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Phenobarbital treatment inhibits the formation of estradiol-dependent mammary tumors in the ACI rat

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PB, phenobarbital; NQO1, NADPH: quinone oxidoreductase; GST, glutathione S-transferase

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Abstract

Exposure of female ACI rats for 28 weeks to 3 mg estradiol (E_2) contained in cholesterol pellets elevated blood E_2 levels and caused palpable mammary tumors in all animals. Co-administration of phenobarbital (PB) in their drinking water reduced the incidence, number and size of MT but did not reduce blood E_2 levels. Inhibition of MT by PB was accompanied by significant changes in total hepatic metabolism of E_2 measured *in vitro*. PB treatment caused approximately a 4-fold increase in hepatic metabolism of E_2 in control and E_2-treated rats. The major NADPH-dependent metabolites of E_2 were 2-OH E_2 and estrone (E_1). PB, either alone or together with E_2, increased microsomal 2-hydroxylation of E_2; formation of E_1 was either unaffected or decreased slightly. PB also increased microsomal metabolism of E_2 to minor metabolites (4-OH-E_2, 6α-OH-E_2, 6β-OH-E_2, 14α-OH-E_2, 6-keto E_1 and 2-OH-E_1) and reduced the formation of the E_2-17β-oleoyl ester and the E_2- 3- and 17-glucuronides. In contrast, when given in combination with E_2, PB increased the formation of both glucuronides. Co-treatment of animals with PB and E_2 increased activities of NADP(H):quinone oxidoreductase and glutathione-*S*-transferase to a greater extent than either compound alone. Collectively, these results show that the multiple actions of PB on hepatic metabolism of E_2 including induction of E_2-hydroxylation, glucuronidation, and antioxidant defense enzymes along with inhibition of E_2 esterification in livers of female ACI rats accompany a marked reduction of E_2-dependent mammary tumors in this model.

Introduction

Phenobarbital (PB) is a known inducer of microsomal hydroxylation of drugs and steroids in rodents and in humans (Conney et al., 1973). Treatment of rodents with PB induces the synthesis of hepatic microsomal enzymes that hydroxylate progesterone, estradiol (E_2), estrone (E_1), deoxycorticosterone , testosterone, Δ^4-androstene-3,17-dione and cortisol (reviewed by Zhu and Conney, 1998). Endogenous estrogens are hydroxylated at multiple positions by several hepatic and non-hepatic microsomal monooxygenase systems (reviewed by Martucci and Fishman, 1993; Zhu and Conney, 1998). The alteration of microsomal hydroxylation of steroids by PB is reflected *in vivo* by enhanced metabolism and altered actions of steroids. For example, pretreatment of rats with PB decreases the uterotropic action of E_2 and E_1 and enhances their metabolism *in vivo* (Levin et al., 1967, Levin et al., 1968). Treatment of rats with PB also inhibits the growth–promoting effect of testosterone on the seminal vesicles (Levin et al., 1974) and decreases the anesthetic action of progesterone and deoxycorticosterone in rodents (Conney et al., 1965).

Oxidative stress arising from redox cycling of catechol estrogens formed during E_2 metabolism has been suggested as an important factor in initiation and progression of many cancers including mammary carcinogenesis (Cavalieri et al., 1997, 2004). In mammals, the liver contains high levels of cytochromes P450 (CYP) which catalyze NADPH-dependent oxidation of estrogens to various hydroxylated or keto metabolites (Martucci and Fishman, 1993; Zhu and Conney, 1998). Reaction of endogenous catechol estrogens with DNA causes formation of depurinating DNA adducts (Cavalieri et al., 1997; Li et al., 2004; Cavalieri et al., 2004) which has been proposed to cause oncogenic mutations (Chakravarti et al., 1995). The carcinogenic

effects of 4-catechol estrogens (E_2 and E_1) in the kidney of castrated male Syrian hamsters (Liehr et al., 1986) reinforce this hypothesis. However, administration of high doses of 2-OH-E_2, 4-OH-E_2 or 4-OH-E_1 to ACI rats failed to cause mammary tumors under conditions where E_2 was highly active (Turan et al., 2004). In addition, the direct injection of estrone-3,4-quinone (the chemically reactive ortho-quinone derived from 4-OH-E_1) under the nipples of the mammary glands in rats failed to cause mammary tumors, whereas injection of a positive control, a diol epoxide of benzo[c] phenanthrene was highly active (El Bayoumy et al., 1996).

In the present study we investigated the effects of PB on the formation of mammary tumors induced by E_2 in ACI rats, an estrogen-sensitive strain which is considered a unique model because of its high sensitivity to estrogen dependent mammary ductal adenocarcinomas (80 to 100% incidence) within a relatively short time period (Harvell et al., 2000; Shull et al., 1997; Li et al., 2002). The main objective of the present study was to determine whether PB-induced increases in E_2-metabolism in the liver corresponded with alterations in the formation of E_2-induced mammary tumors. The liver is a major site of estrogen metabolism, and estrogen circulates primarily as inactive conjugates of E_1 and E_2 produced in the liver, i.e., conjugated E_2 is a transport form of estrogen which is converted back to active estrogen in target tissues such as mammary gland (reviewed in: Pasqualini 2004; Reed et al., 2005; Zhu and Conney, 1998)

Methods

Chemicals. E_2**,** ascorbic acid, NADPH, β-glucuronidase (EC. 3.2.1.3.1), UDP-glucuronic acid (UDPGA), sodium phenobarbital, saccharic acid 1,4-lactone, *p*-nitrophenol, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione, cytochrome c, oleoyl coenzyme A, sodium azide, and Tris-base were obtained from Sigma Chemicals Co. (St. Louis, MO). Glutathione reductase (140.7 U/mg protein) was purchased from Fluka Biochemica (Switzerland), ^{35}S-PAPS from New England Nuclear (Boston, MA), and PAPS (>99% pure) from H. Glatt and R. Landseidel, German Institute of Nutrition (Potsdam, Germany). [2,4,6,7,16,17-^{3}H(N)]-Estradiol (*s.a.*110-170 Ci/mmol) was purchased from NEN Life Science Products Inc. (Boston, MA). Pellets with or without E_2 were purchased from Hormone Pellets Press (Shawnee Mission, KS). AIN-76A diet was purchased from Dyets Inc. (Bethlehem, PA). All other chemicals used were of the highest grade from standard sources.

