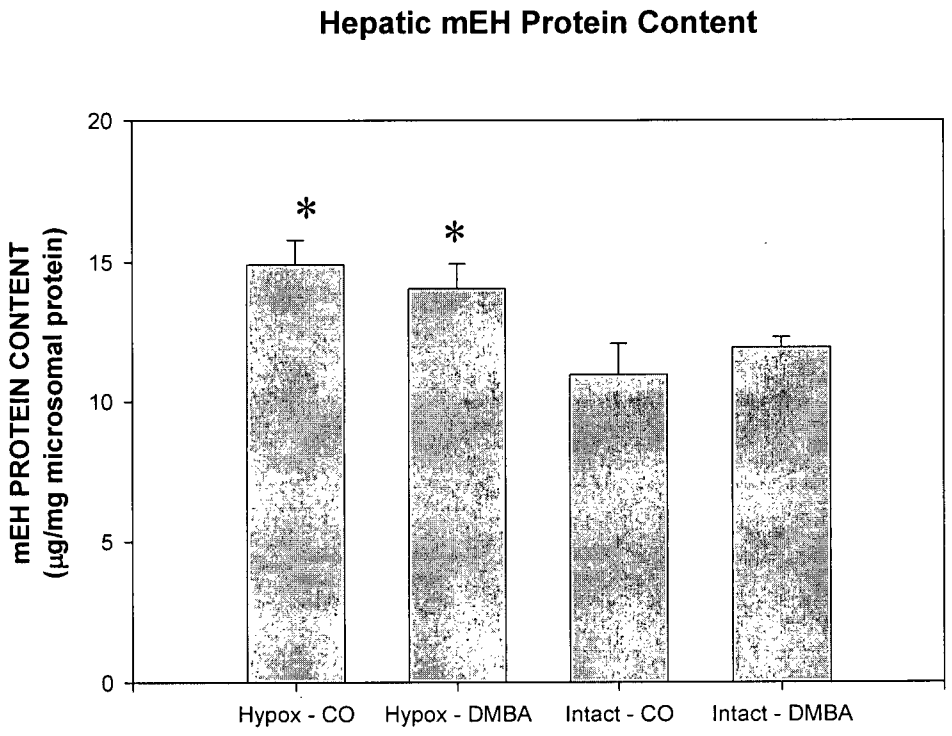


Figure 3.8 Immunoblot showing mammary samples probed with rabbit anti-rat mEH IgG (20 μg/ml). From left to right, bands showing samples of (1) Hypophysectomized rats treated with corn oil, (2) Hypophysectomized rats treated with DMBA, (3) mEH standards of 0.005, 0.01, 0.05, 0.1, 0.25 μg per lane, (4) Intact rats treated with DMBA, (5) Intact rats treated with corn oil. All microsomal samples were loaded at 40 μg of microsomal protein per lane. The membrane was developed with substrate solution for 2.5 minutes.

A.



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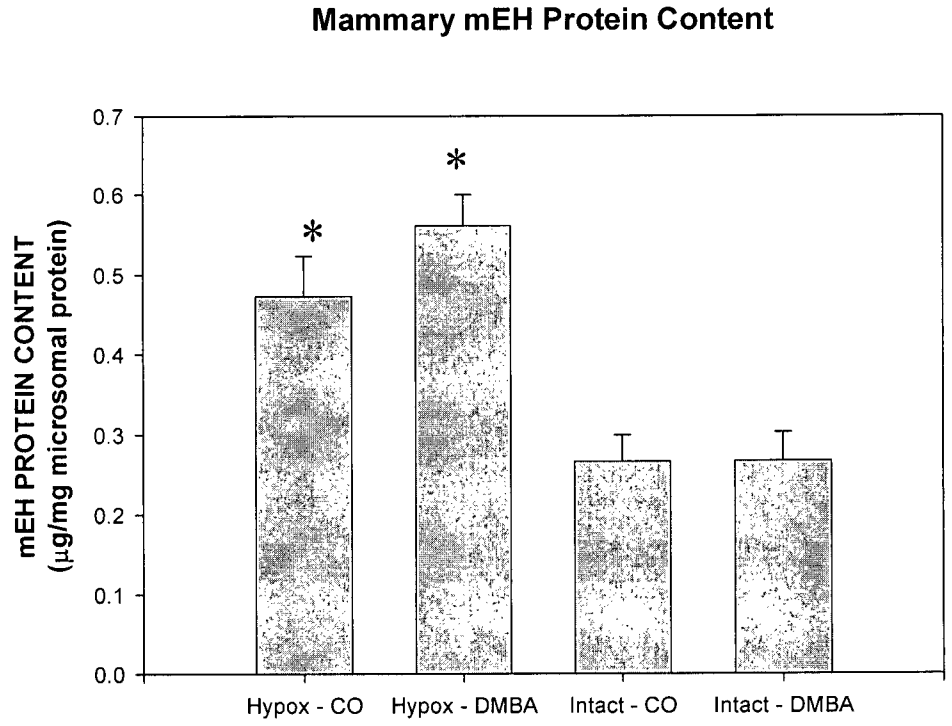

Figure 3.9 Summary of hepatic mEH (A) and mammary mEH (B) protein content in corn oil- or DMBA-treated hypophysectomized and intact rats. Bars are shown as mean ± SEM ($n = 6$). * Significantly different from other treatment groups with a p-value <0.05.

3.2.11 Effects of Hypophysectomy and DMBA Treatment on Hepatic Enzyme Activity

Both methoxyresorufin *O*-demethylase (MROD) and benzo[*a*]pyrene (BaP) hydroxylase assays were well established in the laboratory. Assay conditions and equipments had already been optimized and validated in the past by other students and lab members (Ngui, 1997). Additional optimization experiments were performed to substantiate assay conditions for the samples used in the present study.

3.2.11.1 Validation of MROD assay conditions: Calibration Curve

A range of resorufin concentrations, 0, 10, 25, 75, 100, 500, and 1000 pmol per 10 µl, was used to generate the standard curve of fluorescence intensity versus resorufin concentration (see Figure 3.10). All fluorescence readings of the sample were within the reading of the lowest and highest resorufin standard concentration. A standard curve was generated each time the assay was performed.

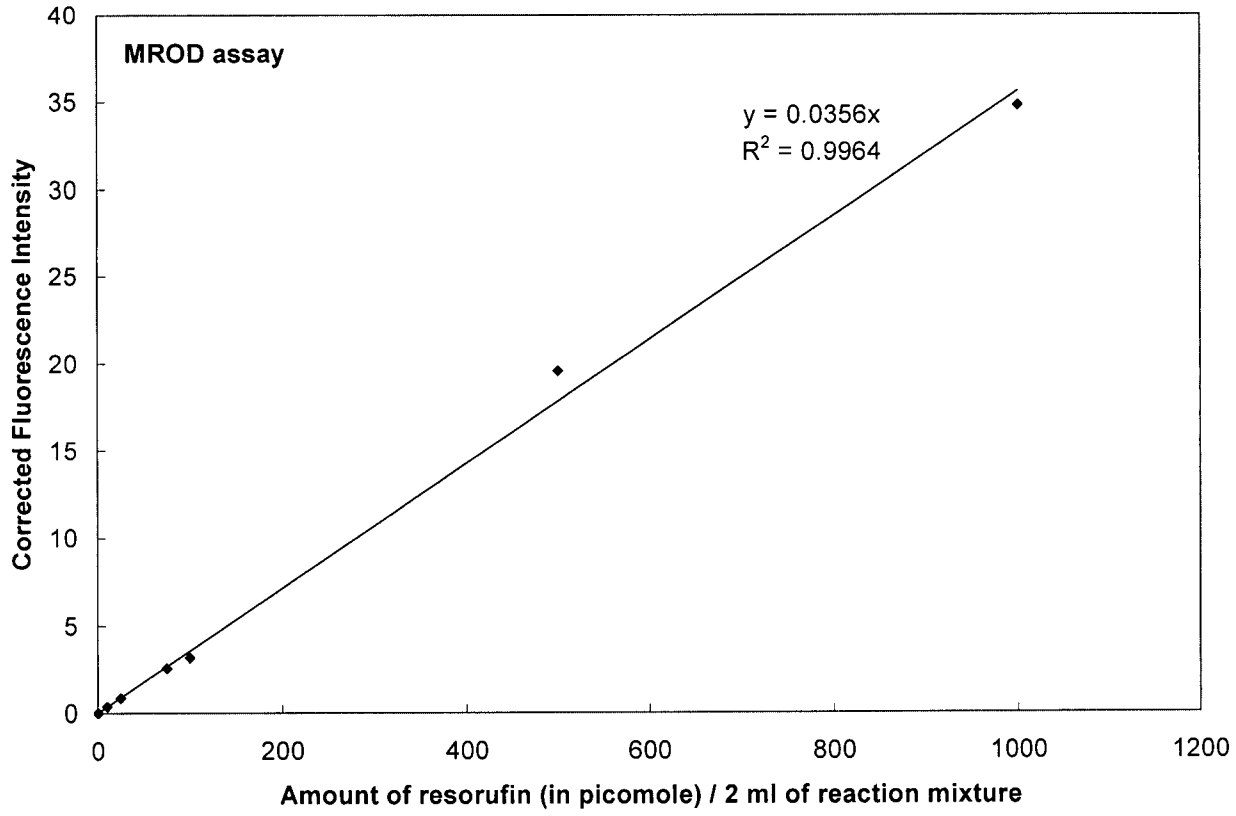

Figure 3.10 Calibration curve for the MROD assay. Varying resorufin concentrations of 0, 10, 25, 75, 100, 500, and 1000 pmol per 2 ml of reaction mixture. Assay performed as described in section 2.9.1. All measurements were performed in duplicate.

