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Estrogen-Induced Breast Cancer: Alterations in Breast Morphology and Oxidative Stress as a Function of Estrogen Exposure

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Abstract

Epidemiological evidence indicates that prolonged lifetime exposure to estrogen is associated with elevated breast cancer risk in women. Oxidative stress and estrogen receptor-associated proliferative changes are suggested to play important roles in estrogen-induced breast carcinogenesis. In the present study, we investigated changes in breast morphology and oxidative stress following estrogen exposure. Female ACI rats were treated with 17 β -estradiol (E₂, 3 mg, s.c.) for either 7, 15, 120 or 240 days. Animals were sacrificed, tissues were excised, and portions of the tissues were either fixed in 10% buffered formalin or snap-frozen in liquid nitrogen. Paraffin-embedded tissues were examined for histopathologic changes. Proliferative changes appeared in the breast after 7 days of E₂ exposure. Atypical ductal proliferation and significant reduction in stromal fat were observed following 120 days of E₂ exposure. Both *in situ* and invasive carcinomas were observed in the majority of the mammary glands from rats treated with E₂ for 240 days. Palpable breast tumors were observed in 82% of E₂-treated rats after 228 days, with the first palpable tumor appearing after 128 days. No morphological changes were observed in the livers, kidneys, lungs or brains of rats treated with E₂ for 240 days compared to controls. Furthermore, 8-iso prostane (8-isoPGF_{2 α}) levels as well as the activities of antioxidant enzymes, such as glutathione peroxidase, superoxide dismutase and catalase, were quantified in the breast tissues of rats treated with E₂ for 7, 15, 120 and 240 days and compared to activity levels in age-matched controls. 8-isoPGF_{2 α} levels displayed time-dependent increases upon E₂ treatment and were significantly higher than control levels at the 15, 120 and 240 day time-points. 8-isoPGF_{2 α} levels observed in E₂-induced mammary tumors were significantly higher than levels found in control mammary tissue from age-matched animals. Similarly, alterations in glutathione peroxidase and superoxide dismutase activities were detected in both mammary and tumor tissue from E₂-treated rats. Taken together, our data reveal that proliferative changes in the breast tissue of ACI rats are associated with increases in 8-isoPGF_{2 α} formation as well as changes

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in the activities of antioxidant enzymes. These oxidative changes appear to be a function of E₂ exposure and occur prior to tumor development.

Keywords

Breast cancer; ACI rat; animal model of breast cancer; estrogen; 17 β -estradiol; histopathology; mammary tumor; oxidative stress

Introduction

In the United States, breast cancer represents the most common neoplasm and the second most frequent cause of cancer death in women (Galati and O'Brien, 2004; Ju *et al.*, 2006). Since 2003, the incidence of breast cancer in the United States has decreased, possibly reflecting reduced utilization of hormone replacement therapy (Ries *et al.*, 2007). In contrast, global registry data demonstrates that breast cancer incidence has been increasing in most parts of the world since 1973 (IARC, 2007). The importance of estrogens in the etiology of breast cancer is widely recognized and the United States government has added steroidal estrogens to the list of known human carcinogens (IARC, 1987; Cavalieri *et al.*, 1997; IARC, 1999; NTP, 2002; Bhat *et al.*, 2003; Cavalieri *et al.*, 2006). Elevated lifetime estrogen exposure is considered a major risk factor for breast cancer, and many known risk factors for the disease are related to a woman's reproductive history (McPherson *et al.*, 2000; Nelson *et al.*, 2002; Rossouw *et al.*, 2002). For example, breast cancer risk factors include early menarche, nulliparity or late pregnancy, late menopause, prolonged oral contraceptive use and hormone replacement therapy (McPherson *et al.*, 2000; Nelson *et al.*, 2002; Rossouw *et al.*, 2002). While a compelling body of data supports a role for estrogen in breast carcinogenesis, the molecular mechanisms underlying the initiation and progression of estrogen-related cancers remain indefinite (Clemons and Goss, 2001; Key *et al.*, 2002).

Estrogens exert their carcinogenic effects by both estrogen receptor (ER)-dependent and ER-independent mechanisms (Liehr *et al.*, 1993; Clemons and Goss, 2001; Bhat *et al.*, 2003; Yager and Davidson, 2006). The ER-dependent pathway involves the activation of the ER by estrogens, leading to the expression of estrogen-responsive genes, as well as stimulation of cell growth and proliferation (Liehr *et al.*, 1993; Clemons and Goss, 2001; Bhat *et al.*, 2003; Cavalieri *et al.*, 2006; Yager and Davidson, 2006). Moreover, estrogens promote the expansion of estrogen-responsive neoplastic cells, resulting in accumulation of genomic damage (Liehr *et al.*, 1993; Clemons and Goss, 2001; Bhat *et al.*, 2003; Cavalieri *et al.*, 2006; Yager and Davidson, 2006). The ER-independent pathway of estrogen-induced breast cancer involves the generation of genotoxic estrogen metabolites, which are highly reactive and damage DNA (Clemons and Goss, 2001; Patel and Bhat, 2004; Yager and Davidson, 2006). 17 β -estradiol (E₂) is metabolized to catechol estrogens 2-hydroxyestradiol (2-OHE₂) and 4-hydroxyestradiol (4-OHE₂) by cytochrome P450 1A1 (Cyp1A1) and cytochrome P450 1B1 (Cyp1B1), respectively (Cavalieri *et al.*, 1997; Cavalieri and Rogan, 2004). While, 2-OHE₂ and 2-methoxyestradiol (2-MeOE₂) have putative chemo-protective characteristics, 4-OHE₂ is highly genotoxic (Liehr *et al.*, 1986; Li and Li, 1987; Ziegler *et al.*, 1997; Badawi *et al.*, 2001; Brueggemeier *et al.*, 2001; LaVallee *et al.*, 2002). Tumorigenic estrogen metabolites such as 4-OHE₂ undergo oxidative metabolism to form electrophilic quinones, which readily react with DNA to produce depurinating adducts (Liehr *et al.*, 1993; Cavalieri *et al.*, 1997; Cao *et al.*, 1998; Patel and Bhat, 2004). In addition, redox cycling of quinones and semiquinones results in the formation of free radicals and reactive oxygen species, thereby creating more opportunities for genetic damage (Liehr *et al.*, 1986; Yager, 2000). Recent studies also suggest that mitochondria may also be involved in the generation of estrogen-associated reactive oxygen species (ROS) (Felty *et al.*, 2005a; Felty *et al.*, 2005b; Roy *et al.*, 2007). The oxidative

stress generated by estrogens is suspected to act in concert with ER-mediated signaling pathways to promote DNA damage and altered expression of genes responsible for controlling cell cycle and proliferation (Liehr *et al.*, 1986; Cavalieri *et al.*, 1997; Yager, 2000; Bhat *et al.*, 2003; Cavalieri and Rogan, 2004).

The ACI rat model of estrogen-induced mammary cancer provides a relevant biological system in which to study the molecular mechanisms underlying human breast cancer. Continuous treatment of ovary-intact female ACI rats with E₂ results in nearly 100% mammary tumor incidence within six to seven months (Shull *et al.*, 1997; Harvell *et al.*, 2000). Mammary tumors have not been observed in ovary-intact female ACI rats not exposed to exogenous E₂ (Shull *et al.*, 1997; Harvell *et al.*, 2000). All mammary tumors observed in this model have been classified as carcinomas, and invasive features have been observed in some (Shull *et al.*, 1997; Harvell *et al.*, 2000). E₂-induced mammary gland tumors in ACI rats and human sporadic breast cancers share many pertinent histopathologic and molecular features, both in early pre-malignant lesions as well as in primary tumors (Makris *et al.*, 1997; Arnerlov *et al.*, 2001; Li *et al.*, 2002a; Li *et al.*, 2002b; Li *et al.*, 2004; Weroha *et al.*, 2006). For example, mammary cancers induced by E₂ in ACI rats are estrogen-dependent and exhibit genomic instability, characteristics commonly observed in human breast cancers (Makris *et al.*, 1997; Arnerlov *et al.*, 2001; Li *et al.*, 2002a; Li *et al.*, 2002b; Li *et al.*, 2004; Weroha *et al.*, 2006).