Animals and treatments. Female ACI rats (7 to 8 weeks old) were obtained from Harlan Sprague-Dawley Laboratory (Indianapolis, IN). The animals were housed individually in an AAALAC accredited barrier facility under controlled temperature, humidity, and lighting conditions and were fed with AIN-76A diet (Dyets Inc., Pennsylvania, PA). Treatment protocols started 4 days after arrival of the animals. Rats received water *ad libitum* or 0.05% PB in their drinking water. A single 20-mg pellet containing 3 mg of E_2 plus 17 mg of cholesterol was implanted subpannicularly in the shoulder region, as previously described (Li et al., 2002). Control animals were implanted with 20-mg cholesterol pellets alone. The rats were palpated for mammary tumors twice weekly, and weighed every two weeks for the duration of the experiment. Animals (N=8-15) were killed by decapitation after 6, 12 or 28 weeks. The

geometric volume of the tumors was determined using the formula: length x width x height x 0.5326, assuming an hemi-ellipsoid shape (Shah et al., 1999).

Serum levels of E_2

Trunk blood collected at decapitation was allowed to clot at 4°C for six hours and centrifuged. The serum was collected and stored at -80°C. Circulating levels of E_2 were determined in whole serum by RIA, using Coat-A Count® Estradiol RIA kits (Diagnostic Products Corporation, Los Angeles, CA). According to the manufacture's instructions, this assay measures both bound and free E_2 in serum.

Tissue Processing. All the animals were subjected to macroscopic pathologic examination when killed and the number, volume and localization of mammary tumors recorded. The mammary glands and the tumors were quickly removed. Portions of these tissues were fixed in Carnoy's solution for 4 h and processed for embedding in paraffin. Sections (6 μm) were prepared from each of the Carnoy's-fixed tissues and stained with hematoxylin and eosin. Selected estrogen target organs including pituitary, adrenals, thymus, uterus, kidneys and liver were removed and weighed.

Preparation of hepatic subcellular fractions. Liver cytosols and microsomes were prepared by differential centrifugation as described previously (Thomas et al., 1983) and stored at –80^{o}C until used. The protein concentration was determined with the BCA^{TM} protein assay kit (PIERCE, Rockford, IL) according to the supplier's instructions using bovine serum albumin as a standard.

Enzyme Assays

NADPH- dependent oxidation of E_2 (CYP450 assay) was carried out using liver microsomes

incubated with 5 mM ascorbic acid, 3 mM magnesium chloride, 50 μM sodium phosphate buffer (pH 7.4) and 25 μM [^{3}H]-E_2 (0.5 μCi) for 20 min at 37 ^{o}C. The enzyme reaction was initiated with 2 mM NADPH and terminated by the addition of 5 ml of ethyl acetate and vortexing. The ethyl acetate extracts were evaporated to dryness under nitrogen. The residue was dissolved in methanol and analyzed for metabolite composition by HPLC as described previously (Suchar et al.,1996; Mesia-Vela et al., 2002).

Fatty acyl-CoA:estradiol acyltransferase was assayed in reaction mixtures containing 50 μM [^{3}H]-E_2 (1 μCi), 100 μM fatty acyl-CoA, 5 mM magnesium chloride in 0.1 M sodium acetate buffer (pH 5.5) in a final volume of 0.5 ml. The reaction was initiated by the addition of liver microsomes (1 mg of protein/ml). After incubation at 37^{o}C for 30 min, the reaction was arrested by placing the tubes on ice, followed by addition of 0.2 ml of ice-cold sodium acetate buffer (pH 5.5) and extraction by vortexing with 4 ml of ethyl acetate (HPLC grade from Fisher Scientific). Dry extracts were redissolved and 90 μl aliquots analyzed by HPLC as described previously (Xu et al., 2002). Metabolite quantification was based on the amount of radioactivity in the metabolite peak as compared to the total radioactivity collected from the HPLC column from each sample.

Glucuronosyltransferase activity was assayed using a modification of a previously described method (Sanchez et al., 2003). The reaction mixture contained 1.0 mg of microsomal protein, 2 mM UDPGA, 5mM $MgC1_2$, 100 μM [^{3}H]-E_2 (0.15 μCi) and 50 mM Tris-HCl buffer pH 8.5 in a final volume of 150 μl. The reaction was initiated by addition of UDPGA. Incubations proceeded at 37^{o}C for 15 min and were terminated by placing them on ice and adding 50-μl ice-cold acetonitrile. The reaction mixtures were then vortexed and centrifuged at 3000 x g for 5

min. 10 µl of the supernatants were used for the determination of E_2 and E_2-glucuronides by HPLC. The HPLC system consisted of a Shimadzu SCL-10A system controller with a Shimadzu SIL-10A auto injector, two LC-l0AD pumps, SPD-10A UV-Vis detector set at 280 nm and an Eclipse XDB C18 (4.6mm × 150 mm) column (MacMod Analytical, Chadds Ford, PA). The solvent system consisted of solvent A: 0.1% acetic acid in water and solvent B: containing 20% methanol, 80% acetonitrile and 0.1% acetic acid. E_2 and its 3- and 17-hydroxyglucuronides were eluted with a 30 min linear gradient from 25 to 90% B. Metabolite quantification was based on the amount of radioactivity in the metabolite peak as compared to the total radioactivity collected from the HPLC column from each sample. The retention time of metabolites and E_2 agreed with corresponding UV-absorbing peaks of standards. The glucuronides were identified by their co-elution with authentic standards or by determination of E_2 after hydrolysis in the presence of β-glucuronidase (200 U/ml).