3.2.11.2 Validation of MROD assay conditions: Saturating Substrate Concentration

MROD activity was measured in hepatic microsomes of one sample from every treatment group using a range of substrate concentrations, 0, 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, 2 mM. The treatment groups were 1) Intact + CO, 2) Intact + DMBA, 3) Hypox + CO, and 4) Hypox + DMBA. The effect of substrate concentration on resorufin formation at 5 minutes is shown in Figure 3.11. The results suggested that a substrate concentration of 2.5 μM methoxyresorufin (final concentration) was the optimal substrate concentration for all treatment groups. At substrate concentrations above 0.5 μM, resorufin formation decreased.

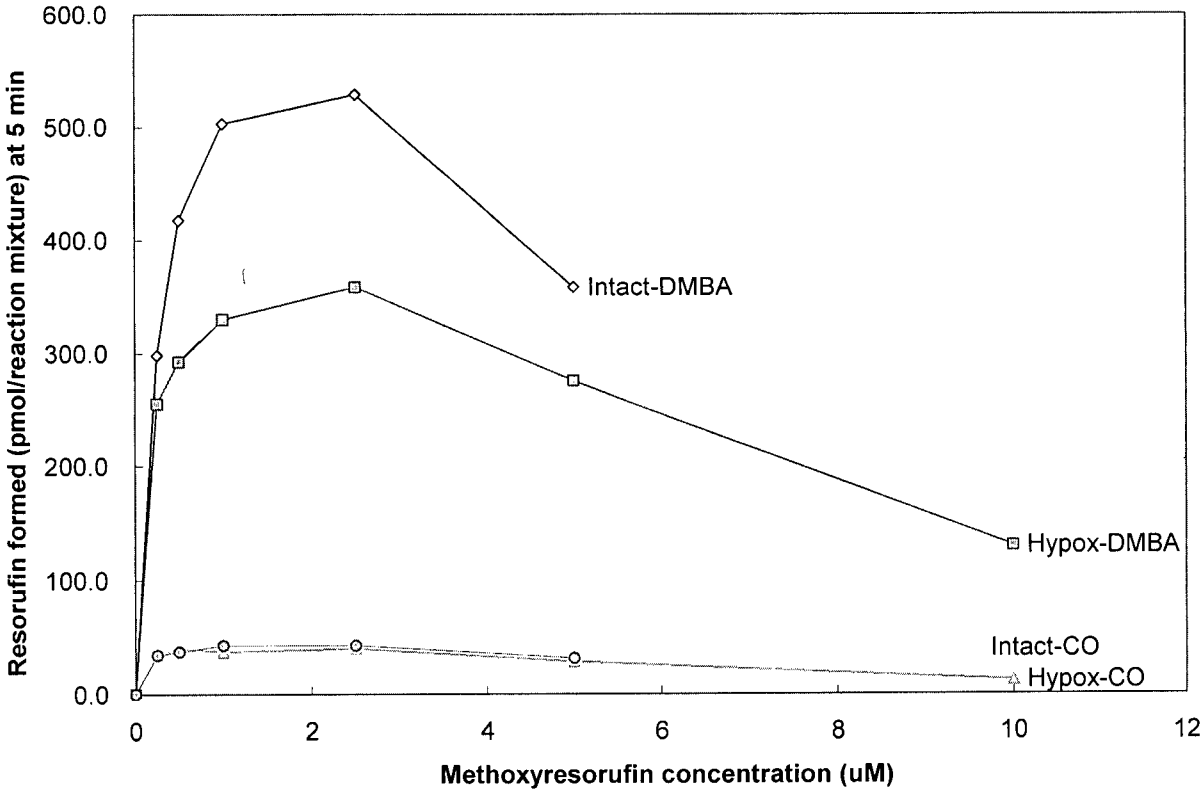

Figure 3.11 Effect of varying substrate concentrations on resorufin formation in hepatic microsomes (2 mg protein per ml) in reaction mixture containing 1.93 ml of 0.1 M HEPES buffer and varying final methoxyresorufin concentrations 0, 0.25, 0.5, 1, 2.5, 5, and 10 μM. Assay performed as described in section 2.9.1. All measurements were performed in duplicate.

3.2.11.3 Validation of MROD assay conditions: Reaction Time

Varying reaction time for resorufin formation was investigated in rat hepatic microsomes. The MROD activity was measured in a sample prepared from DMBA-treated hypophysectomized rat. Reaction time was investigated under two final substrate concentrations of 1 and 2.5 μM. The results shown in Figure 3.12 suggested that product formation is linear between 0 to 16 minutes. A reaction time of 5 minutes was selected for measuring MROD activities in the samples.

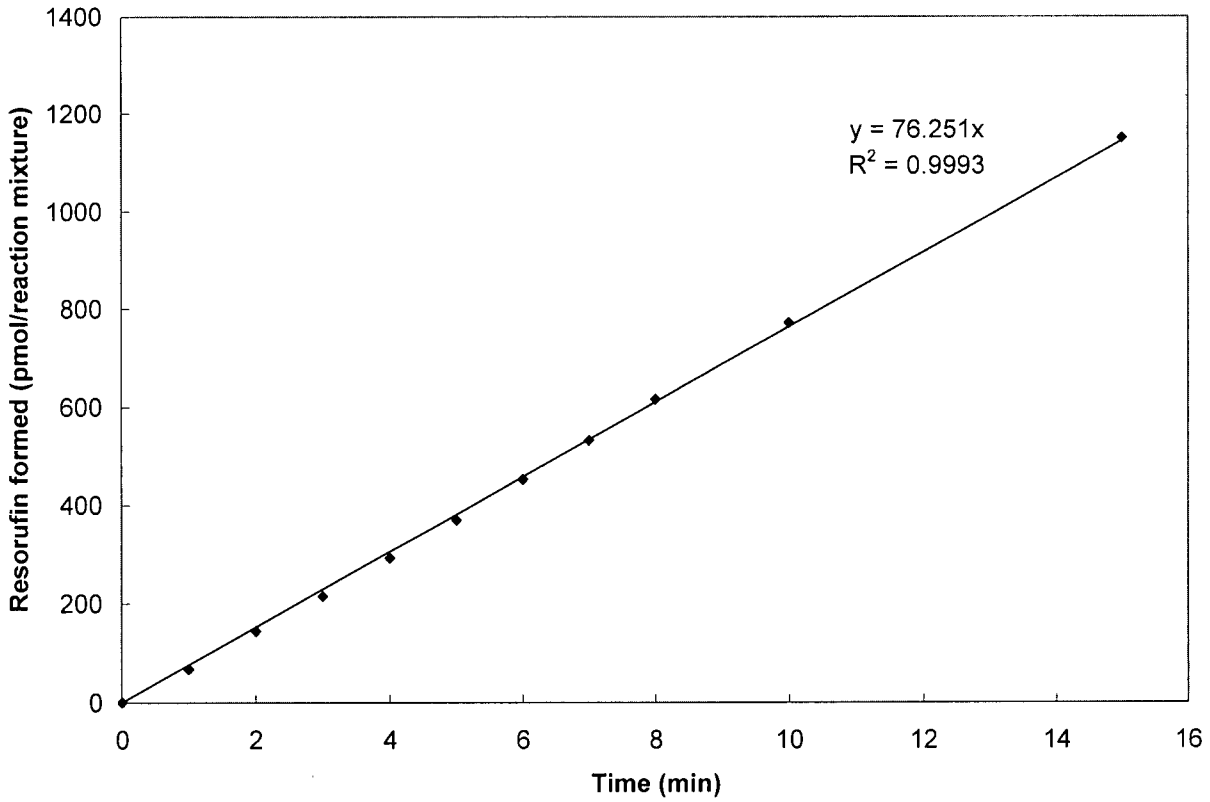

Figure 3.12 The formation of resorufin over time from 0 to 16 minutes in hepatic microsomes (2 mg protein per ml) in reaction mixture containing 1.93 ml of 0.1 M HEPES buffer and 2.5 μM methoxyresorufin (final concentration). Assay performed as described in section 2.9.1. All measurements were performed in duplicate.