The objective of the current study was to investigate the occurrence of changes in breast morphology and oxidative stress following estrogen exposure *in vivo* using the ACI rat model of breast carcinogenesis. Female ACI rats were treated with E₂ (3 mg, s.c.) for 7, 15, 120 and 240 days. Animals were examined daily for palpable tumors. At the end of the experiments, animals were sacrificed and various tissues, including breast (an estrogen-target tissue) and liver (a non-target tissue), were excised and examined for changes in levels of oxidative stress markers and histopathologic alterations. Our findings indicate that proliferative changes in the breast tissue of E₂-stimulated rats are associated with increases in 8-isoPGF_{2α} formation and alterations in the activities of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx). Thus, changes in oxidative stress markers seem to be a function of duration of exposure to E₂ and precede tumor development.

Materials and Methods

Tumor development/estrogen treatment of rats and histopathologic analysis

Female ACI rats, 4 weeks of age (Harlan Sprague Dawley, Indianapolis, IN) were housed in the Columbia University animal facility under controlled temperature, humidity, and lighting conditions. The animals were fed an AIN-76A phytoestrogen-free diet (Dyets, Bethlehem PA) and water was given *ad libitum*. Animals were randomly distributed into two groups—control and E₂-treatment. After a one-week acclimatization period, rats in the E₂-treatment group were implanted subcutaneously with E₂ pellets (3 mg E₂ + 17 mg cholesterol) and rats in the control group were implanted with cholesterol pellets (17 mg cholesterol). E₂ and cholesterol pellets were prepared using a pellet press as described previously (Han and Liehr, 1994; Wang and Liehr, 1995; Bhat *et al.*, 2003). Eight separate sub-groups of rats, containing at least 10 rats per group, were treated with either E₂ or cholesterol pellets for 7, 15, 120 or 240 days. At the end of each of these time periods, animals were anesthetized using isoflurane and sacrificed. Mammary tissues (both tumor and normal) as well as liver, uterus, kidney, lung and brain tissues were removed and snap-frozen in liquid nitrogen for future analyses. Frozen tissues were stored at -70°C. A portion of the excised tissues was stored in 10% buffered formalin for histopathologic and immunohistochemical analyses. Tumor incidence and the number of tumor nodules were counted at the time of dissection. The formalin-fixed tissue was embedded in paraffin, and sections of 4- to 5-μm thickness were cut. Paraffin-embedded sections of the mammary, liver, brain, uterus, kidney, lung and ovary were stained with hematoxylin and eosin

for histopathologic evaluation by a pathologist. Tumors in the breast fat pads were identified grossly at the time of animal dissection. Tumor size was measured in millimeters and the volume (length \times breadth \times height) of tumor in cubic millimeters was obtained.

Determination of 8-iso-Prostane F_{2 α} (8-isoPGF_{2 α})

Total 8-iso-Prostane F_{2 α} (8-isoPGF_{2 α}) levels in the mammary and liver tissue of female ACI rats were quantified using a direct 8-isoPGF_{2 α} enzyme immunoassay kit obtained from Assay Designs (Ann Arbor, MI) according to the suppliers instructions as described previously (Bhat *et al.*, 2003). Fifty to 100 mg rat liver or mammary tissue was homogenized in cold PBS (pH 7.4) containing 0.005% butylated hydroxytoluene. Tissue homogenates were prepared in 2 ml round-bottom microcentrifuge tubes by using a TissueLyser (Qiagen, Valencia CA). Homogenization was carried out in the TissueLyser at 29 cycles per second for 4 minutes. Protein concentrations from the neutralized homogenates were determined using the Pierce BCA protein assay kit (Pierce, Rockford IL). 8-isoPGF_{2 α} esters in 100 μ l of the total liver or mammary homogenate were hydrolyzed by incubation with 25 μ l of 10 N NaOH at 45°C for 2 hours. The reaction mixture was cooled on ice for 5 minutes, neutralized with 25 μ l of 12 N HCl and centrifuged in a microcentrifuge for 5 minutes 4°C. The clear neutralized supernatant was transferred into a new microcentrifuge tube and 50 μ l of the hydrolyzed/neutralized sample was used for the 96-well format 8-isoPGF_{2 α} assay. Samples were incubated with the 8-isoPGF_{2 α} antibody for 18 hours at 4°C. After incubation, the contents of the wells were emptied and washed with wash buffer. Wash buffer was then removed from the wells and the color was developed by incubation with 200 μ l of p-nitrophenyl phosphate for 45 minutes at room temperature. The reaction was stopped by the addition of 50 μ l of stop solution and the plate was read at 405 nm. Standard curves were run on each plate and were generated by measuring the optical density of 160-100,000 pg/ml of 8-isoPGF_{2 α} standards that were processed simultaneously with the unknown samples. Data are expressed as mean 8-isoPGF_{2 α} pg/mg protein \pm standard error of the mean. Fold changes were calculated by comparing 8-isoPGF_{2 α} levels detected in the mammary or liver tissue of E₂-exposed animal tissues to levels in the mammary or liver tissue of age-matched control animals.

Measurement of superoxide dismutase (SOD) activity

SOD activity in the mammary tissue of ACI rats was measured using a commercially-available kit from Cayman Chemical Company (Catalog Number 706002, Ann Arbor MI). Briefly, SOD activity was quantified using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. At the time of dissection, liver and mammary tissues were rinsed in PBS (pH 7.4) in order to remove red blood cells and clots. Tissues were subsequently snap-frozen in liquid nitrogen and stored at -80°C. At the time of analysis, approximately 50 mg of mammary or liver tissue was homogenized in 50 mM phosphate buffer containing 1 mM EDTA, 210 mM mannitol and 70 mM sucrose. Homogenization was performed using a TissueLyser (Qiagen, Valencia CA) for 4 minutes with the frequency set to 30 cycles per second. After homogenization, homogenates were centrifuged at 1500 \times g for 5 minutes at 4°C. The supernatant was removed and the protein concentration was measured using the BCA Protein Assay (Pierce, Rockford IL). Prior to measurement of SOD activity, mammary tissue homogenates were diluted with phosphate buffer to a concentration of 85 μ g/ml. Liver tissue homogenates were diluted to a concentration of 8.5 μ g/ml. Optimization of the assay showed these concentrations to produce absorbances within the linear range of the standard curve. Analysis of SOD activity in the tissue homogenates was carried out according to the manufacturer's instructions in 96-well format. Standard curves were run on each plate and were generated by measuring the optical density of seven samples with SOD activity ranging from 0-0.25 units/ml. The absorbance of the sample and standard wells were measured at 405 nm using a plate reader. SOD activity is reported as units/mg protein. One unit of SOD is defined as the amount of enzyme needed to cause 50% dismutation of the superoxide radical.

Fold changes in SOD activity were calculated by comparing SOD activity in the mammary or liver tissue of E₂-treated ACI rats to SOD activity measured in the mammary or liver tissue of age-matched controls.