Cytosolic sulfotransferase (SULT) activity was determined using $PAP^{35}S$ as cofactor. The incubations were carried out with 20 mM Tris.HCl, pH 7.5, 4 mM $MgCl_2$, 10µM $PAP^{35}S$ (0.02 µCi) and 5 µM *p*-nitrophenol in 100 µl (Foldes and Meek, 1973).

Cytosolic NADPH quinone oxidoreductase (NQO1) was measured by reduction of cytochrome c (50 µM) in the presence of liver cytosol, 10 µM menadione and 1 mM NADPH. The reaction was monitored at 550 nm. The reactions were carried out in 100 mM potassium phosphate buffer pH 7.7 containing 0.04% triton X-100 at 25^{o}C (Jaiswal et al., 1988). Activity of dicumarol-inhibitable menadione reductase was determined using an extinction coefficient of 21/mM/cm for cytochrome c.

Cytosolic glutathione *S*-transferase (GST) activity was measured in the presence of liver cytosolic protein, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 1 mM reduced glutathione and 100 mM potassium phosphate buffer pH 6.5, at 25^{o}C. Conjugation of CDNB with glutathione was monitored at 340 nm. Specific activity was calculated using an extinction coefficient of 9.6/mM/cm (Habig et al., 1974)**.**

Cytosolic glutathione peroxidase (GPx) activity was measured using hydrogen peroxide as the substrate and was monitored by the decrease in absorbance at 340 nm. Specific activity was calculated using an extinction coefficient for NADPH of 6.22/mM/cm (Flohe and Gunzler, 1984).

Statistical analysis of data

Data are presented as the mean ± S.E. Differences between means were assessed by ANOVA followed by Bonferroni hoc post test, $P < 0.05$.

Results

Tissue wet weights and histopathology in PB and E_2 –treated ACI rats. Administration of E_2 increased pituitary weight by 3.5 and 4.8-fold after 6 and 12 weeks of treatment, respectively. These E_2-dependent increases in pituitary weights were not reduced by co-administration of PB (Table 1). A small increase in kidney weight (36%) and a reduction of thymus weight was also observed in the group treated with E_2 for 6-12 weeks (Table 1). Relative liver weight was increased about 1.5-fold by E_2 at both experimental periods (Table 2). Administration of PB also enhanced the E_2-induced increase in the relative liver weight, indicating differential effects on liver by both drugs. A 2 to 2.6-fold increase in the microsomal protein/liver after 6 and 12 weeks of PB treatment reflected the induction of microsomal protein by PB treatment (Table 2). PB alone or in combination with E_2 slightly increased adrenal weights at 6 weeks (Table 1). No alteration of body or uterine weight was seen during PB or E_2 treatment. It is noteworthy that PB did not alter E_2- dependent increases in pituitary weights (Table 1). Significant losses in body weight were noted at about 20 weeks when large palpable mammary tumors were seen in E_2-treated rats. In addition, pathology studies showed key changes in liver and mammary tissue. In liver, there was an increase of mitotic figures and scattered single cell degeneration caused by E_2 treatment alone. These effects diminished with time of exposure. Administration of PB alone induced enlargement of hepatocytes and vacuolar degeneration that increased markedly with the time of exposure to the drug. In contrast, for co-administered PB and E_2, the effects of PB were predominant with a reduction of the scattered single cell degeneration induced by E_2. In mammary samples, pronounced hyperplasia of mammary ductal cells due to E_2- treatment was observed. No quantitative difference could be observed in the extent of hyperplasia induced by

E_2 in relation to time of exposure, but some of the samples from the 12 week exposure group showed major ductal changes and atypia. PB treatment alone did not alter the morphology of mammary tissue. Mammary tumors classified as mammary ductal adenocarcinomas (MDAs) were histologically similar in animals treated with E_2 alone or E_2 plus PB.

Effect of PB on the incidence, multiplicity and size of mammary tumors in E_2-treated ACI rats. The first mammary tumor appeared during the 15^{th} week in E_2- treated rats. Fifty percent of the animals had mammary tumors by the 26^{th} week and 100% of the animals had mammary tumors by week 28. Although PB administration did not substantially alter the initial onsent of E_2-induced mammary tumors, it reduced to 53% the number of rats with mammary tumors at the end of the experiment (28 weeks) (Fig. 1). Treatment of the rats with PB had a dramatic inhibitory effect on the number and size of E_2-induced mammary tumors observed at 28 weeks (96-97 % inhibition; Fig. 2).

Serum E_2 levels. Treatment of female ACI rats with E_2 increased normal serum E_2 levels by 9 and 6-fold after 6 and 12 weeks of treatment, respectively. PB alone did not alter the serum level of total E_2 in normal or E_2-treated rats at any of the periods of the study (Fig 3).

Effect of PB administration on the NADPH-dependent oxidative metabolism of E_2 by liver microsomes. 2-OH- E_2 and E_1 were the major metabolites formed during the incubation of E_2 with control liver microsomes and NADPH (Table 3). Treatment of the rats with PB for 6 and 12 weeks increased the formation of 2-hydroxy-E_2 per liver by 4.7 and 6.6 fold, respectively when compared with E_2-treated rats. Treatment of the rats with PB for 6 and 12 weeks prevented the increase in formation E_1 induced by chronic E_2 treatment. In addition to the above results, PB

administration also caused substantial increases in the metabolism of E_2 to 6α-hydroxy-E_2, 6β-hydroxy-E_2, 14α-hydroxy-E_2, 4-hydroxy-E_2, 6-keto-E_2 and 2-hydroxy-E_1 per liver (calculated from tables 2 and 3). Since the later compounds are relative minor metabolites, the overall major effect of PB was to enhance the 2-hydroxylation of E_2 and also the 2-hydroxylation of E_1.

Effect of PB administration on the esterification of E_2 by fatty acylCoA: E_2-acyltransferase in liver microsomes. Treatment of rats with E_2 for 6 or 12 weeks had little or no effect on liver microsomal formation of E_2-17β-oleoyl ester when compared with control rats (calculated from Tables 2 and 4); however, co-administration of PB and E_2 reduced the esterification of E_2 by fatty acylCoA:E_2-acyltransferase significantly at both 6 and 12 weeks (Table 4). PB treatment alone decreased esterification of E_2 only after 12 weeks of treatment.