3.2.11.4 Effects of hypophysectomy and DMBA treatment on MROD activity

In the assay, rat hepatic microsomal samples were incubated with methoxyresorufin under optimal assay conditions and formation of resorufin was measured as described in section 2.9.1. Mean MROD activities of each treatment group are summarized in Table 3.7. According to the results, hypophysectomy did not affect MROD activity as there was no difference in MROD activity in hepatic microsomes prepared from corn oil-treated intact and hypophysectomized rats. On other hand, DMBA induced MROD activity in hepatic microsomes prepared from both intact and hypophysectomized animals. MROD activity had a 17-fold induction in DMBA-treated hypophysectomized rats compared against uninduced rats and a 14-fold induction in intact rats. The increase in MROD activity agrees with the induction of hepatic CYP1A2 enzymes by DMBA. A similar result was observed when activities were expressed per nmol of total CYP. The results demonstrated that hypophysectomized rats contain the necessary CYP enzymes for MROD activity and the enzymes are responsive to DMBA induction.

Table 3.7 Effects of hypophysectomy and DMBA treatment on hepatic microsomal MROD activities

Treatment	Resorufin formed (pmol/min/mg protein)	Resorufin formed (pmol/min/nmol CYP)
Hypox + CO	46 ± 6	51 ± 11
Hypox + DMBA	790 ± 58 †	748 ± 62 ∫
Intact + CO	64 ± 1	97 ± 4
Intact + DMBA	896 ± 51 †	920 ± 50 §

Values are shown in mean ± SEM (n = 6).

† Significantly different from Hypox, Intact + CO with a p-value <0.05.

∫ Significantly different from Hypox, Intact + CO, and Intact + DMBA with a p-value <0.05.

§ Significantly different from Hypox, Intact + CO, and Hypox + DMBA with a p-value <0.05.

3.2.11.5 Validation of BaP Hydroxylase assay condition: Inter-assay variation

To eliminate day-to-day variations, a standard curve was generated each time the assay was performed. The product standards underwent the same extraction procedure as the samples to eliminate any discrepancies caused by the extraction methods.

3.2.11.6 Validation of BaP Hydroxylase Assay: Reaction Time

Varying reaction time for BaP hydroxylase assay was investigated in rat hepatic microsomes. BaP hydroxylation activity was measured in a sample prepared from corn oil-treated intact rat. Reaction time was investigated from 0 to 10 minutes. The results shown in Figure 3.13 suggested that fluorescence intensity which is a direct measurement of product formation is linear between 0 to 10 minutes. A reaction time of 4 minutes was selected for measuring BaP hydroxylation activities in the samples.

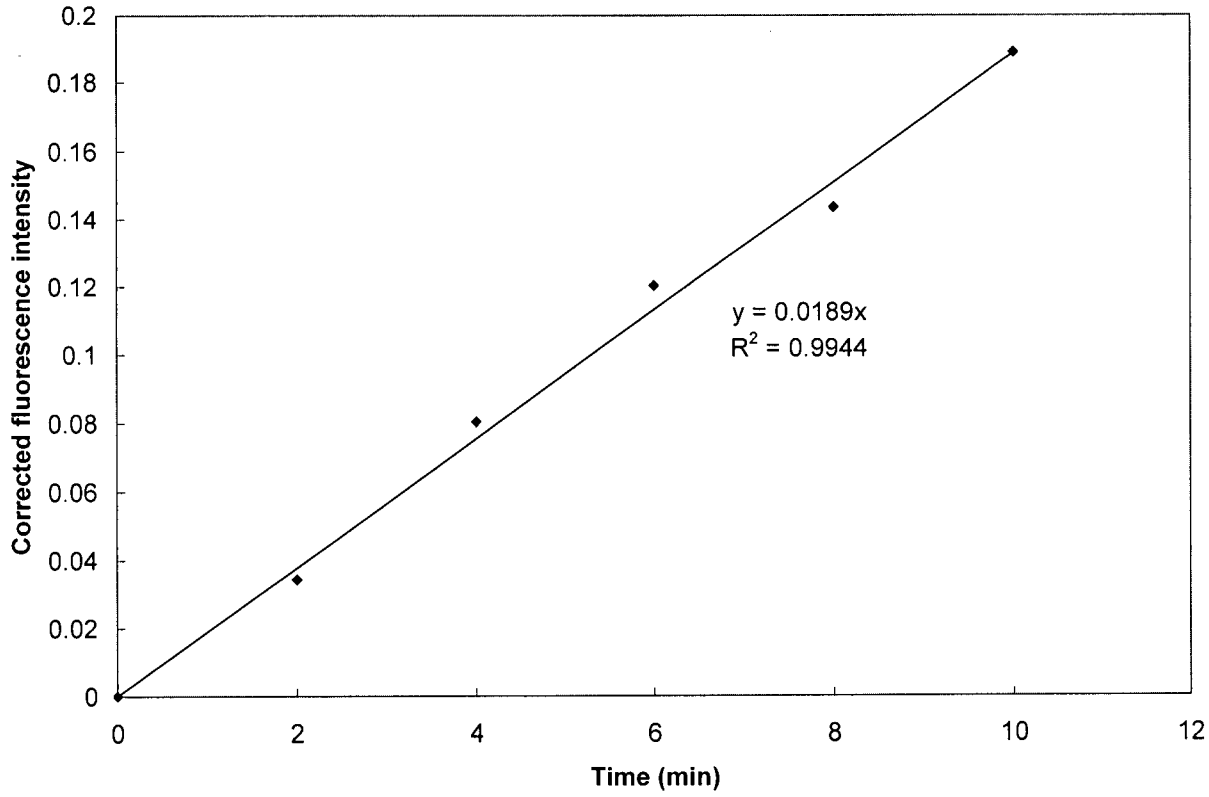

Figure 3.13 The formation of OH-BaP over time from 0 to 12 mintues in hepatic microsomes (2 mg protein per ml in reaction mixture containing 500 µl of 100 mM KPO_4 buffer, 430 µl distilled water and 80 µM benzo[*a*]pyrene (final concentration). Assay performed as described in section 2.9.2. All measurements were performed in duplicate.

3.2.11.7 Effects of hypophysectomy and DMBA treatment on BaP Hydroxylation activity

Rat hepatic microsomal samples were incubated with BaP under optimal assay conditions and formation of BaP metabolites was measured using Shimadzu spectrofluorophotometer as described in section 2.9.2. BaP is metabolized into many hydroxyl derivatives by CYP enzymes with the major product as 3-OH BaP. Other minor metabolites are formed at the following relative percentages: 9-OH-BaP, 3-13%, BaP-9,10-diol, 15-25%, BaP-7,8-diol, 12-14%, BaP-4,5-oxide, 8%, and BaP quinones, 14-17% (Yang 1978). Hepatic BaP hydroxylation activity in rats is mediated by several CYP enzymes. Purified recombinant human CYP1A1 and CYP1B1 exhibit the highest BaP hydroxylation activities versus recombinant CYP2A6, CYP2C9, CYP2C19, and CYP3A4 (Shimada 2004). Mean BaP hydroxylation activities of hepatic microsomal samples in each treatment group are summarized in Table 3.8. BaP hydroxylation activity is expressed as both nmol of OH-BaP formed per min per mg of protein and also per nmol of total CYP. Between the BaP hydroxylation activity of the control animals, no difference is observed in either intact or hypophysectomized rats. DMBA treatment induced BaP hydroxylation activity in intact and hypophysectomized rats. BaP hydroxylase activity (expressed as per mg of protein) had a 3-fold induction in the DMBA-treated hypophysectomized rats, whereas BaP hydroxylase activity had a 5-fold induction in the DMBA-treated intact rats. The magnitude of induction in BaP hydroxylase activity is significantly different in the liver microsomes prepared from DMBA-treated intact and hypophysectomized animals. Although the activities were different between DMBA-treated hypophysectomized and intact rats, the results suggested that hypophysectomized rats contain the CYP enzymes needed for the hydroxylation of BaP and DMBA was able to induce these CYP enzymes in the livers of

intact and hypophysectomized rats.

Table 3.8 Effects of hypophysectomy and DMBA treatment on hepatic microsomal BaP hydroxylase activities.

Treatment	OH-BaP formed (pmol/min/mg protein)	OH BaP formed (pmol/min/nmol CYP)
Hypox + CO	65 ± 10	190 ± 20
Hypox + DMBA	180 ± 20 †	430 ± 50 †
Intact + CO	62 ± 10	230 ± 20
Intact + DMBA	330 ± 30 ‡	850 ± 80 ‡

Values are shown in mean ± SEM (n = 6).

† Significantly different from Hypox, Intact + CO, Intact + DMBA with a p-value <0.05.

‡ Significantly different from Hypox, Intact + CO, Hypox + DMBA with a p-value <0.05.

4. DISCUSSION

Rats are a widely used animal model for studying human breast cancer as the histology of mammary tumors in rats closely resembles human breast cancer (Russo *et al.*, 1990). A single treatment with DMBA has been reported to be highly effective at inducing mammary cancer in intact virgin rats. However, DMBA is not as effective in inducing cancer in hormonally-depleted (i.e. ovariectomized or hypophysectomized) rats (Huggins *et al.*, 1958). The present study investigated the effect of hypophysectomy and DMBA treatment on mammary tumorigenesis and on the expression of hepatic and mammary CYP1 and mEH enzymes in female Sprague-Dawley rats.