Quantification of catalase (CAT) activity

CAT activity in the mammary tissue of ACI rats was quantified using a kit commercially-available from Cayman Chemical Company (Catalog Number 707002, Ann Arbor MI). This method is based on the reaction of the CAT enzyme with methanol in the presence of an optimal concentration of H₂O₂. The formaldehyde produced was measured spectrophotometrically at 540 nm using 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which changes from colorless to purple following oxidation. At the time of analysis, approximately 50 mg of mammary or liver tissue was homogenized in 50 mM phosphate buffer containing 1 mM EDTA, 210 mM mannitol and 70 mM sucrose. Homogenization was carried out as described above. After homogenization, homogenates were centrifuged at 10,000 × g for 15 minutes at 4°C. The supernatant was removed and the protein concentration was measured as described above. Prior to measurement of CAT activity, mammary tissue homogenates were diluted with phosphate buffer to a concentration of 75 µg/ml. Liver homogenates were diluted to a concentration of 8.5 µg/ml. Optimization of the assay showed these concentrations to produce absorbances within the linear range of the standard curve. Analysis of CAT activity in the tissue homogenates was performed according to the manufacturer's instructions in 96-well format. Standard curves were run on each plate and were generated by measuring the optical density of seven standards. CAT activity is reported as nmol/min/mg protein. Fold changes in CAT activity in E₂-treated rats were calculated by comparing CAT activity in the mammary or liver tissue of E₂-treated rats to that of mammary or liver tissue from age-matched controls.

Determination of glutathione peroxidase (GPx) activity

GPx activity in the mammary tissue of ACI rats was determined by using a kit available from Cayman Chemical Company (Catalog Number 703102, Ann Arbor MI). The GPx Assay kit measures GPx activity by way of a coupled reaction with glutathione reductase. Oxidized glutathione is produced during reduction of an organic hydroperoxide by GPx, and is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP⁺ causes a decrease in absorbance at 340 nm. The rate of decrease in the absorbance of the samples at 340 nm is directly proportional to the GPx activity in the sample. At the time of analysis, approximately 50 mg of mammary or liver tissue was homogenized in 50 mM phosphate buffer containing 1 mM EDTA, 210 mM mannitol and 70 mM sucrose. Homogenization was carried out as described above. After homogenization, homogenates were centrifuged at 10,000 × g for 15 minutes at 4°C. The supernatant was removed and the protein concentration was measured as described above. Prior to measurement of GPx activity, mammary tissue homogenates were diluted with phosphate buffer to a concentration of 150 µg/ml. Liver homogenates were diluted to a concentration of 8.5 µg/ml. Optimization of the assay showed these concentrations to produce absorbances within the linear range of the assay. Analysis of GPx activity in the tissue homogenates was carried out according to the manufacturer's instructions in 96-well format. Seven time points, spanning 7 minutes, were obtained in order to accurately assess the decrease in absorbance at 340 nm. GPx activity was reported as nmol/min/mg protein. Fold changes in GPx activity were calculated by comparing GPx activity in the mammary or liver tissue of E₂-treated ACI rats to that of mammary or liver tissue from age-matched control rats.

Statistical Analyses

All data were analyzed using Sigma Plot 8.0 (Systat Software, San Jose CA). Tumor incidence was calculated by dividing the number of animals with tumors by the total number of animals in the experimental group. The average number of tumor nodules per tumor-bearing animal was calculated by dividing the sum of the tumor nodules in all tumor-bearing animals by the total number of tumor-bearing animals. The average number of tumor nodules per rat is expressed as the mean \pm the standard error. Average tumor size was calculated as the mean of the tumor sizes present in tumor-bearing animals and is expressed as mean \pm standard error. 8-isoPGF_{2 α} , SOD, GPx and CAT assays were all performed in duplicate using samples from at least 7 different animals in each of the treatment groups. The unpaired t-test analysis was used to calculate p values for comparisons of 8-iso-PGF_{2 α} levels, SOD, GPx and CAT activity between estrogen-treated rats and age-matched controls. P values < 0.05 were considered significant.

Results

Mammary tissue from female ACI rats displayed morphological changes following E₂ exposure

A number of morphological changes were observed in the mammary tissue of rats treated with E₂. Mammary tissue from 100% of rats exposed to E₂ for 7 days revealed hyperplastic lobular units that expanded into the stromal fat (data not shown). Progressive increases in lobular hyperplasia, elongation of ducts and compression of stromal fat were observed in the mammary tissue of all animals treated for 15 days with E₂ (Figure 1). In contrast, mammary tissue from 100% age-matched cholesterol-treated control rats showed normal tubulolobular architecture, consisting of branched ducts terminating in alveolar buds (Figure 1). The branched tubuloalveolar units are evenly distributed in the abundant fat pad of the mammary gland (Figure 1). While proliferative changes in mammary tissue following prolonged exposure to E₂ have been previously reported, early changes, such as those present after 7 or 15 days E₂ exposure, have not yet been reported in the literature. Further expansion of the hyperplastic lobules and significant reduction in stromal fat was observed in mammary tissue from 100% of rats treated with E₂ for 120 days (data not shown). In addition to hypertrophic and hyperplastic changes, E₂ treatment resulted in the appearance of malignant lesions. Cystic papillary carcinoma was observed in the mammary tissue from ~8% of rats after 120 days E₂ exposure (Figure 2). Mammary tissue from 100% of control rats remained unchanged after 120 days and was morphologically similar to tissue from animals in the 7- and 15-day control groups (data not shown). After 240 days treatment with E₂, mammary tissue from 100% of the rats in the E₂ treatment group showed advanced expansion of hyperplastic lobules, dilated ducts containing inspissated secretions and further compression of stromal fat (Figure 3). *In situ* carcinomas were present in mammary glands from ~80% of rats treated with E₂ for 240 days, and invasive carcinomas were present in mammary glands from ~40% of rats treated with E₂ for 240 days (Figure 3). No morphological changes were observed in the liver, kidney, lung or brain tissues of any of the E₂-treated rats compared to age-matched control rats (data not shown). Approximately 9% of E₂-treated animals displayed squamous metaplasia of the uteri (data not shown). No vasculo-lymphatic invasion, mammary lymph node involvement, or distant organ metastases (lung, liver) were identified in any of the tissues examined.

Female ACI rats exposed to E₂ developed mammary tumors

The incidence of mammary tumors in female ACI rats treated with E₂ reached 82% (9/11) following 240 days exposure. None of the animals in the control group developed tumors. The first palpable mammary tumor appeared in the E₂ group after 128 days exposure. The number of tumor nodules in tumor-bearing animals ranged from 1 to 6, with an average of 2.9 ± 1.4

nodules per tumor-bearing rat. The average tumor size in E₂-treated rats was 1086 ± 472 mm³.

8-isoPGF_{2α} levels were elevated as a function of E₂ exposure in female ACI rats

Following exposure to E₂ for 15, 120 or 240 days, mammary 8-isoPGF_{2α} levels were significantly increased over levels detected in age-matched control rats (Figure 4). 8-isoPGF_{2α} levels were 1.9-fold higher in rats exposed to E₂ for 15 days compared to age-matched control animals (p < 0.05). Similarly, 2.5 and 5-fold elevations in 8-iso-PGF_{2α} levels were observed in rats treated with E₂ for 120 and 240 days compared to age-matched controls, respectively (p < 0.05). In addition, 8-isoPGF_{2α} levels were increased 6-fold in tissue from E₂-induced mammary tumors relative to age-matched control mammary tissue (p < 0.05) (Figure 4). Increases in 8-isoPGF_{2α} levels as a function of E₂ exposure have not been previously reported in an animal model of estrogen-induced mammary cancer.