Effect of PB administration on the glucuronidation of E_2 by liver microsomes.

Treatment of rats with E_2 for 6 or 12 weeks did not alter microsomal glucuronidation of E_2 per mg microsol protein at any experimental time; however, treatment with PB reduced the formation of both E_2-glucuronides (3- and 17-) by 45- and 34%, respectively, at 12 weeks. When given in combination with E_2, PB increased formation of both glucuronides by 1.2- to 1.5-fold per mg protein after 6 and 12 weeks, respectively (calculated from Tables 2 and 4).

Effect of E_2 and PB administration on sulfotransferase and antioxidant enzymes in the liver.

Hepatic sulfotransferase: E_2 administration for 6 or 12 weeks decreased p-nitrophenol sulfonation per mg cytosolic protein by ~ 30% (Table 5). Administration of PB had little or no effect on p-nitrophenol sulfonation (SULT1A1) activity when given alone or together with E_2

(Table 5).

Antioxidant enzymes: Although administration of PB caused a modest decrease in hepatic GPx activity per mg cytosolic protein and E_2 treatment was inactive, the administration of E_2 or PB for 6 or 12 weeks caused large increases in hepatic NQO1 and GST activity (Table 6). Administration of PB for 6 or 12 weeks caused a 3.7- to 4.2- fold increase in NQO1 activity per mg cytosolic protein, and administration of E_2 alone increased this activity 2.6- to 3.7-fold (Table 6). Administration of PB for 6 and 12 weeks elevated GST activity 2.7- to 2.9-fold per mg cytosolic protein; and administration of E_2 alone increased this activity1.9- to 2-fold (Table 6).

The administration of a combination of PB and E_2 increased in NQO1 activity 68 to 84% (4.9- to 6.3-fold) when compared with the activity observed after administration of E_2 alone.

Administration of a combination of PB and E_2 also increased GST activity beyond that seen with either compound alone (3.1- to 3.9-fold per mg cytosolic protein, Table 6). In summary, administration of E_2 or PB alone caused large increases in the levels of the antioxidant enzymes, NQO1 and GST per mg cytosolic protein, and the effects of the combined administration of PB and E_2 were even larger than after administration of E_2 or PB alone.

Discussion

The frequency, size and number of mammary tumors induced in female ACI rats chronically treated with E_2 were reduced dramatically by PB given in the drinking water (Figs 1 and 2). The reduction of E_2-dependent mammary tumors noted in animals exposed to PB indicates that very potent mechanisms of protection are activated by this compound in ACI rats. Surprisingly, the dramatic decrease in mammary tumors in PB treated rats did not correlate with levels of serum E_2 measured in animals given E_2 chronically (Fig. 3). The failure of pituitary and uterine weights, which normally change in response to the "estrogenicity" of serum *in vivo*, to change in rats co-treated with E_2 and PB is in accord with the finding that total E_2 levels were essentially the same in serum from both groups of animals. Further studies to characterize bound and free forms of E_2 and its metabolites in serum and mammary tissue of rats treated with E_2 alone or in combination with PB over the course of mammary tumor generation are needed in future studies employing the ACI rat model.

The inhibition of E_2-dependent tumorigenesis by PB was accompanied by significant changes in both the oxidation of E_2 and by smaller changes in conjugation reactions. In addition, PB treatment had a remarkable stimulatory effect on two antioxidant defense enzymes, NQO1 and GST, which may be particularly important for the protection of PB against mammary tumorigenesis in the ACI rat model. Since blood E_2 levels were unchanged after PB, it is possible that E_2 synthesis was enhanced to compensate for increased E_2 metabolism. Further studies to clarify the relationship between alterations in hepatic metabolism of E_2 and the profile of E_2 metabolites in blood over the course of mammary tumor formation in the ACI rat model are

clearly warranted.

Chemoprevention of E_2-induced mammary tumors and modulation of microsomal oxidation of E_2 by PB.

The chemopreventive effect of PB likely involves multiple mechanisms including an increase in microsomal oxidation triggered by PB. The strong stimulatory effect of PB on the hepatic formation of E_2-hydroxylated E_2-metabolites including some that are formed only in trace amounts if at all in control animals (4-hydroxy-E_2, 6α-hydroxy-E_2, 14α-hydroxy-E_2, 6β-hydroxy-E_2 and 6-keto-E_1) is noteworthy. The formation and pattern of induction of hydroxylated metabolites of E_2 by PB was similar to findings in other rat strains (Suchar et al., 1996) which differ from the ACI rat in sensitivity to E_2-induced mammary tumors. The major hydroxylated metabolite produced in the liver of the ACI rat is 2-hydroxy-E_2. This metabolite, which is potentially chemopreventive against mammary tumors, was markedly induced in liver microsomes by PB in rats also treated with E_2. A number of studies indicated that the 2-methoxy derivative of this metabolite formed by catechol O-methyl transferase has strong antiproliferative and proapoptotic actions in a variety of human cancer cell lines including human breast cancer lines *in vitro* (Lottering et al., 1992; Pribluda et al., 2000; Liu and Zhu, 2004). Furthermore, 2-methoxy-E_2 has strong antiangiogenic and inhibitory effects on growth of mammary tumors in mice *in vivo* (Klauber et al., 1997; Fotsis et al., 1994). Recently, synergistic inhibitory effects of 2-methoxy-E_2 and microtubule-disrupting agents were observed on the proliferation of human breast cancer cells (Han et al., 2005). Thus, the marked stimulatory effect of PB on the 2-hydroxylation of E_2 may contribute to the inhibitory effect of PB on E_2 induced mammary tumors in the ACI rat. The other hydroxylated metabolites, some of which (e.g. 4-hydroxy-E_2)