Previous studies reported that hypophysectomized rats failed to develop mammary cancer following treatment with 3-MC. Both 3-MC and DMBA require metabolic activation to become carcinogenic. Specifically, DMBA requires CYP1 and mEH enzymes to form the carcinogenic metabolite, DMBA-3,4-diol epoxide. Our hypothesis was that hypophysectomy would reduce mammary tumor incidence in DMBA-induced rats. Hypophysectomized rats may lack specific CYP enzymes required for DMBA bioactivation. For the second hypothesis, our study aimed to test CYP enzyme expression and activity for the bioactivation pathway of DMBA between hypophysectomized and intact rats. Two experiments were performed. Study 1 investigated the effect of a single DMBA treatment on mammary tumorigenesis in intact and hypophysectomized rats. Study 2 analyzed the expression of hepatic and mammary CYP and mEH enzymes in hypophysectomized rats. In addition, a separate study examined body weight and mortality in hypophysectomized rats given intermittent DMBA treatment (see Appendix I).

4.1 Effect of Hypophysectomy on Growth and Organ Development

Body weight of the animals was measured throughout the study as an indicator of the animals' health. Hypophysectomized animals exhibited little or no weight gain when compared to intact animals. Hypophysectomized rats exhibited a mean weight gain of 33 ± 14 g over 120 days, whereas intact rats had an average weight gain of 163 ± 29 g. Growth hormone is one of the pituitary hormones that stimulates endogenous production of IGF-1 (insulin-like growth factor-1) leading to somatic growth stimulation. Body and some organ weights are under the influence of growth hormone and IGF-1 (Guler *et al.*, 1988). Monitoring the body weights of intact and hypophysectomized animals also provides an indication of the success of the surgical procedure of hypophysectomy.

Hypophysectomy affects organ development differentially. Guler *et al.* (1988) reported that kidney and spleen weights were reduced in hypophysectomized rats and that administration of IGF-1 to these rats restored organ weights to their normal range. In our study, liver, ovary and uterus were excised and weighed at the time the rats were terminated. There was no difference in relative liver weight but relative ovarian and uterine weights were significantly lower in hypophysectomized rats when compared to intact rats. Development of the ovaries and uterus is under the influence of LH and FSH, which are secreted by the pituitary gland. The administration of recombinant human FSH to hypophysectomized animals was shown to restore ovarian and uterine weights to values that are similar to organ weights in intact animals (Mannaerts *et al.*, 1994). Furthermore, FSH also induced follicular growth to preovulatory stages, induced ovarian estradiol production, and induced endometrial proliferation in hypophysectomized rats.

Mammary gland development is also under the influence of pituitary hormones. In rats,

the mammary glands are distributed in pairs along the milk line, with one pair located in the cervical, two pairs in the thoracic, one pair in the abdominal and two pairs in the inguinal regions (Russo *et al.*, 1990). Mammary gland development is a progressive process which begins during the neonatal period. The mammary gland, before puberty, is characterized by branches of ducts with terminal end buds (TEBs). At puberty (between days 35 and 42 of age), which is defined by the onset of the estrous cycle, TEBs in the mammary gland begin to differentiate to alveolar buds and lobules and lobule formation accumulates over multiple estrous cycles (Masso-Welch *et al.*, 2000). Similar to the development of the human breast, the lobules in virgin female rats are predominantly type 1 lobules with little branching and differentiation. During pregnancy and lactation, the lobules increase significantly in size and in the number of alveoli to a stage called type 3 and type 4 lobules (Russo *et al.*, 1990). Experimental evidence shows the importance of ovarian and pituitary hormones in mammary gland development (Reece *et al.*, 1936, Nathanson *et al.*, 1939). The proliferation of mammary epithelium is induced by ovarian hormones, estradiol and progesterone. However, mammary gland proliferation can only respond to ovarian hormones if the pituitary gland is intact. The mammary gland shows almost complete atrophy in rats following hypophysectomy (Nathanson *et al.*, 1939). Ovarian hormones alone have little or no mammogenic activity (Lamote *et al.*, 2004). Pituitary hormones that have been shown to be involved in the stimulation of mammary duct growth include growth hormone, prolactin, and adrenal corticoids (Nandi *et al.*, 1995, Lamote *et al.*, 2004), but the underlying mechanism for each hormone remains unsolved. A study using hypophysectomized monkeys further demonstrated the complex role of the pituitary gland in mammary mitogenesis (Kleinberg *et al.*, 1985). In that study, it was demonstrated that hypophysectomy prevented full estradiol-induced mammary development. Mammary gland development was significantly

decreased in estradiol-treated hypophysectomized monkeys compared to estradiol-treated intact monkeys. Thus, it is evident that the pituitary plays an important role in mammary gland development in rodents and primates.

4.2 Effect of DMBA Treatment on Mammary Tumorigenesis

A single intragastric dose of 20 mg of DMBA is conventionally used to induce mammary cancer in Sprague-Dawley rats. The type of mammary tumor induced by DMBA in rats is primarily papillary carcinoma, a type of malignant lesion that originates in the mammary ducts (Russo and Russo, 2000). DMBA induces tumors with latencies that range between 8 and 21 weeks. Final tumor incidence is close to 100% if the animals were monitored for 180 days post-treatment (Russo and Russo, 1996). The susceptibility of the mammary gland to DMBA-induced carcinogenesis is strongly age-dependent. Susceptibility is maximal when the carcinogen is administered to animals between the ages of 45 to 55 days, the age of sexual maturity. A decline in tumor incidence and number of tumors per animal is observed when the animal is treated after this period (Sinha *et al.*, 1983). In our study, rats were treated with DMBA at approximately 60 days of age. The animals were monitored for mammary tumors for 120 to 127 days following DMBA treatment before the termination of the animals. The study period was not extended beyond 127 days because mammary tumors in some of the rats grew to a size that affected the animal's mobility. In addition, a few tumors were starting to ulcerate through the adjacent skin at 127 days after treatment. Thus, in compliance with guidelines of the university animal care committee, we decided to terminate all rats at 120 to 127 days after DMBA treatment. At 120 to 127 days post DMBA treatment, intact rats had a 55% tumor incidence with the first palpable tumor at 8 weeks post DMBA treatment. Tumor incidence (55%) was less than the 80 to 100% value reported in the literature. This could be

as a result of the following reasons: 1) the study period for my study was only 120 to 127 days, whereas previous studies monitored the animals for 180 days or longer and 2) the rats in my study were dosed at approximately 60 days of age; however, rats are the most susceptible to carcinogenic chemicals between 45 to 55 days of age. In terms of tumor multiplicity, there was an average of 2.5 tumors per tumor-bearing rat. DMBA-induced mammary incidence, latency, and multiplicity were consistent with previous data obtained in our laboratory (Tai *et al.*, 2006).

4.3 Effects of Hypophysectomy and DMBA Treatment on Mammary Tumorigenesis

We initially hypothesized that hypophysectomized rats treated with DMBA would develop fewer mammary tumors than intact rats. A single intragastric dose of 20 mg of DMBA was given to both intact and hypophysectomized adult female virgin Sprague-Dawley rats. None of the DMBA-treated hypophysectomized rats developed mammary tumors during the monitoring period, whereas DMBA-treated intact animals had a 55% mammary tumor incidence. Our results highlight the importance of the pituitary in the initiation and development of mammary tumors in DMBA-treated rats. A previous study demonstrated that treatment with 3-MC induced mammary cancer in 67% of ovariectomized rats versus 100% in intact rats, whereas none of the 3-MC-treated hypophysectomized rats developed mammary tumors (Huggins *et al.*, 1959). Furthermore, Sterental *et al.* (1962) determined that both ovariectomy and hypophysectomy caused mammary cancer regression; however, estrogen administration failed to reactivate tumor growth in hypophysectomized rats whereas estrogen administration reactivated tumor growth in ovariectomized rats. Our study together with previous studies demonstrates that both ovarian and pituitary hormones are involved in

the regulation of breast cancer, but an intact pituitary appears to be an essential component in mammary tumor growth and development. Further experiments are needed to identify the individual or a combination of pituitary factors that are involved in mammary tumorigenesis.