Activities of antioxidant enzymes SOD and GPx were altered in response to E₂ exposure

SOD activity levels in the mammary tissue of rats treated with E₂ for 7, 15 and 120 days were not significantly different from SOD activity levels found in age-matched controls (Figure 5). However, mammary tissue from rats treated with E₂ for 240 days, displayed a 3.1-fold increase in SOD activity relative to age-matched controls (p < 0.05) (Figure 5). Moreover, SOD activity was 1.8 times higher in mammary tumor tissue than in age-matched control mammary tissue (p < 0.05) (Figure 5). No differences in SOD activity levels were detected between E₂-treated and control rat livers (Table 1). GPx activity levels in the mammary tissue of ACI rats treated with E₂ for 7, 15, 120 and 240 days did not differ significantly from levels detected in age-matched control rats (Figure 6). In contrast, GPx activity in E₂-induced mammary tumors was elevated 2.5-fold over levels detected in age-matched control rats (Figure 6). GPx activity levels in the liver tissue of E₂-treated rats after 240 days was significantly higher than levels observed in control rats (Table 1). CAT activity levels in mammary tissue from rats treated with E₂ for 7, 15, 120 or 240 days were not significantly different from levels observed in age-matched control rats (data not shown). In E₂-induced mammary tumors, CAT activity was not significantly different from activity levels found in control mammary tissue.

Discussion

In the present study, we used the ACI rat model of breast cancer to examine the biochemical and histopathologic changes that occur in mammary tissue following estrogen exposure. Specifically, we set out to assess the contribution of oxidative stress to the pathogenesis of estrogen-induced breast tumorigenesis. We examined both proliferative changes and oxidative stress markers as a function of length of exposure to E₂ in order to discern the association between oxidative stress and exposure to estrogen. In addition, we quantified oxidative and proliferative changes during the pre-neoplastic and neoplastic phases of E₂ exposure. While proliferative changes in mammary tissue following prolonged exposure to E₂ have previously been reported, early changes, such as those that occur after 7 or 15 days of E₂ exposure, have not yet been reported in the literature. The findings of the present study describe the progression from normal mammary tissue to mammary cancer in the ACI rat model. These data suggest a progression from normal mammary tissue to proliferative tissue, such as atypical ductal hyperplasia, later progressing to tumor formation and malignancy. The initial proliferative stage was observed following 7 to 15 days of E₂ exposure. The proliferative process became atypical after 120 days of E₂ exposure, and the first palpable tumor appeared after 128 days of E₂ treatment.

Levels of 8-isoPGF_{2α} and activities of antioxidant enzymes including SOD, CAT and GPx were analyzed in the mammary tissue of E₂-treated and control ACI rats in order to evaluate

oxidative stress. Oxidative stress is characterized by an imbalance between ROS and antioxidant defense systems that control the production of free radicals and ROS (Cracowski *et al.*, 2002). Oxidative stress and subsequent oxidative damage to biomolecules, such as DNA, protein and lipids, has been identified as a salient feature of numerous acute and chronic diseases, including cancer (Cracowski *et al.*, 2002; Bhat *et al.*, 2003; Patel and Bhat, 2004; Milne *et al.*, 2008). 8-isoPGF_{2α}, an isoprostane produced from arachidonic acid during lipid peroxidation, was used as a marker of oxidative stress in ACI rat tissue. Quantification of 8-isoPGF_{2α} is recognized as a reliable method of assessing oxidative stress and oxidative damage *in vivo* (Montuschi *et al.*, 2004; Milne *et al.*, 2007). Free radical-induced peroxidation of membrane lipids can disturb the physical properties of the lipid membrane, and thus interferes with normal cellular functioning (Montuschi *et al.*, 2004; Milne *et al.*, 2008). The observation that 8-isoPGF_{2α} levels increased in a time-dependent manner following E₂ exposure suggests that E₂ induces oxidative stress, in particular lipid peroxidation, prior to tumor development. After just 15 days treatment with E₂, 8-isoPGF_{2α} levels were elevated nearly 2-fold over levels observed in age-matched controls, indicating that lipid peroxidation represents an early event following E₂ exposure. By 240 days, 8-isoPGF_{2α} levels in E₂-exposed mammary tissues were substantially increased, reaching levels 5-times higher than those observed in control mammary tissue (Figure 4). Moreover, E₂-induced mammary tumors displayed significant elevations in 8-isoPGF_{2α} levels relative to control mammary tissue. These changes are indicative of lipid peroxidation and possible cellular damage in the mammary gland as a result of E₂ exposure. Elevations in 8-isoPGF_{2α} appeared to be confined to E₂-target tissues, as no changes in this marker were detected in the liver tissue of E₂-treated rats relative to controls (data not shown).

Antioxidant enzymes represent an important defense mechanism against oxidative stress. SOD, GPx and CAT activities were assessed in E₂-exposed and control ACI rats as a means of further assessing oxidative stress and the response to E₂ exposure. SOD catalyzes the dismutation of superoxide to generate hydrogen peroxide, while CAT is responsible for the decomposition of hydrogen peroxide to oxygen and water. Similarly, GPx eliminates hydrogen peroxide and organic peroxides by way of reduced glutathione. The relationship between SOD and the process of carcinogenesis remains uncertain. Studies have reported that MnSOD protein levels increase with tumor grade and other investigations have correlated increased MnSOD activity with de-differentiation (Tsanou *et al.*, 2004; Kumaraguruparan *et al.*, 2005; Kattan *et al.*, 2008). Similarly, Western blotting of human breast cancer tissues revealed that MnSOD expression was 1.5-fold higher in cancer tissue compared to adjacent normal tissue (Er *et al.*, 2004). *In vitro* experiments have shown increased MnSOD activity in non-aggressive and aggressive breast cancer cells relative to non-tumorigenic mammary epithelial cells (Mukhopadhyay *et al.*, 2004). In contrast, MnSOD over-expression has been associated with decreased cell growth and survival *in vitro* and with decreased tumor growth in xenograft models of breast cancer (Weydert *et al.*, 2006). Using the ACI rat model of breast cancer, we have shown that SOD activity was significantly increased in mammary tissue from rats exposed to E₂ for 240 days as well as in mammary tumor tissue (Figure 5). This finding implies a compensatory mechanism in response to oxidative stress that may represent a step in the process of tumorigenesis. Moreover, up-regulation of antioxidant enzymes may confer a selective advantage for the growth of tumor cells compared to corresponding normal cells.

Further supporting a role for oxidative stress in the pathogenesis of E₂-induced mammary cancer, we detected a significant increase in GPx activity in the mammary tumor tissue of E₂-exposed rats (Figure 6). While GPx activity in non-tumor mammary tissue was not significantly impacted by E₂ exposure at any of the time points tested, our data suggest the possibility of an early increase in GPx activity after 7 days treatment, followed by a leveling off of GPx activity after prolonged E₂ exposure (Figure 6). Increases in SOD activity without parallel increases in GPx and CAT activity may result in elevated hydrogen peroxide production without sufficient detoxifying enzymes, thus magnifying the burden of oxidative stress in the cell.

Previous reports reveal higher GPx activity in human breast tumors compared to adjacent normal tissue, and microarray studies have shown that GPx2 is commonly over-expressed in mammary carcinomas. *In vitro* experiments have found that suppressing GPx2 is associated with growth inhibition in rat and human mammary cancer cell lines (di Ilio *et al.*, 1985; Singh *et al.*, 1990; Gromadzinska *et al.*, 1997; Iscan *et al.*, 1998; Naiki-Ito *et al.*, 2007). However, the level of GPx in tumor tissue does not seem to be altered in all cases (Singh *et al.*, 1990). The lack of elevation in GPx activity perhaps reflects that this anti-oxidant pathway is only triggered late in the process of carcinogenesis. Hence, at early time-points (i.e. during proliferation and atypical ductal proliferation) the GPx anti-oxidant mechanism is unaffected.

Taken together, our data indicate that oxidative stress plays an important role in the development of estrogen-induced mammary cancer. Increases in lipid peroxidation and up-regulation of antioxidant enzymes precede tumor development and occur in a manner dependent on duration of exposure to E₂.