may be tumorigenic (Liehr 1997), were also increased to a variable extent by PB. Transport of some of these metabolites either in their free or conjugated form to mammary tissue may contribute to mammary tumorigenesis either by direct genomic effects or via oxidative stress in mammary tissue. It is unlikely that circulating 4-hydroxy-E_2, 4-hydroxy-E_1 or 16α-hydroxy-E_2 contribute to the carcinogenic effects of E_2 in the ACI rat since these metabolites of E_2 were not tumorigenic under conditions described above where E_2 caused mammary tumors (Turan et al., 2004). In addition, the direct injection of estrone-3,4-quinone (the chemically reactive ortho-quinone derived from 4-OH-E_2) under the nipples of the mammary gland in CD rats failed to cause mammary tumors (El-Bayoumy et al., 1996). It is of interest that injection of 4-hydroxy-E_2 or E_2-3,4-quinone into the mammary gland of the female ACI rat resulted in 4-hydroxy-E_2-1-N_3-adenosine and 4-hydroxy-E_2-1-N_7-guanine adducts in DNA (Li et al., 2004). It will be of interest to determine whether these injections result in mammary cancer.

Chemoprevention of E_2-induced mammary tumors and modulation of E_2 conjugation by PB

E_2-3- and 17- glucuronidation, major inactivation pathways of E_2, were modestly increased when PB was co-administered with E_2. In contrast, glucuronidation of E_2 was reduced by PB-treatment alone at 12 weeks. Reduction in the capacity of liver to form E_2-glucuronides following chronic exposure to PB may increase amounts of active E_2 available for transport to E_2-target tissues such as mammary tissue. However, when PB was given concomitantly with E_2, the formation of E_2-3- and 17-glucuronide was modestly increased suggesting enhanced inactivation of E_2 in these animals. Reduction of microsomal fatty acylCoA: E_2 acyltransferase activity in PB-treated rats would reduce the formation of fatty acyl esters of E_2, which are

postulated to be long term storage forms of E_2 in fatty tissues such as the breast (Xu et al., 2002). Further, hepatic cytosolic sulfotransferase (SULT1A1), that may be involved in the conversion of E_2 to conjugates that serve as transport and storage forms, was also decreased slightly by treatment with combinations of PB and E_2.

Chemoprevention of E_2-induced mammary tumors and modulation of antioxidant pathways by PB and E_2.

The observation that administration of PB alone or together with E_2 strongly induced the activity of NQO1 and GST in liver after 6 and 12 weeks of treatment is intriguing because these activities may be linked to detoxification of reactive oxygen species generated by redox cycling of E_2-catechols; and increased levels of these antioxidant enzymes have been associated with anticarcinogenic effects (Ramos-Gomez et al., 2001). It is very important to determine whether the actions of PB observed in liver also occur in mammary tissue. The effects of PB on NQO1 and GST in liver were opposite to those on GPx and were in accord with the idea that regulation of expression of GPx differs from that of NQO1 and GST (Radjendirane et al., 1998; Esposito et al., 2000). It is also of considerable interest that administration of E_2 alone increases the level of antioxidant enzymes, a finding observed earlier by our group (Sanchez et al., 2003; Mesia-Vela et al., 2004). Moreover, the induction of NQO1 and GST in response to chronic treatment with E_2 seems to be specific for the ACI rat (Sanchez et al., 2003). The stimulatory effect of PB administration alone or together with E_2 on NQO1 and GST activity suggests that PB induction of antioxidant enzymes may play a role in the protective effect of PB on E_2-induced breast cancer.

In conclusion, the growth and multiplicity of E_2-dependent mammary tumors in the ACI

rat is markedly reduced by PB in the drinking water. This reduction of mammary tumors in the ACI rat is accompanied by multiple effects of PB on hepatic metabolism including: induction of E_2-hydroxylation via NADPH-dependent oxidations (predominantly an increase in 2-hydroxy E_2 formation), alteration in the formation of E_2 conjugates, and by the induction of important antioxidant defense activities. Such changes induced by PB may reduce the incidence of mammary tumors by: 1) enhancing the formation of a metabolite (2-methoxy E_2) that is inhibitory to mammary tumor growth, 2) decreasing the formation of fatty acid esters of E_2 metabolites that serve as long term depot forms of hormone in mammary tissue, 3) maintaining the formation E_2 glucuronidation in animals treated chronically with the hormone, and 4) elevation of antioxidant defense enzymes. Thus, PB-induced alterations in hepatic E_2-metabolism leading to the inactivation of E_2 and to the increased activity of antioxidant enzymes need to be considered as determinants for E_2-dependent mammary tumor formation in the ACI rat model.

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Footnotes

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Legends for Figures

Fig. 1. **Inhibition of mammary adenocarcinomas by phenobarbital in the ACI rat.** E_2 was delivered in cholesterol pellet implants. Animals (8-11 per group) were examined twice a week for tumors and were killed when the tumors reached 3 cm^2 or at the end of the experiment (28 weeks). PB treatment reduced the incidence of mammary tumors by 47 %.

Fig. 2. Reduction of multiplicity and growth of E_2-induced mammary adenocarcinomas by phenobarbital in the ACI rat. Phenobarbital reduced the multiplicity (tumors/rat; **A**) and growth (tumor size/rat; **B**) of E_2-induced mammary tumors by >95%. The data presented are average values ± S.E. from 8-11 animals per group killed at 28 weeks of treatment.

** Statistically significant (P <0.05) when compared to E_2 treated group (unpaired Student t Test).*

Fig. 3. Serum E_2-levels after chronic treatment of ACI rats with E_2. Data are presented as means ± S.E. of 8-11 animals per group.