4.4 Adverse Effects Associated with DMBA Treatment

In the present study, we assessed the effect of DMBA treatment on body weight and mortality. DMBA treatment had a short-term effect on body weight. Both intact and hypophysectomized animals experienced a decline in body weight for 4 days after the administration of a single intragastric dose of 20 mg of DMBA. In addition, this DMBA dose caused the deaths of 18 DMBA-treated hypophysectomized animals within the first week of treatment. Acute toxic effects of DMBA include lethargy, diarrhea, decreased food consumption, and adrenal necrosis that develop within a day after DMBA treatment (Carter *et al.*, 1988). Previous studies reported that administration of DMBA at a dosage of 27 mg per 100 g of body weight caused death in one-half of the treated rats (Huggins *et al.*, 1961). At this dose, adrenal necrosis was found 3 days after intragastric administration of DMBA and adrenal regeneration began 6 or 7 days post-DMBA treatment (Huggins *et al.*, 1961). The researchers involved in that study concluded both intravenous and intragastric administrations of DMBA cause adrenal necrosis, diarrhea, as well as weight loss in rats. Although weight loss was seen in both intact and hypophysectomized rats treated with 20 mg of DMBA in our study, the same DMBA treatment only caused deaths in hypophysectomized rats. We conclude that hypophysectomized animals are more susceptible to the toxic effects of 20 mg of DMBA because DMBA treatment produced adrenal necrosis, diarrhea, and reduced food consumption (i.e. physiological stresses) that these rats could not overcome because they

lacked the pituitary hormones needed to restore homeostasis.

A different DMBA dosing approach that would be tolerable while preserving mammary carcinogenicity was investigated in the hypophysectomized rats (see Appendix I). The 20 mg dosage of DMBA was divided into four 5 mg doses given intermittently to hypophysectomized rats over a 2-week period. Previous studies demonstrated that divided DMBA dosing was as effective as a single 20 mg dose of DMBA for inducing mammary cancer in rats (Hollingsworth *et al.*, 1998). Hollingsworth *et al.* (1998) administered 5 mg of DMBA (dissolved in 1 ml of corn oil) weekly for a total dosage of 20 mg DMBA to intact Sprague-Dawley female rats (n = 9). In that study, 7 of 9 rats (77.8%) developed malignant tumors, with an average of 2.3 tumors per affected rat. Using the intermittent dosing approach, no significant change in body weight was observed between each DMBA dose and no death was reported in the treated hypophysectomized rats in our study. The acute toxicity of DMBA appears to be dose dependent. Our results demonstrated that hypophysectomized rats can tolerate DMBA administered as four doses of 5 mg each. Although hypophysectomized rats tolerated the divided DMBA dosing regimen, the animals were more difficult to handle on the days they were to receive the third and fourth intragastric doses. The animals had experienced unpleasant adverse effects after the first and second doses of DMBA and they began to reject upcoming doses. For future experiments, the total 20 mg dosage of DMBA could be divided into two doses of 10 mg each given once a week to hypophysectomized rats to induce mammary tumors.

4.5 Bioactivation of DMBA in Hypophysectomized Rats

Both DMBA and 3-MC require metabolic activation by CYP and mEH enzymes to form carcinogenic metabolites (Christou *et al.*, 1989, Shou and Yang, 1990, Shimada and Fujii-Kuriyama, 2004). Failure of the hypophysectomized rats to develop mammary tumors could be as a result of down-regulation of specific CYP and mEH enzymes, which are needed to metabolically activate DMBA. Hepatic metabolism in rats is known to be influenced by gonadal and pituitary hormones. Specifically, expression of hepatic CYP2A, CYP2C, CYP2B, CYP3A, and CYP2E1 enzymes is altered in hypophysectomized rats (Oinonen *et al.*, 1995, Agrawal and Shapiro, 1997, Chen *et al.*, 1999). Expression of extrahepatic CYP enzymes is also regulated by gonadal and pituitary hormones. In our laboratory, we demonstrated that LH and FSH play a role in the regulation of testicular CYP1B1 expression (Leung *et al.*, 2005). Hence, the loss of pituitary hormones in the hypophysectomized animals could lead to an alteration of hepatic and mammary expression of CYP and mEH enzymes required for DMBA activation and ultimately to an inability to develop mammary tumors.

To the best of my knowledge, there are no previous studies that examined the biotransformation of DMBA in hypophysectomized female rats. A previous report compared hepatic metabolism of DMBA between male, female, and ovariectomized Sprague-Dawley rats (Vater *et al.*, 1991). DMBA was infused into the liver and the production of the carcinogenic metabolite, DMBA-3,4-dihydrodiol, was measured in the perfusate. DMBA-3,4-dihydrodiol appeared in the perfusate at higher rates in intact and ovariectomized female rats than in the male rats. The results suggested that the hepatic enzyme composition was different between male and female rats and a possible explanation for a lower incidence

of DMBA-induced mammary tumors observed in male as opposed to female rats (Dao, 1962). In the present study, we measured the expression and activity of CYP and mEH enzymes, which have been shown to be involved in the bioactivation of DMBA (Christou *et al.*, 1995, Shimada and Fujii-Kuriyama, 2004), in intact and hypophysectomized female rats.

4.6 Effect of Hypophysectomy on CYP1 and mEH Enzyme Expression

CYP1 and mEH enzyme expression was measured in hepatic and mammary microsomes prepared from Sprague-Dawley female rats treated with a single intragastric dose of 20 mg of DMBA or an equivalent volume of corn oil. Rats were terminated 24 hours post treatment. Enzyme induction by PAHs such as 3-MC was demonstrated to be maximal at 24 hours post treatment (Conney, 1967). Liver and mammary gland were excised for microsome preparation.

The CYP1A family enzymes are well documented to be involved in the bioactivation of DMBA (Guengerich, 1990). Moreover, formation of the potent carcinogenic DMBA diol-epoxide metabolite requires the presence of mEH. The main site for DMBA biotransformation is the liver, which is involved when the compound goes through first pass metabolism. Liver contains the majority of enzymes required for the biotransformation of xenobiotics and endogenous compounds. There is evidence that hepatic metabolic activation of nitrosamine or PAHs produce short-lived electrophiles that could damage DNA in extrahepatic tissue (Williams and Phillips, 2000). Therefore, the activities and level of CYP1A and other enzymes in the liver were examined. In rats, DMBA induces tumor formation primarily in the mammary gland. Recent research suggested that bioactivation of mammary carcinogens proceeds through a primary metabolic step carried out by hepatic metabolism, followed by complete metabolic activation to the ultimate DNA-reactive

metabolite in the breast (Williams and Phillips, 2000). Although the relative contribution of hepatic and mammary carcinogen activation have not been determined, levels of enzymes in the mammary glands are also important in influencing the carcinogenicity of DMBA.

Hepatic CYP1A1 and CYP1A2 protein expression was measured in liver microsomes prepared from intact and hypophysectomized rats. The CYP1A1 protein band was undetectable in liver microsomes from corn oil-treated intact and hypophysectomized rats, whereas the CYP1A2 protein band was easily detected. The results demonstrated that basal expression of hepatic CYP1A1 was lower than basal expression of hepatic CYP1A2. Our study results agree with previous data where the expression of CYP1A2 and CYP1A1 protein in control Sprague-Dawley female rat liver is 31 ± 9 and 9 ± 4 pmol per mg microsomal protein, respectively (Walker *et al.*, 1999). No difference in hepatic CYP1A2 expression was found between hypophysectomized and intact rats.

Expression of hepatic CYP1B1 protein was not examined in this study because hepatic CYP1B1 protein levels in uninduced rats have been shown to be below the limit of detection (Walker *et al.*, 1999). CYP1B1 protein has only been detected in liver of rats given chronic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) treatment at doses more than 35.7 ng/kg/day for 30 weeks (Walker *et al.*, 1999).

Basal mammary CYP1A1, CYP1A2, and CYP1B1 protein expression was also examined in corn oil-treated rats using the immunoblot technique. However, mammary CYP1A1, CYP1A2, and CYP1B1 protein was detected inconsistently on the immunoblots. By approximation of the amount of microsomal protein sample loaded in the immunoblot, mammary levels of CYP1A1 and CYP1A2 enzymes were more than 2 to 5 times lower than in the liver. CYP1A, 2A, 2B, 2E1, 3A, 4A proteins or mRNA were also found in the breast of human and rat, but the level of expression of these enzymes was lower than in the liver

(Hellmold *et al.*, 1995). As demonstrated by our results and other studies, mammary CYP 1A1, 1A2, and CYP1B1 protein expression was often too low to be detected using immunoblot analysis (Christou *et al.*, 1995). Other studies examined the expression of mammary CYP1A and CYP1B1 enzymes in rat mammary tissue and human breast epithelial and tumor cells by measuring mRNA levels through reverse transcription-PCR (RT-PCR) (Spink *et al.*, 1998, Badawi *et al.*, 2000). In terms of mRNA expression levels, mammary CYP1A2 had the highest expression followed by CYP1A1 and CYP1B1 in the control rat. The level of CYP1A2 mRNA in the mammary gland of the control rat is 43-fold higher than CYP1A1 and 13000-fold higher than CYP1B1. Measuring CYP enzymes by mRNA level could provide information on the relative expression of individual CYP enzyme in tissue. However, the level of mRNA expression does not always reflect the level of protein expression (Iba *et al.*, 1999). Our results indicate that hypophysectomy does not affect CYP1 enzyme expression in hepatic and mammary tissues.