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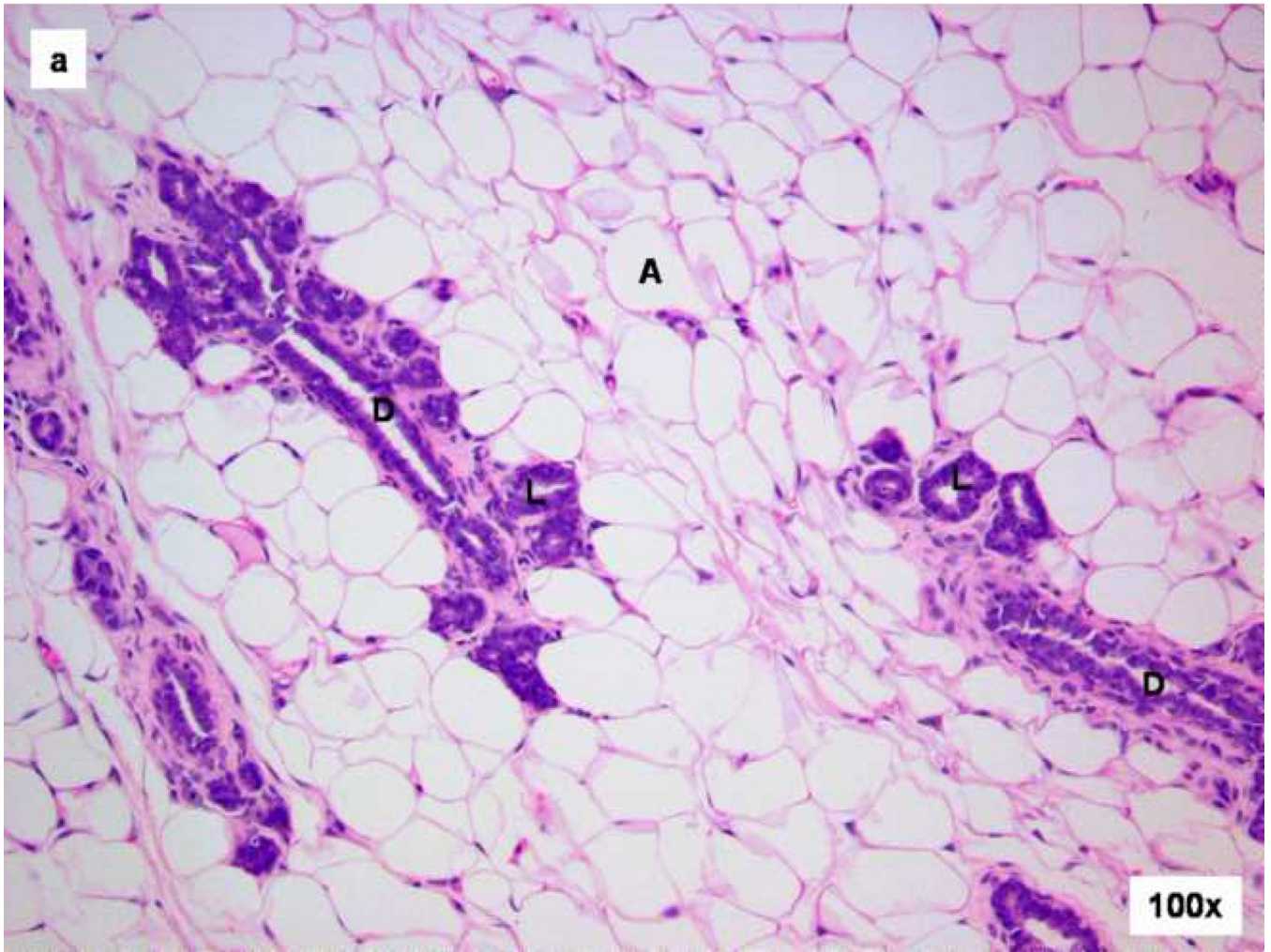
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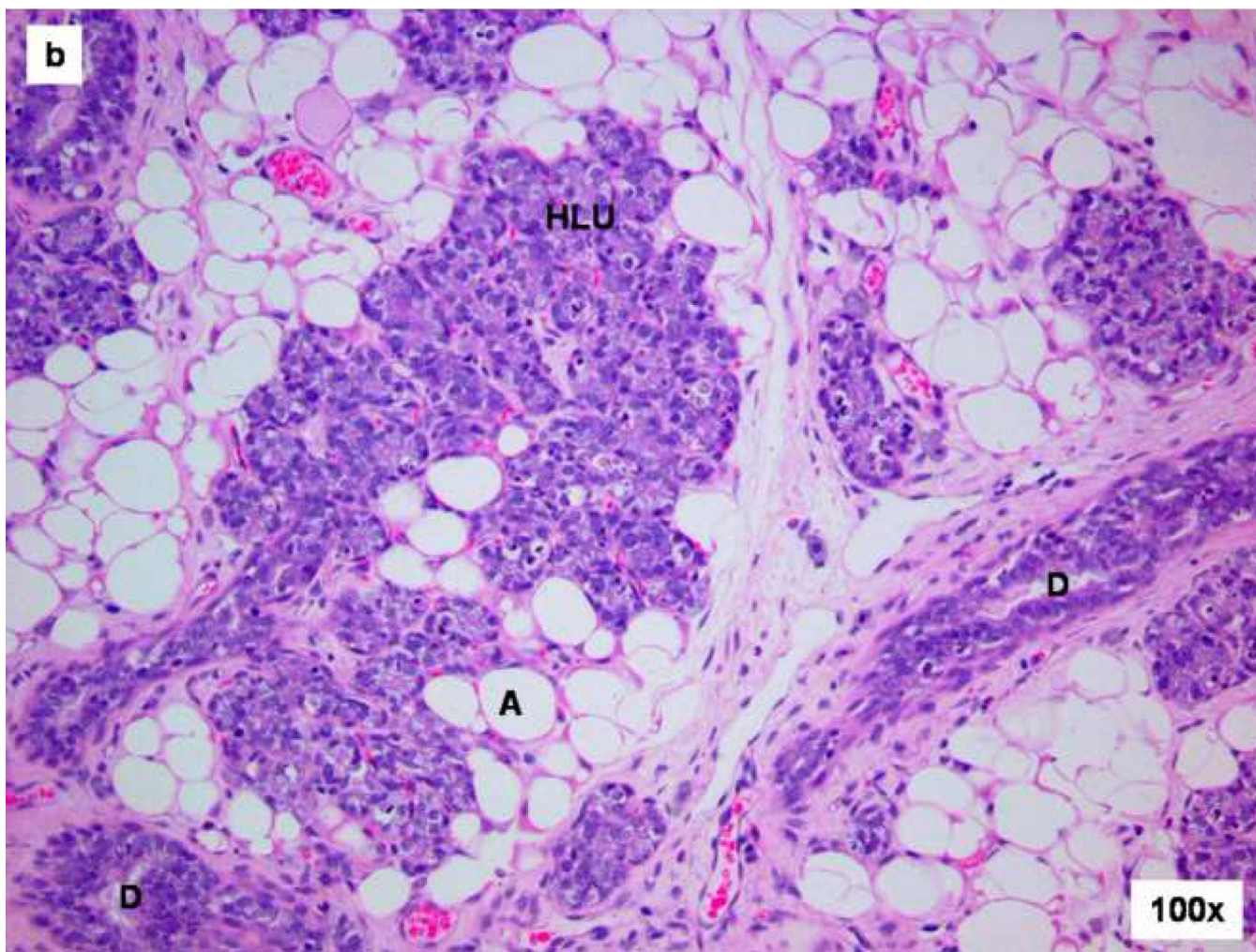


Figure 1. Comparison of control and E₂-treated mammary gland after 15 days

Female ACI rats were treated with an E₂ pellet (s.c., 3 mg E₂ + 17 mg cholesterol) for 15 days. Control rats were implanted with a pellet containing 17 mg cholesterol only. Histopathologic analyses were performed on mammary tissue from at least 4 control and 4 E₂-treated animals. This figure shows mammary tissue representative of the morphology generally observed in control and E₂-treated animals. a) The mammary gland of a control ACI rat shows normal tubulolobular architecture (L), consisting of branched ducts (D) terminating in alveolar buds. The branched tubuloalveolar units are more or less evenly distributed in the abundant fat pad (A) of the mammary gland. b) The mammary glands of a rat treated with E₂ for 15 days showed proliferative changes characterized by a progressive time-dependent elongation of ducts (D) as well as proliferation and expansion of the terminal lobular units (HLU) with compression of the surrounding fat pad (A).