** Statistically significant (P<0.001) when compared with groups not treated with E_2 (unpaired Student's t Test).*

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Table 1. Time course-effects on organ weights of female ACI rats receiving phenobarbital alone or in combination with estradiol

Treatment	***Weight***						
	Body (g)	Pituitary (mg)	Adrenals (mg)	Thymus (mg)	Uterus (mg)	Kidneys (g)	Liver (g)
6 weeks							
Water	159 ± 3	9.1 ± 0.6	52 ± 2	251 ± 12	397 ± 43	1.1 ± 0.05	5.3 ± 0.12
0.05% Phenobarbital	157 ± 3	8.3 ± 0.5	66 ± 3*	243 ± 17	373 ± 16	1.2 ± 0.01	6.2 ± 0.12
E_2	164 ± 3	32 ± 0.7*	55 ± 1	142 ± 4*	418 ± 33	1.5 ± 0.03*,†	7.8 ± 0.31*
0.05% Phenobarbital + E_2	158 ± 5	29 ± 2*	66 ± 1*,#	127 ± 8*	453 ± 28	1.5 ± 0.04*,†	8.0 ± 0.30*
12 weeks							
Water	173 ± 3	8.6 ± 0.2	59 ± 2	191 ± 5	406 ± 25	1.2 ± 0.03	5.2 ± 0.2
0.05% Phenobarbital	173 ± 5	8.8 ± 1	70 ± 3*	204 ± 15	403 ± 24	1.3 ± 0.05	6.0 ± 0.2
E_2	171 ± 3	44 ± 5*	58 ± 2	107 ± 13*	532 ± 47	1.5 ± 0.04*	6.6 ± 0.07
0.05% Phenobarbital + E_2	168 ± 2	38 ± 2*	59 ± 2	112 ± 5*,†	431 ± 11	1.4 ± 0.03*	7.3 ± 0.1

3 mg of E_2 was delivered in a 20 mg cholesterol pellet. 0.05% sodium phenobarbital was given in the drinking water. Control animals received water and a 20 mg cholesterol pellet only. Data are presented as means ± S.E. of 8-11 animals per group.

* Statistically different from water control group,

Statistically different from E2 treated group, † Statistically different from PB treated group (ANOVA followed by Bonferroni hoc post test) ($P < 0.05$)

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Table 2. Effect of treatment of female ACI rats with PB or E_2 for 6 or 12 weeks on liver weight and on liver microsomal protein per rat.

Treatment	*Percent liver weight*	*Microsomal protein per g lier (mg/g)*	*Microsomal protein per liver (mg/liver)*
6 weeks			
Water	3.4 ± 0.05	13 ± 1	67 ± 6
0.05% Phenobarbital	4.0 ± 0.05*	22 ± 2*	137 ± 12*
E_2	4.7 ± 0.1*	10 ± 0.7	75 ± 6
0.05% Phenobarbital + E_2	5.1 ± 0.08*,#	18 ± 1*,#	146 ± 20*,#
12 weeks			
Water	2.9 ± 0.07	12 ± 0.7	62 ± 4
0.05% Phenobarbital	3.5 ± 0.005*	27 ± 3*	162 ± 15*
E_2	3.9 ± 0.07*	10 ± 2	64 ± 10
0.05% Phenobarbital +E_2	4.4 ± 0.07*,#	25 ± 1*,#	184 ± 10*,#

3 mg of E_2 was delivered in a 20 mg cholesterol pellets. 0.05% sodium phenobarbital was given in the drinking water. Control animals received water and a 20 mg cholesterol pellet only. Percent liver weight = (liver weight /body weight) x 100

Data are presented as means ± S.E. of six animals per group

* Statistically different from water control group

Statistically different from E_2 treated group (ANOVA followed by Bonferroni hoc post test) ($P < 0.05$)

Table 3. Effect of phenobarbital administration on the NADPH-dependent oxidation of estradiol by liver microsomes from ACI rats

Treatment	Estradiol metabolites formed (pmol/mg protein/min)									E_2 metabolized	
	6α-OH-E_2	***14α-OH-E_2***	***E_3***	***6β-OH-E_2***	***6-keto-E_1***	***4-OH-E_2***	***2 -OH- E_2***	***2-OH- E_1***	***E_1***		
6 weeks										pmol/mg/min (%)[a]	nmol/liver/min
Water	0 ± 0	6 ± 0.6	16 ±1	2 ± 0.7	7 ± 3	9 ± 2	123 ± 11	24 ± 7	134 ± 13	392 ± 23 (17%)	28 ± 3
0.05% Phenobarbital	6 ± 2*	19 ± 2	27 ± 6	33 ± 3*	25 ± 3*	59 ± 1*	314 ± 13*	114 ± 18*	122 ± 20	884 ± 56* (29%)	115 ± 11*
E_2	0 ± 0	5 ± 1	15 ± 2	1 ± 0.5	8 ± 2	11 ± 2	149 ± 10	32 ± 6	232 ± 7*	530 ± 28 (25%)	40 ± 4
0.05% Phenobarbital + E_2	4 ± 1*,#	34 ± 5*,#	28 ± 5	42 ± 3#	29 ± 3*	36 ± 2#	434±37*,#	98± 8*,#	108 ± 15#	1053 ± 39*,# (38%)	145 ± 12*,#
12 weeks											
Water	0 ± 0	9 ± 1	17 ± 1	2 ± 1	10 ± 3	7 ± 1	169 ± 12	41 ± 7	140 ± 28	473 ± 33 (24%)	30 ± 3
0.05% Phenobarbital	8 ± 1*	21 ± 4*	26 ± 2	33 ± 3*	26 ± 3*	32 ± 4*	418 ± 27*	114 ±19*	94 ± 10*	1002 ±33* (31%)	166 ± 16*
E_2	0 ± 0	6 ± 1	17 ± 2	1 ± 0.5	13 ± 3	11 ± 3	182 ± 36	102 ± 26	309 ± 42*	644 ± 64 (32%)	51 ± 10
0.05% Phenobarbital + E_2	4 ± 1*,#	26 ± 4*,#	27 ± 1	38 ± 2*,#	25 ± 5*	36 ±3*,#	427± 2*,#	156±14*,#	90 ± 15*,#	1096 ± 42*,# (37%)	179 ± 15*,#

3 mg of E_2 was delivered in a 20 mg cholesterol pellets. 0.05% sodium phenobarbital was given in the drinking water. Control animals received water and a 20 mg cholesterol pellet. Liver microsomes (0.5 mg protein) were incubated at 37 ^{o}C for 20 min with 50 μM [^{3}H]-E_2, 2 mM NADPH, 5 mM ascorbic acid. Each value is mean ± S.E. obtained from liver microsomes from 6 ACI rats.