Basal mEH protein levels were measured in liver and mammary microsomes of hypophysectomized and intact female rats. Hepatic mEH protein expression was approximately 1.3-fold higher in corn oil-treated hypophysectomized rats than control intact rats. Mammary mEH protein levels were approximately1.7-fold higher in the corn oil-treated hypophysectomized rats than corn oil-treated intact rats. Comparing mEH expression level in liver and mammary tissue, mEH protein expression is more than 30 times higher in liver than in the mammary gland. Our study was the first to measure the expression of mEH protein in mammary tissue of hypophysectomized female rats. The results demonstrated an up-regulation of mEH enzymes in liver and mammary tissue by hypophysectomy suggesting that the regulation of mEH is influenced by pituitary hormones as proposed by other researchers (Delinger and Vesell, 1989, Inoue *et al.*, 1995).

4.7 Effect of DMBA Treatment on CYP1 and mEH Enzyme Expression

Both CYP1A1 and CYP1A2 protein levels were induced in the livers of hypophysectomized and intact rats after a single intragastric dose of 20 mg of DMBA. Our results were consistent with other studies, which demonstrated that PAHs and TCDD are capable of inducing CYP1 enzymes through the Ah receptor (Walker *et al.*, 1999, Badawi *et al.*, 2000). CYP1A2 and CYP1A1 enzymes expressed in hepatic tissue were induced by DMBA treatment as shown by immunoblot analysis. The immunoblot data also showed that basal CYP1A2 and CYP1A1 protein levels were similar in the livers of intact and hypophysectomized rats. DMBA appears to induce CYP1A1 to a greater extent than CYP1A2 in the liver. Badawi *et al.* (2000) measured CYP1 enzyme mRNA levels in untreated and chlorinated hydrocarbon-treated rat livers. In that study, mRNA levels of CYP1A1, CYP1A2, and CYP1B1 were induced in the liver of rats administered TCDD, dieldrin, or 2,4-dichlorophenoxyacetic acid. There are no published studies that investigated the induction of hepatic or mammary CYP1 enzymes by a single dose intragastric dose of 20 mg of DMBA. In a previous study, DMBA was administered to mice as a single intraperitoneal injection at dosages of 5 to 100 mg per kg of body weight and induction of hepatic mRNA levels of CYP1A1, CYP1A2, and CYP1B1 was observed. The study also stated that DMBA, in comparison with other PAHs such as 3-MC, benzo[*k*]fluoranthrene, and BaP, is only a moderate inducer of CYP1A1 and CYP1B1 in mouse liver (Shimada *et al.*, 2003).

In mammary tissue, CYP1A1, CYP1A2, and CYP1B1 enzyme expression was induced by DMBA treatment as shown by immunoblot analysis. We were unable to quantify the basal expression of mammary CYP1 protein because band intensity was below the limit of

quantitation. By visual inspection, the expression of mammary CYP1A2, CYP1A1, and CYP1B1 were induced by DMBA treatment in hypophysectomized and intact rat. The level of mammary CYP1A2 is the highest, followed by CYP1A1 and CYP1B1. Our study demonstrated that hypophysectomized animals are capable of inducing CYP1A1, CYP1A2, and CYP1B1 expression in liver and mammary tissue.

Microsomal EH protein levels were not induced by DMBA treatment in liver and mammary tissue of hypophysectomized and intact female Sprague-Dawley rats. A previous study reported a modest induction of mEH in the livers of rats administered *trans*-stilbene oxide (a synthetic proestrogen), phenobarbital, Aroclor 1254, or 3-MC (Thomas *et al.*, 1981). However, our results showed mEH expression was unaffected by DMBA treatment in liver or mammary tissue. In the study by Thomas *et al.* (1981), rats were treated with daily intraperitoneal injections of the chemical for 4 days and the animals in our study were treated with a single intragastric dose of DMBA. Thus, it is possible that the dose of DMBA and the duration of treatment in our study were insufficient to elicit induction of mEH.

4.8 Methods to Measure DMBA Bioactivation

In the present study, we did not directly measure DMBA bioactivation in intact and hypophysectomized female Sprague-Dawley rats. It would be ideal to measure DMBA biotransformation directly in the liver and mammary tissue to identify possible differences between hypophysectomized and intact rats. There are different approaches to determine the bioactivation of DMBA *in vivo* and *in vitro*. Previous studies incubated DMBA with rat liver or mammary microsomes in a reaction mixture and oxidative metabolites of DMBA were measured and identified using HPLC (high pressure liquid chromatography) (Tamulski *et al.*,

1973, Yang *et al.*, 1975, Wong *et al.*, 1980). Vater *et al.* (1991) analyzed the formation of DMBA metabolites using non-recirculating liver perfusion, where the liver was left *in situ* and the common bile duct, portal vein, and vena cava were cannulated for perfusion. DMBA in the perfusion medium was then infused into the liver and the perfusate was collected for HPLC analysis of DMBA metabolite formation. Alternatively, formation of DMBA-DNA adducts in the collected sample could be analyzed using HPLC as previously described by Cai *et al.* (1997) and Kleiner *et al.* (2002).

The DMBA metabolites can be resolved by HPLC and can be detected by UV or fluorescence spectroscopy and identified by comparison of their retention times with those of synthetic reference compounds. DMBA metabolites were identified by comparing the chromatographic properties of the reference compounds. Some, but not all, synthetic DMBA metabolites are available commercially. At this time, a DMBA biotransformation assay has not been established in this laboratory. For the present study, we analyzed the potential for DMBA bioactivation in hypophysectomized and intact rats indirectly by measuring the activities of hepatic CYP1 enzymes that are documented to be involved in the bioactivation of DMBA.

4.9 Effects of Hypophysectomy and DMBA Treatment on Enzyme Activities

MROD and BaP hydroxylase assays were used as a measure of CYP1A-mediated enzyme activities in hepatic microsomes. MROD and BaP hydroxylase activities were not measured in mammary microsomes due to inadequate sample size for analysis. Approximately 150 µl of microsome was prepared from mammary tissues per rat. The protein concentrations in the mammary samples were between 5 to 10 mg of microsomal

protein per ml. However, each immunoblot or enzyme activity assay required 20 to 40 µl of microsomal sample. In our study, we decided to use the mammary microsome samples for immunoblot analysis to determine CYP1 and mEH protein levels.

MROD and ethoxyresorufin *O*-deethoxylase (EROD) assays have been used as an indicator of CYP1A2 and CYP1A1 activity, respectively. Burke *et al.* (1994) determined that the demethylation of methoxyresorufin is catalyzed primarily by CYP1A2 in liver microsomes prepared from control rats and 3-MC-treated rats. Ethoxyresorufin was a selective probe for CYP1A1 in liver microsomes prepared from 3-MC-treated rats. Benzo[*a*]pyrene is a PAH that is often used as a substrate for analyzing microsomal CYP1A. BaP is oxidized into many hydroxyl derivatives by CYP enzymes with 3-OH BaP as the major product. Minor metabolites are formed in the following relative percentages: 9-OH-BaP, 3-13%, BaP-9,10-diol, 15-25%, BaP-7,8-diol, 12-14%, BaP-4,5-oxide, 8%, and BaP quinones, 14-17% (Yang and Kicha, 1978). Analogous to DMBA, BaP requires CYP and mEH enzymes for activitation to carcinogenic metabolites via the formation of the bay region diol epoxide. Purified recombinant human CYP1A1 and CYP1B1 exhibit higher BaP hydroxylase activities than recombinant CYP1A2, CYP2A6, CYP2C9, CYP2C19, and CYP3A4 enzymes (Shimada 2004). Using purified rat hepatic CYP enzymes, CYP1A1 exhibited the highest catalytic activity toward hydroxylation of BaP at the 3 and 9 positions. Other enzymes such as CYP2C11, CYP2C6, CYP2B1, CYP1A2, and CYP2A1 also exhibit BaP hydroxylation activity but are less efficient (Ryan and Levin, 1990).

Results of the present study show that MROD activity was similar in liver microsomes prepared from hypophysectomized rats and intact rats. DMBA treatment induced a 14-fold increase in MROD activity in the intact rats and a 17-fold increase in MROD activity in the hypophysectomized rats. Therefore, DMBA treatment elicited an increase in MROD activity,

which could be as a result of induction of hepatic CYP1A2 expression.

According to our results, hepatic BaP hydroxylase activity was not affected by hypophysectomy in corn oil-treated female rats. A previous study reported on the effect of hypophysectomy and castration on hepatic BaP hydroxylase activity in male Sprague-Dawley rats (Al-Turk *et al.*, 1981). That study showed a reduction in BaP hydroxlase activity in hepatic microsomes prepared from hypophysectomized male rats. However, our results demonstrated that hypophysectomy of female rats did not affect BaP hydroxylase activity. Because hydroxylation of BaP is mediated by several CYP enzymes in uninduced rats, the difference in results between our study and that of Al-Turk *et al.* (1981) can be explained by a sex difference in CYP enzyme expression in hypophysectomized male and female rats. Some hepatic CYP enzymes such as CYP2C11 are expressed predominantly or solely in male rats, whereas other enzymes such as CYP2C12 are expressed only in female rats (Shapiro *et al.*, 1995).