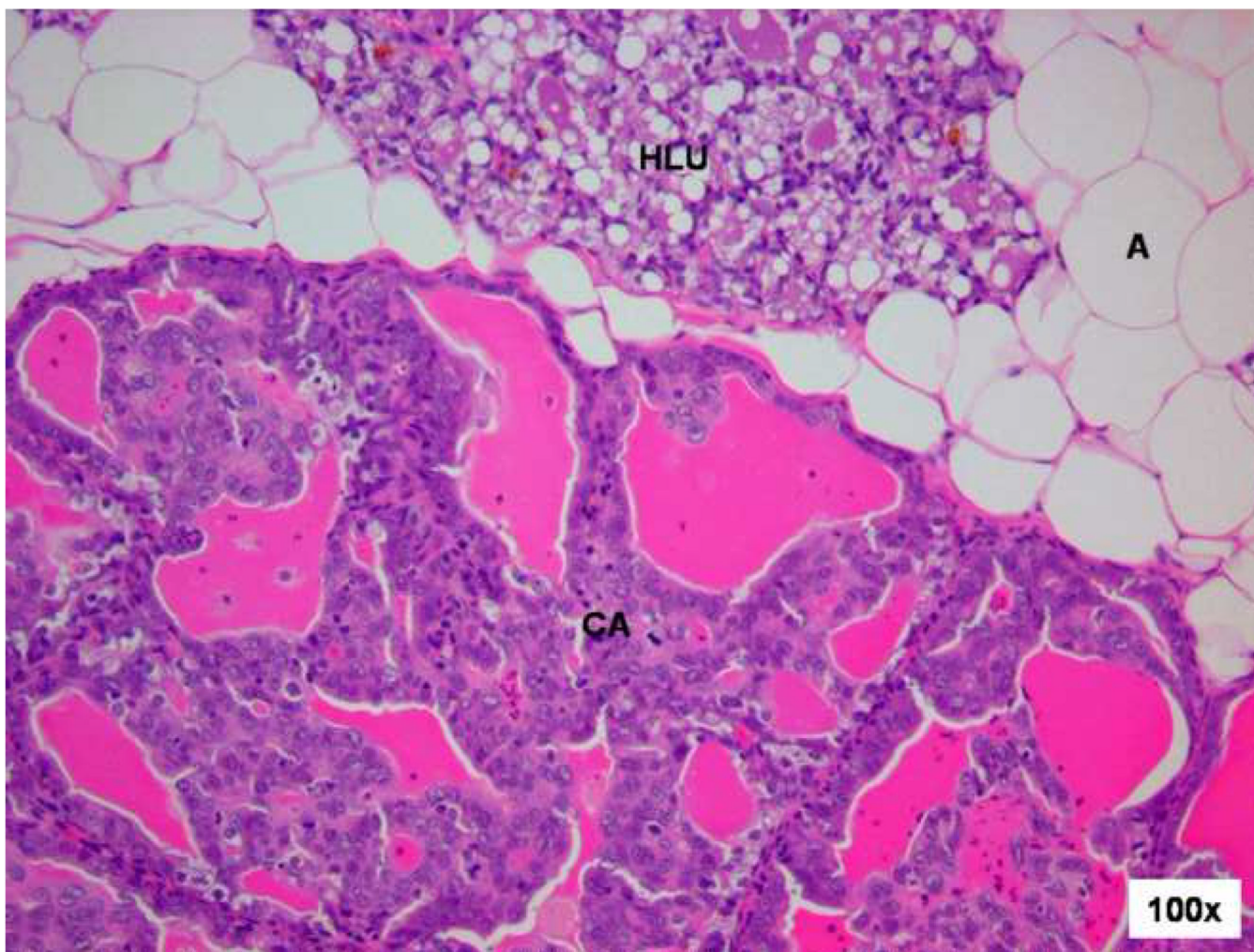
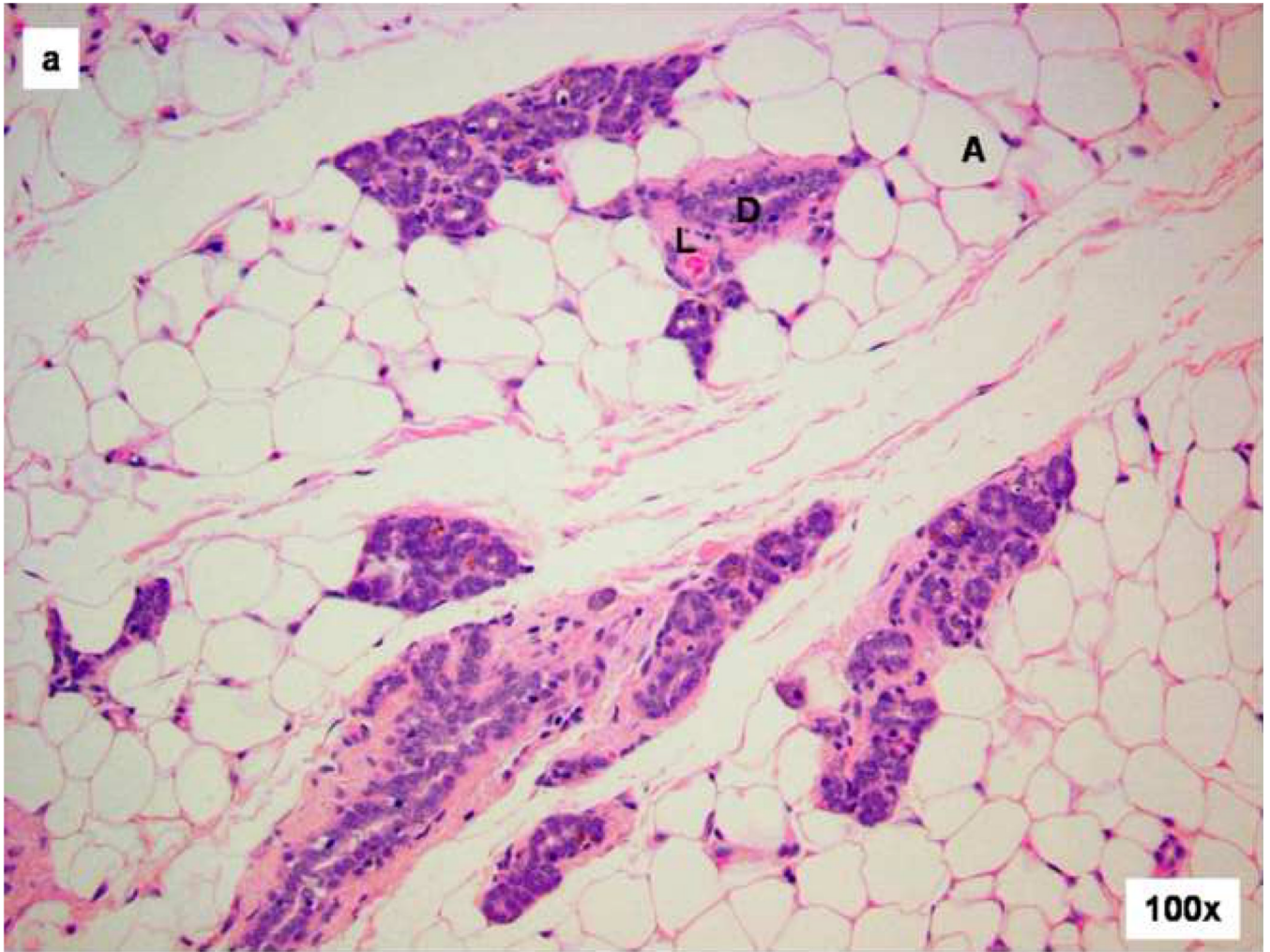