Percentage of E_2 metabolized

* Statistically different from water control group

Statistically different from the E_2 treated group (ANOVA followed by Bonferroni hoc post test) ($P < 0.05$)

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Table 4. Effect of treatment of ACI rat with PB or E_2 on the microsomal conjugation of estradiol by liver microsomes.

Treatment	*Conjugate formed (pmol/mg/min)*		
	Esterification	***Glucuronidation***	
	E_2-oleoyl-ester	***E_2-3-glucuronide***	***E_2 -17-glucuronide***
6 weeks			
Water	13 ± 1	138 ± 7	91 ± 8
0.05% Phenobarbital	21 ± 10	126 ± 14	117 ± 17
E_2	17 ± 4	118 ± 6	64 ± 2
0.05% Phenobarbital + E_2	8 ± 0.2*,#	171 ± 16 #	138 ± 14#
12 weeks			
Water	16 ± 3	117 ± 11	82 ± 7
0.05% Phenobarbital	5 ± 1*	64 ± 8*	54 ± 4*
E_2	14 ± 2	132 ± 12	75 ± 9
0.05% Phenobarbital + E_2	7 ± 1*,#	139 ± 18†	120± 14#,†

3 mg of E_2 was delivered in a 20 mg cholesterol pellet. 0.05% sodium phenobarbital was given in the drinking water. Control animals received water and a 20 mg cholesterol pellet. Liver microsomes (1 mg/ml protein) were incubated as described in Material and Methods section. Data are mean ± S.E. obtained from microsomes from six animals per group

* Statistically different from water control group,

Statistically different from E_2 treated group,

† Statistically different from PB treated group (ANOVA followed by Bonferroni hoc post test) ($P < 0.05$)

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Table 5. Effect of treatment of ACI rats with PB or E_2 on sulfotransferase (SULT 1A1) activity in liver cytosol

Treatment	**p-** ***nitrophenol sulfate formed*** ***(pmol/mg/min)***
6 weeks	
Water	147 ± 12
0.05% Phenobarbital	134 ± 7
E_2	93 ± 4 *
0.05% Phenobarbital + E_2	82 ± 1*
12 weeks	
Water	134 ± 18
0.05% Phenobarbital	111 ± 8
E_2	98 ± 12*
0.05% Phenobarbital + E_2	66 ± 5 *

3 mg of E_2 was delivered in a 20 mg cholesterol pellets. 0.05% sodium phenobarbital was given in the drinking water. Control animals received water and a 20 mg cholesterol pellet. Liver cytosols (25 μg) were incubated at 37 ^{o}C for 30 min with 5 μM *p*-nitrophenol as substrate as described in Material and Methods section. Data are presented as mean ± S.E. of five animals per group.

* Statistically different from water control group

$^{\#}$ Statistically different from E_2 treated group (ANOVA followed by Bonferroni hoc post test) ($P < 0.05$)

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Table 6. Effect of treatment of ACI rats with PB or E_2 on antioxidant enzyme activity in liver cytosol

Treatment	*Enzyme Activity (nmol/mg/min)*		
	NQO1	***GPx***	***GST***
6 weeks			
Water	713 ± 21	1527 ± 24	1001 ± 70
0.05% Phenobarbital	2986 ± 155*	1092 ± 49*	2668 ± 191*
E_2	1888 ± 69*	1471 ± 90	2001 ± 125*
0.05% Phenobarbital + E_2	3476 ± 152*,#	1156 ± 39#	3056 ± 160*,#
12 weeks			
Water	446 ± 33	1020 ± 60	879 ± 59
0.05% Phenobarbital	1670 ± 181*	787 ± 67	2549 ± 379*
E_2	1664 ± 88*	1029 ± 189	1665 ± 83*
0.05% Phenobarbital + E_2	2793 ± 116*,#	738 ± 83	3462 ± 428*,#

3 mg of E_2 was delivered in a 20 mg cholesterol pellets. 0.05% sodium phenobarbital was given in the drinking water. Control animals received water and received a 20 mg cholesterol pellet only. Data are presented as means ± S.E. of six animals per group

* Statistically different from water control group

Statistically different from E_2 treated group (ANOVA followed by Bonferroni hoc post test) ($P < 0.05$)