Hepatic BaP hydroxylase activity was induced by DMBA treatment in hypophysectomized and intact rats. However, hepatic BaP hydroxylase activity was significantly higher in DMBA-treated intact rats than in DMBA-treated hypophysectomized rats. Several factors can affect the hydroxylation of BaP including inherited influences such as strain, age, CYP enzyme expression and polymorphisms, and NADPH cytochrome P450 reductase levels. BaP hydroxylation at the 3 or 9 position is mediated primarily by CYP1A1 (Ryan and Levin *et al.*, 1989). According to the results obtained for hepatic CYP1A1 expression, there was no significant difference in the expression of hepatic CYP1A1 protein between DMBA-treated hypophysectomized and intact rats. NADPH cytochrome P450 reductase, which catalyzes electron transfer from NADPH to CYP, is an essential component for all CYP-mediated hydroxylation reactions. Although our study did not measure the

expression of NADPH cytochrome P450 reductase in the liver, it was documented previously that hypophysectomy affected the expression and activity of NADPH cytochrome P450 reductase in male and female Fischer rats (Waxman *et al.*, 1989). However, MROD activity, which also requires the activity of NADPH cytochrome P450 reductase, was similar between hypophysectomized and intact rats, suggesting a reduction in BaP hydroxylase activity is not due to altered NADPH cytochrome P450 reductase level or activity. The results seen in the BaP hydroxylase activities could be affected by other factors such as the expression of other hepatic CYP enzymes such as CYP2C and CYP2B which also contribute to the hydroxylation of BaP. The levels of these enzymes were not examined in this study.

In our study, mEH enzyme activity was not examined. EH activity in hepatic microsomes can be determined by the radiometric method of Schmassmann *et al.* (1976) using [^{3}H]benzo[*a*]pyrene-4,5-oxide as the substrate. Inoue *et al.*, (1995) reported mEH expression and activity were induced by hypophysectomy in female mice. There was a 77% increase in mEH enzyme activity in hypophysectomized female mice compared against intact female mice. It was also reported previously that there is a sex-related and age-dependent difference in mEH activities in the Sprague-Dawley rats. Hepatic mEH activity demonstrated a developmental pattern in female rats, where mEH activity was low at the neonatal stage, followed by a rapid rise in activity at puberty and remained stationary for up to 90 days (Denlinger and Vesell, 1989). Male rats followed a similar age-development pattern in mEH activity, but the activity in adult male was significantly higher than adult female rats. Hormonal influences on the developmental pattern of mEH were also examined in that study. The administration of testosterone propionate to female rats increased hepatic mEH activity to a level similar to the adult male rats (Denlinger and Vesell, 1989). The data agree with our results and implicate a strong hormonal influence on both mEH expression and activity.

Additional experiments would be needed to identify the specific hormone or hormones that are involve in the regulation of mEH expression.

4.10 Evaluation of Hormones and DMBA-Induced Mammary Tumorigenesis

Hormones are an important factor in mammary gland development. Rat mammary gland expresses hormone receptors such as estrogen receptor, progesterone receptor, prolactin receptor, and also growth hormone receptors. Normal ductal branching and proliferation requires the presence of estrogen (Bocchinfuso and Korach, 1997) and progesterone (Humphrey *et al.*, 1997) and an intact pituitary (Reece *et al.*, 1936). It is evident that mammary gland growth is under the control of both the ovary and pituitary gland (see Figure 4.1).

We speculate that the initiation and progression of DMBA-induced mammary cancer in rats depends on the interaction of multiple mammogenic and lactogenic factors. There have been numerous studies and reports investigating the role of ovarian hormones and pituitary factors on mammary tumorigenesis. Estradiol administered at low doses stimulates the growth of DMBA-induced mammary tumors (Huggins *et al.*, 1958). However, the development and growth of these tumors are inhibited by high doses of 17β-estradiol (Huggins *et al.*, 1958). Some researchers have hypothesized that estrogens bind to estrogen receptor to stimulate tumor growth (Lewis *et al.*, 2004). Others have speculated that estrogens are mammogenic because of their stimulatory effects on prolactin secretion, where prolactin secretion is stimulated by low and inhibited by large doses of estrogens (Russo and Russo, 1998). Plaut *et al.* (1993) demonstrated that prolactin is essential for mammary lobulo-alveolar development in whole organ culture and that the mammary gland is more sensitive to prolactin than growth hormone in lobulo-alveolar development. The same study

also demonstrated that growth hormone does not mediate mammary gland development through binding to prolactin receptor nor is it mediated by IGF-1. Nevertheless, the mechanism of action of individual hormone in mammary tumor initiation and progression is still unclear.

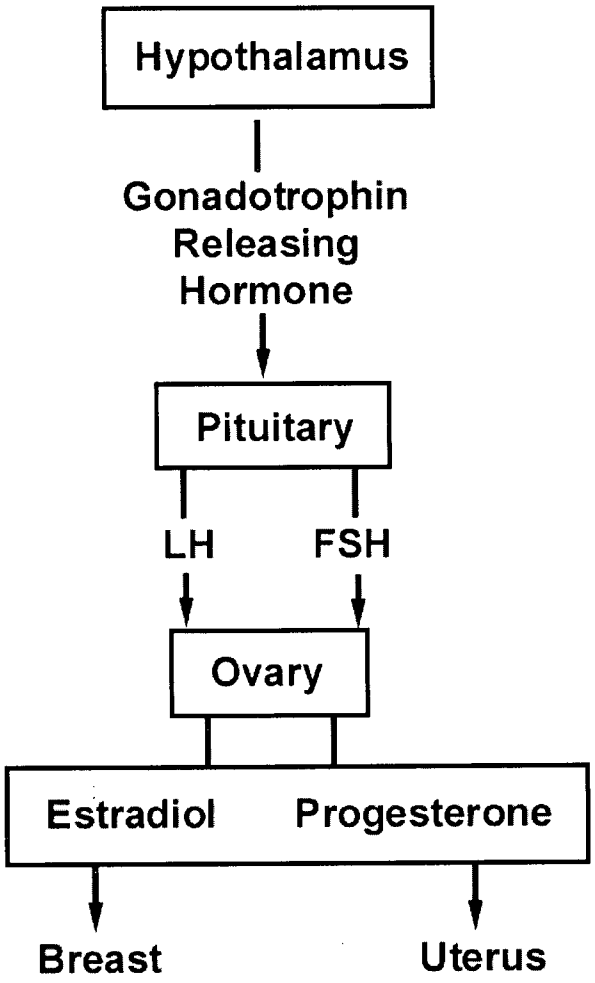

Figure 4.1 The pituitary releases gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) through the secretion of the gonadotrophin release hormone by the hypothalamus. The gonadotrophins then act on the ovary to stimulate the release of ovarian hormones, estradiol and progesterone to promote growth of the breast and uterus.

4.11 Summary

1. A single dose of 20 mg of DMBA caused a 66% mortality rate in the hypophysectomized rats. Deaths were related to the acute toxic effects of DMBA. When 20 mg of DMBA was given as four divided doses of 5 mg each, no deaths was observed in the hypophysectomized rats.

2. Hypophysectomized adult virgin female rats treated with a single intragastric dose of 20 mg of DMBA did not develop mammary tumorigenesis among the surviving rats, whereas 55% of the intact female rats treated with DMBA developed mammary tumors. Mammary tumor latency was approximately 8 weeks post DMBA treatment and there was an average of 2.5 tumors per tumor-bearing rat.

3. Basal hepatic CYP1A1 and CYP1A2 expression was unaffected by hypophysectomy. Hepatic CYP1-mediated activities measured by MROD and BaP hydroxylase assays were also similar between intact and hypophysectomized rats. Mammary CYP1A1, CYP1A2, and CYP1B1 protein levels in corn oil-treated rats were below the detection limit as assessed by immunoblot analysis. DMBA treatment of intact and hypophysectomized rats induced hepatic CYP1A1 and CYP1A2 and mammary CYP1A1, CYP1A2, and CYP1B1 enzyme expression. Hepatic CYP1-mediated enzyme activities were also increased in liver microsomes of animals treated with DMBA.

4. Hepatic and mammary mEH protein expression was significantly higher in hypophysectomized rats when compared to intact animals. DMBA treatment had no additional effect on hepatic and mammary mEH protein expression. The expression of

rat hepatic and mammary mEH appears to be regulated by pituitary hormones.

4.12 Conclusion and Future Directions

Hypophysectomized rats failed to develop mammary tumors induced by DMBA. It was demonstrated in a previous study that tumors were induced by 3-MC in ovariectomized rats but at a decreased incidence compared to intact rats, whereas a complete failure of tumor induction was seen in hypophysectomized rats (Huggins *et al.*, 1958). A factor or a combination of factors controlled by the pituitary gland appears to be an essential component in the initiation and development of breast cancer.