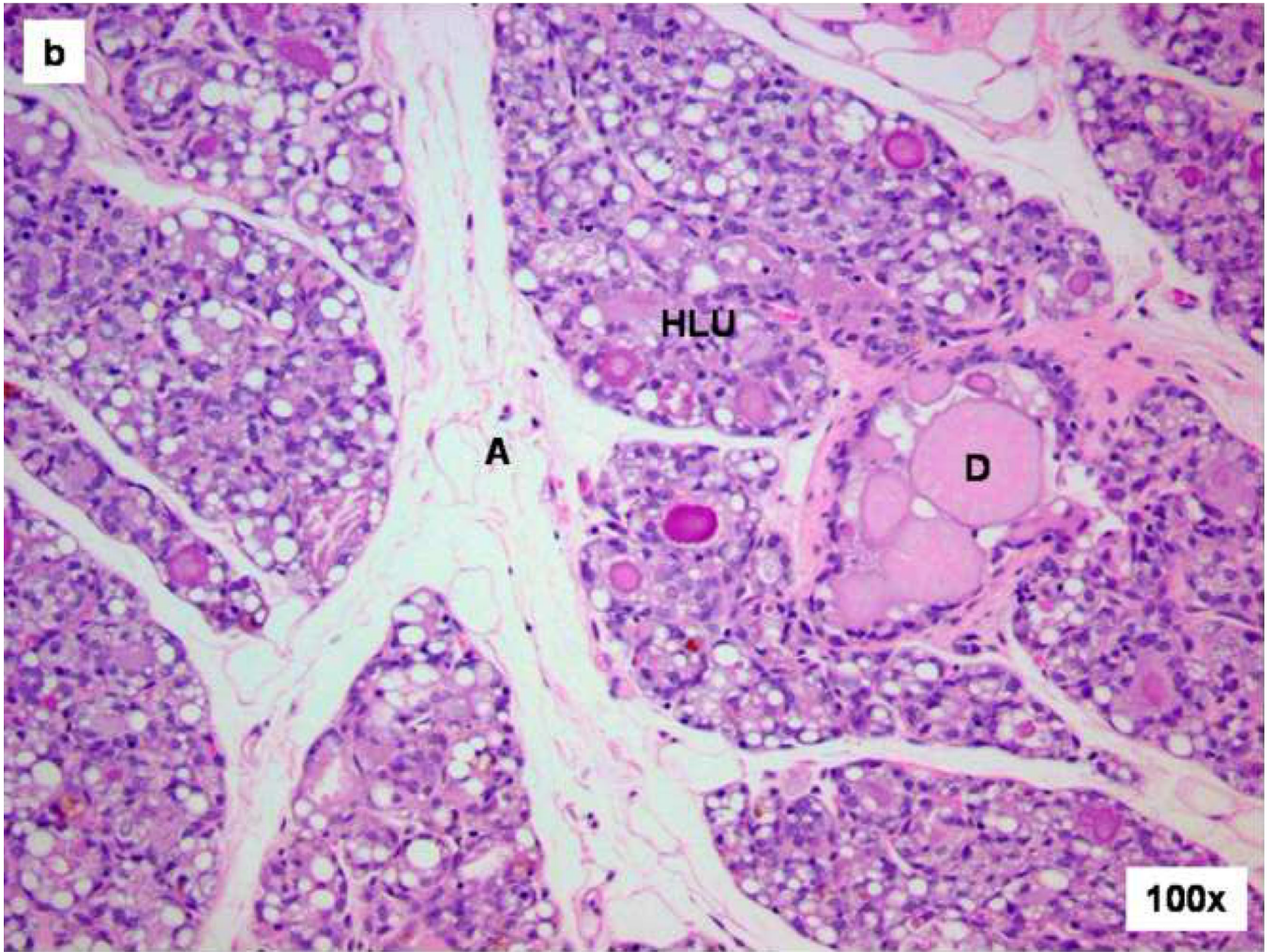