Figure 1

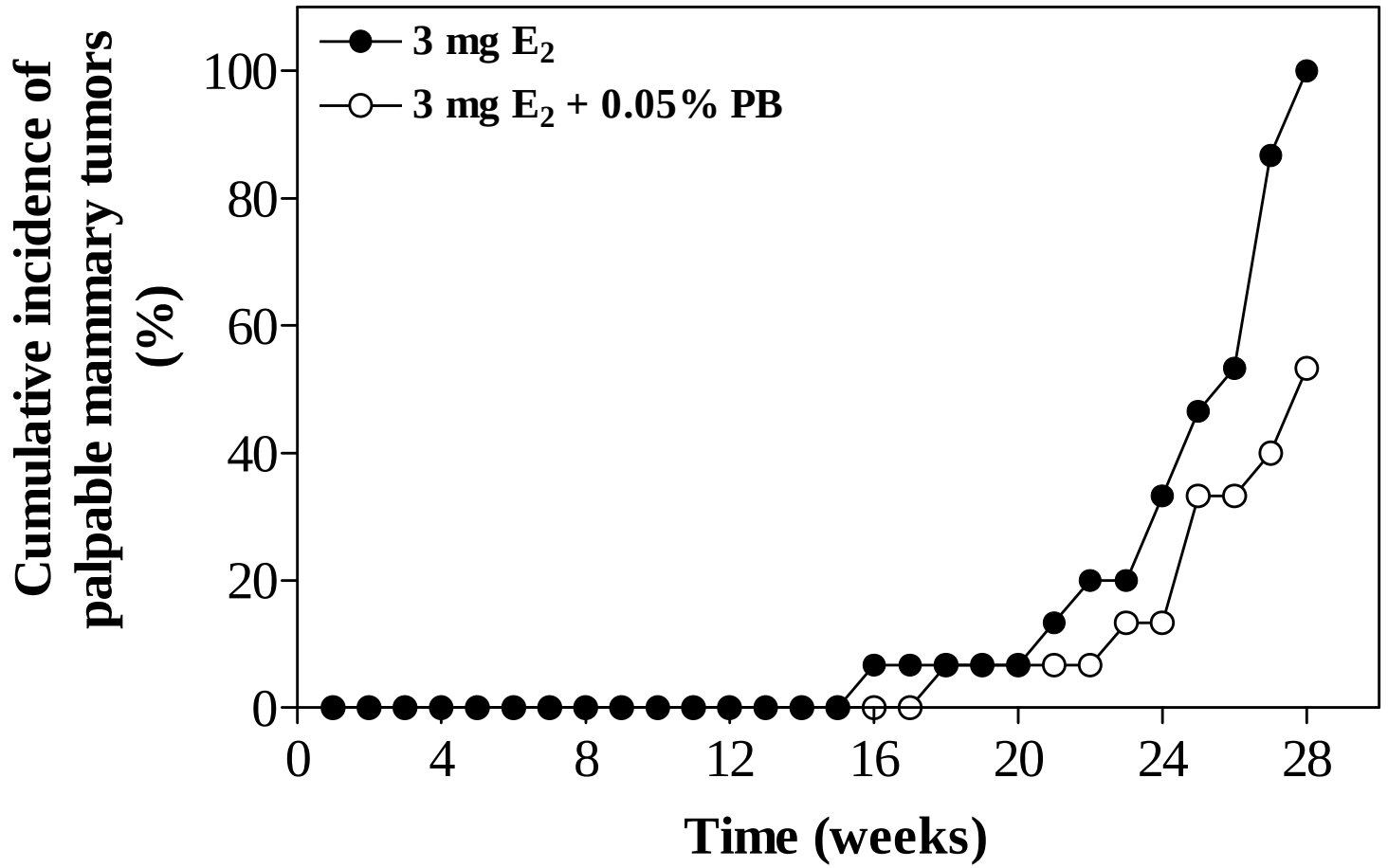

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Figure 2

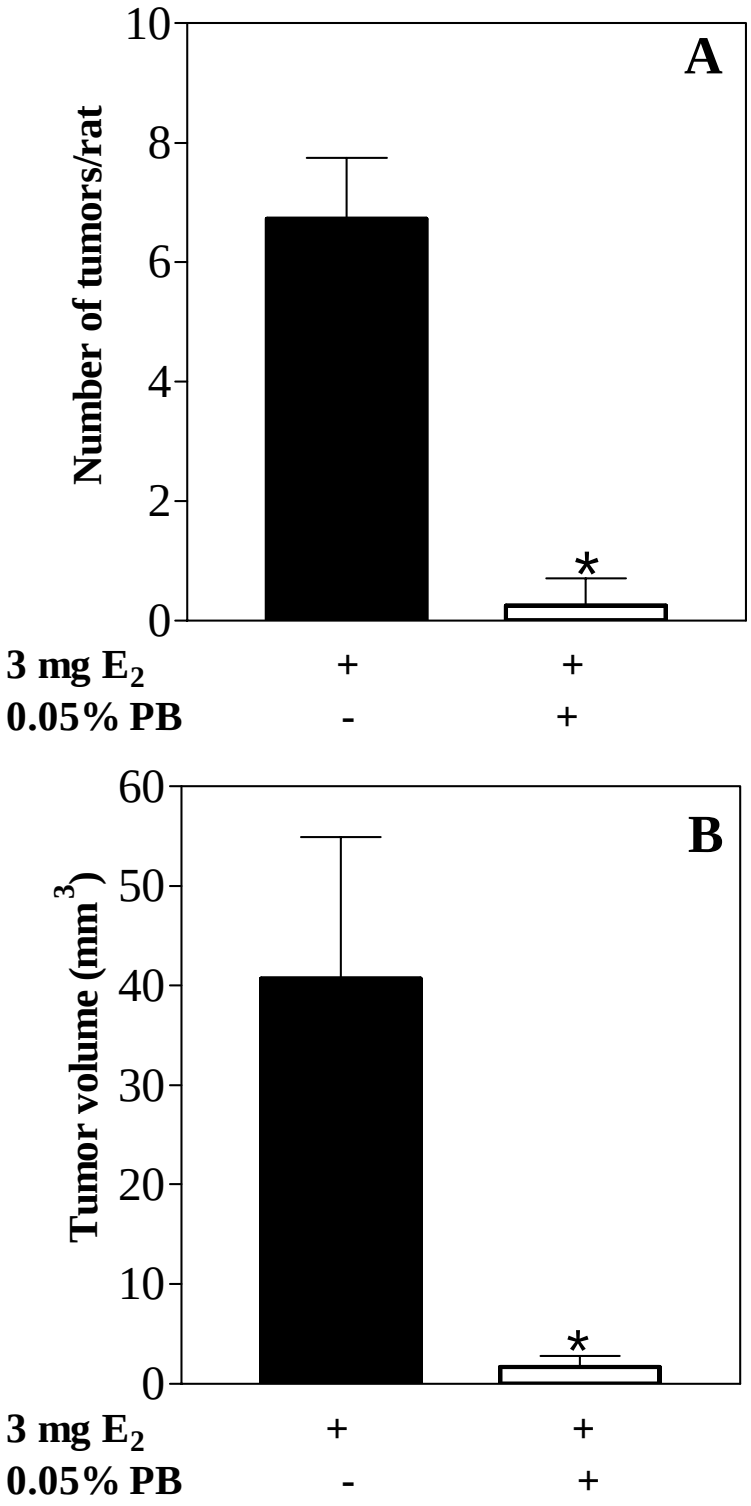

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Figure 3