The expression and activity of CYP1 and mEH enzymes needed for the bioactivation pathway of DMBA were measured between hypophysectomized and intact rats. Both CYP1 and mEH enzymes play important roles catalyzing the reactions that transform DMBA to the carcinogenic diol-epoxide metabolite. The formation of DMBA-3,4-diol, in particular, is limited by the availability of mEH (Christou *et al.*, 1989). Although the present study did not directly measure DMBA bioactivation in intact and hypophysectomized rats, our results demonstrated that hypophysectomy did not affect the expression or induction of hepatic CYP1A1 and CYP1A2 or the expression of mammary CYP1A1, CYP1A2, and CYP1B1. Hepatic CYP1 enzyme activities in hypophysectomized rats were also measured and no significant difference was found between intact and hypophysectomized animals. Thus, the failure of hypophysectomized animals to develop mammary tumors following treatment with DMBA is not due to the bioactivation process of DMBA.

Mammary tumorigenesis has a strong hormonal component. Estradiol, progesterone, growth hormone, and prolactin are well documented regulators of breast cancer growth.

Hypophysectomized rats failed to develop mammary tumors is most probable due to a suppression of ovarian and pituitary hormones. These hormones are required for normal mammary gland development; they are also essential for mammary tumor development and growth. However, it remains uncertain as to how these hormones work individually or in conjunction with each other to initiate and promote tumor growth. For future studies, we are aiming to administer individual hormones to hypophysectomized rats to examine mammary tumor initiation and progression.

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6. APPENDIX

6.1 Appendix I: Mortality in Hypophysectomized Rats Given Intermittent DMBA Doses

6.1.1 Introduction

Study 1 had shown that hypophysectomized rats are more susceptible to the acute toxic effect of a single intragastric dose of 20 mg of DMBA (results shown in Table 3.3). Since the acute toxic effects of DMBA are likely to be dose-dependent, administering lower amounts of DMBA with intermission may reduce mortality while conserving the carcinogenic effects. In this experiment, we proposed a DMBA dosing regimen similar to the method described by Hollingsworth *et al.* (1998) to induce mammary tumors in hypophysectomized rats.

6.1.2 Experimental Design

Fourteen hypophysectomized rats were treated with four doses of 5 mg of DMBA dissolved in 1 ml of corn oil (a total of 20 mg of DMBA) over a 2-week period. The timing and interval of each dose is shown in Figure 6.1. Body weights of the animals were measured daily during the study period. All deaths were recorded. The animals were terminated by decapitation 24 hours after the last DMBA dose.

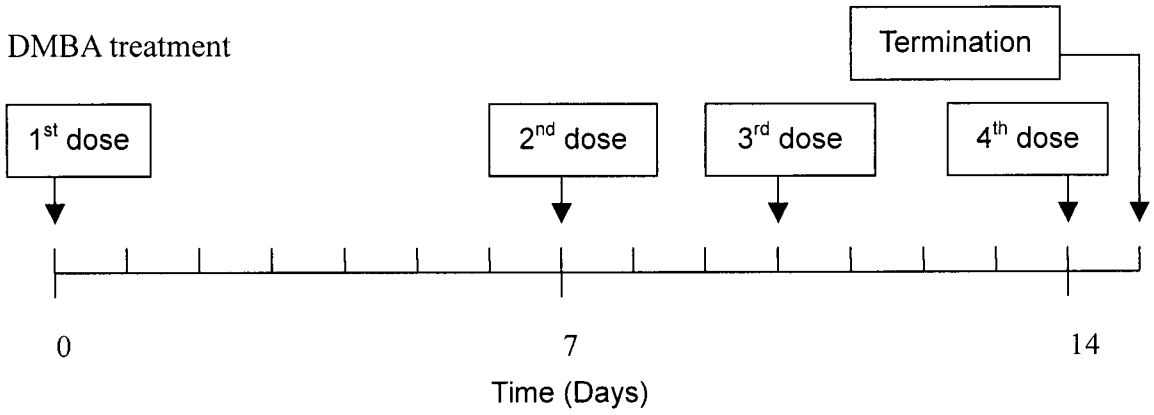

Figure 6.1 Experimental Design: Mortality in hypophysectomized rats given intermittent DMBA doses.

6.1.3 Effect of Intermittent DMBA Treatment on Body Weight

Body weight was monitored daily and throughout the treatment period. The daily change in body weight is illustrated in Figure 6.2. Similar to the results seen in Study 1, hypophysectomized rats demonstrated little or no weight gain during the monitoring period. The animals had an average body weight of 136 ± 12 g in the beginning of the study and an average of 149 ± 36 g at termination. Between each 5 mg dose of DMBA, no significant weight loss was observed in the animals.

6.1.4 Effect of Intermittent DMBA Treatment on Mortality

The results of Study 1 demonstrated that more than 66% of hypophysectomized rats died after a single intragastric dose of 20 mg of DMBA. In contrast, no deaths were observed in the hypophysectomized rats given 20 mg of DMBA as intermittent doses. Hypophysectomized rats were able to tolerate 20 mg DMBA administered as four divided doses of 5 mg each.

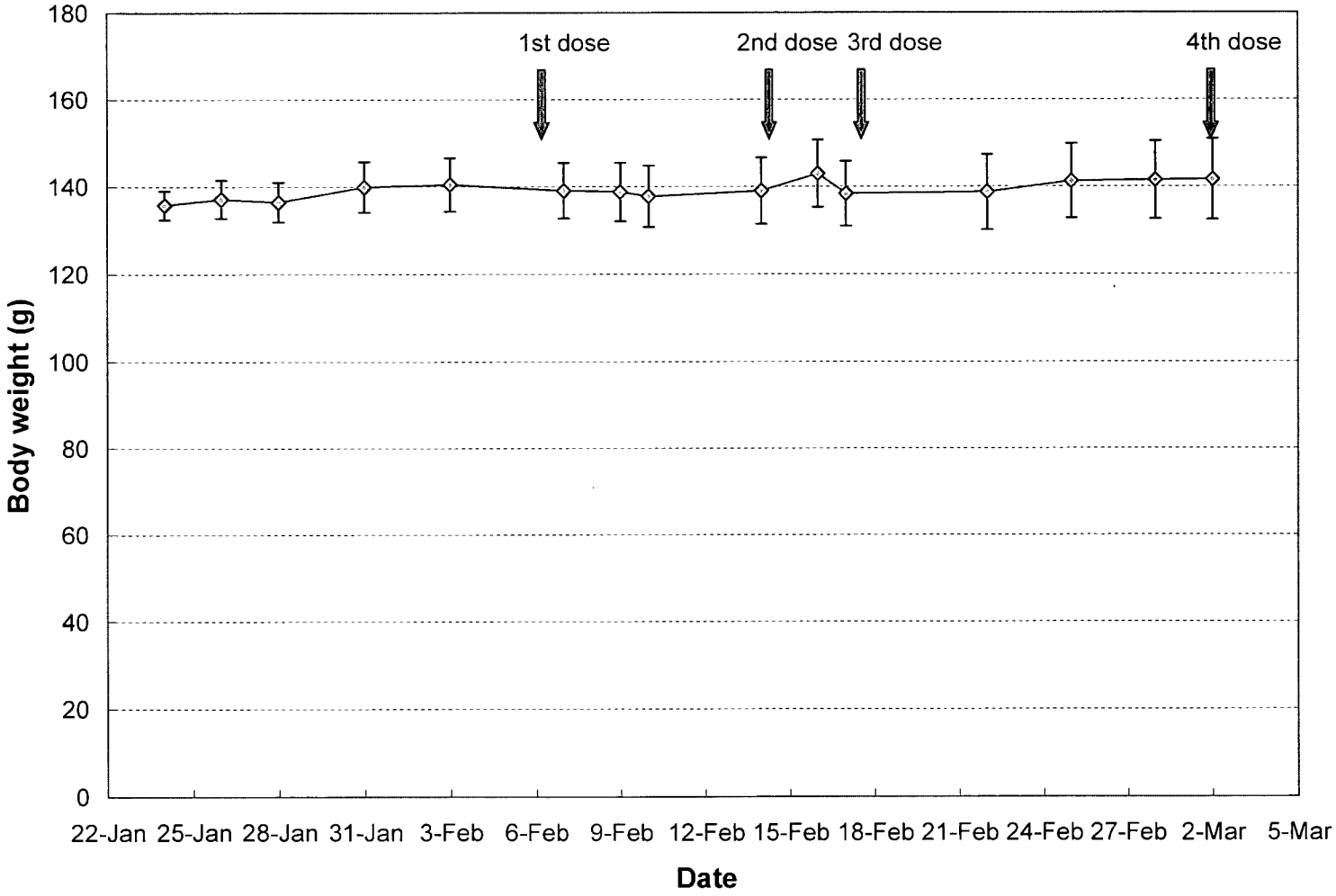

Figure 6.2 Average body weight of rats during the experimental period (n = 14). Values are expressed as mean ± SEM.