Figure 2. Cystic papillary carcinoma in E₂-treated mammary gland after 120 days treatment
Female ACI rats were treated with an E₂ pellet (s.c., 3 mg E₂ + 17 mg cholesterol) for 120 days. The ductal structures are markedly dilated (compare with uninvolved hyperplastic lobular unit (HLU) at the top of image). The neoplastic epithelium grows into the lumen forming papillae and rigid arches devoid of fibrovascular cores. The neoplastic cells are enlarged and generally uniform in size. In addition to the hypertrophic and hyperplastic changes, E₂ treatment results in the appearance of malignant lesions. The mammary tissue of one animal showed cystic papillary carcinoma (CA) following 120 days of E₂ treatment.





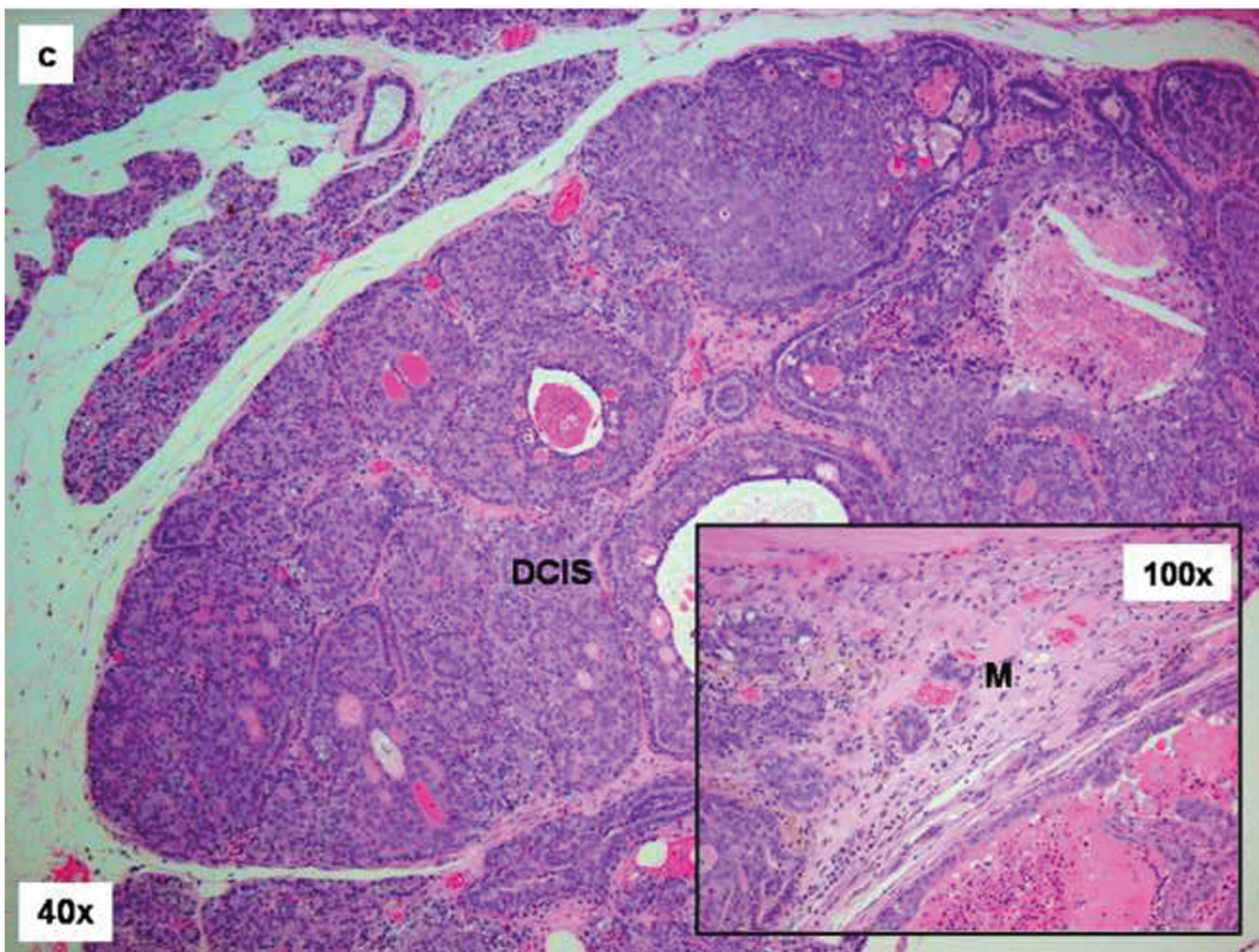


Figure 3. Comparison of control and E₂-treated mammary gland after 240 days

Female ACI rats were treated with an E₂ pellet (s.c., 3 mg E₂ + 17 mg cholesterol) for 240 days. Control rats were implanted with a pellet containing 17 mg cholesterol only. Histopathologic analyses were performed on mammary tissue from all control and E₂-treated animals. This figure shows mammary tissue representative of the morphology generally observed in control and E₂-treated animals. a) The mammary gland of control ACI rats shows normal lobular architecture (L) with branched ducts (D) and normal distribution of fat tissue (A). The control mammary tissue at 240 days is unchanged relative to earlier time-points. b) The E₂-stimulated mammary gland shows increased proliferation with dilated ducts containing inspissated secretions (D) and increased proliferation and expansion of terminal lobular units (HLU) accompanied by compression of and expansion into the surrounding fat tissue (A). c) Histomorphologic analysis revealed carcinomas, both *in situ* (DCIS) and micro-invasive, measuring 10 or more mm in the largest dimension. The carcinomas showed a mixed morphology, with solid patterns with or without comedo-necrosis predominating, but cribriform and cystic-papillary patterns were also present. (40 × image) When present, the invasion was limited to foci of micro-invasion. 100x Inset) Microinvasive foci (M) were characterized by breakdown of the basal membrane of the *in situ* lesion by a proliferation of atypical small glands which invaded the surrounding stroma surrounded by a prominent desmoplastic reaction.

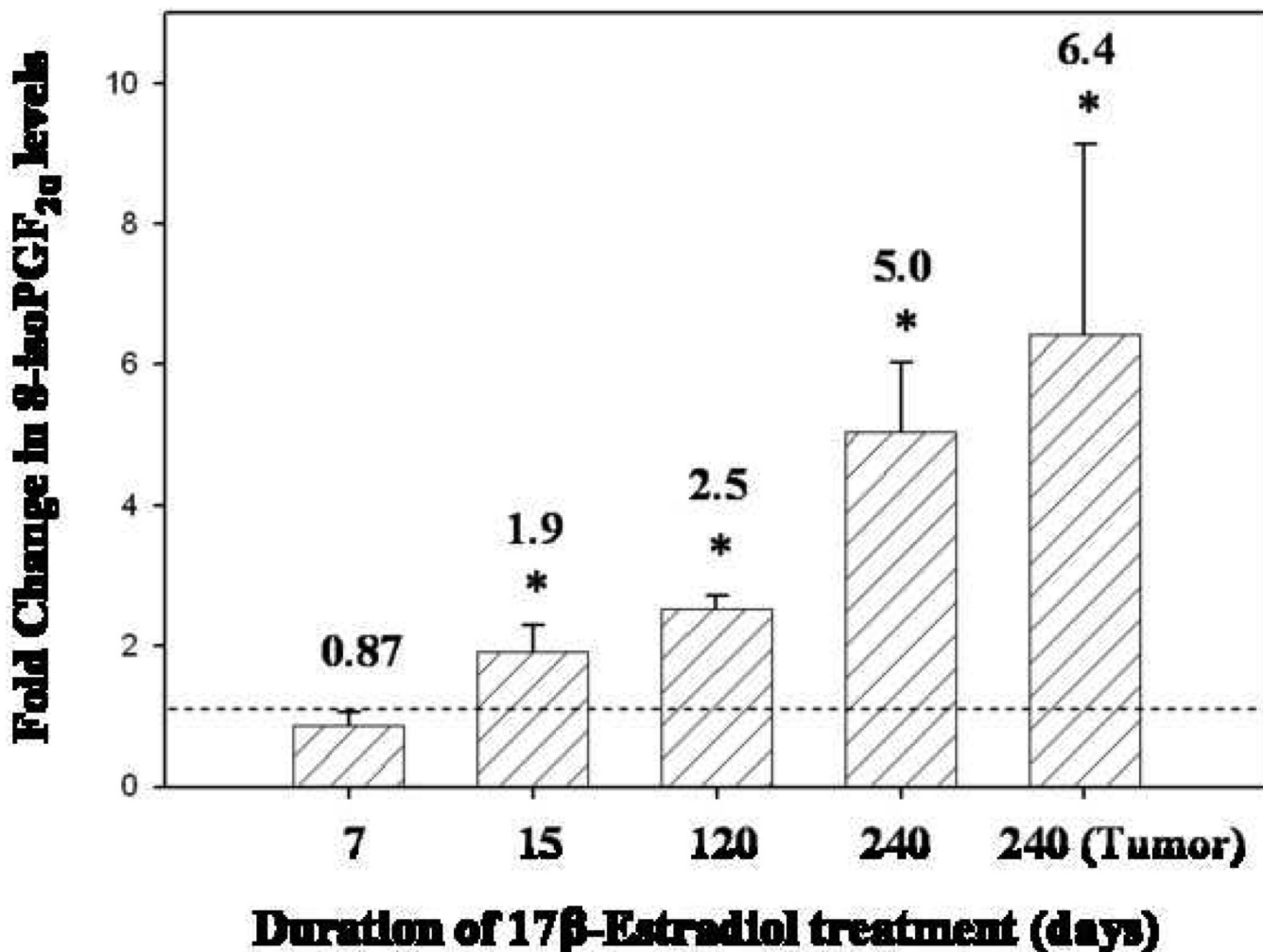


Figure 4. 8-isoPGF_{2α} levels in ACI rat mammary tissue are increased in a time-dependent fashion after E₂ exposure

Female ACI rats were treated with E₂ as described in the methods section. 8-isoPGF_{2α} levels were measured in mammary tissue from control and E₂-treated rats from the 7, 15, 120 and 240 day treatment groups. 8-isoPGF_{2α} levels were assessed in mammary tumor tissue as well. Fold changes were calculated for E₂-treated animals relative to age-matched controls. Fold change data are reported as an average of values obtained for at least 7 different animals ± SE. The absolute values of 8-isoPGF_{2α} (pg 8-isoPGF_{2α} per mg protein ± SE) in mammary tissue from control rats in the 7, 15, 120 and 240 day groups were 1590 ± 300, 511 ± 87, 1320 ± 370 and 492 ± 100, respectively. * indicates a p value <0.05 compared to respective age-matched controls.

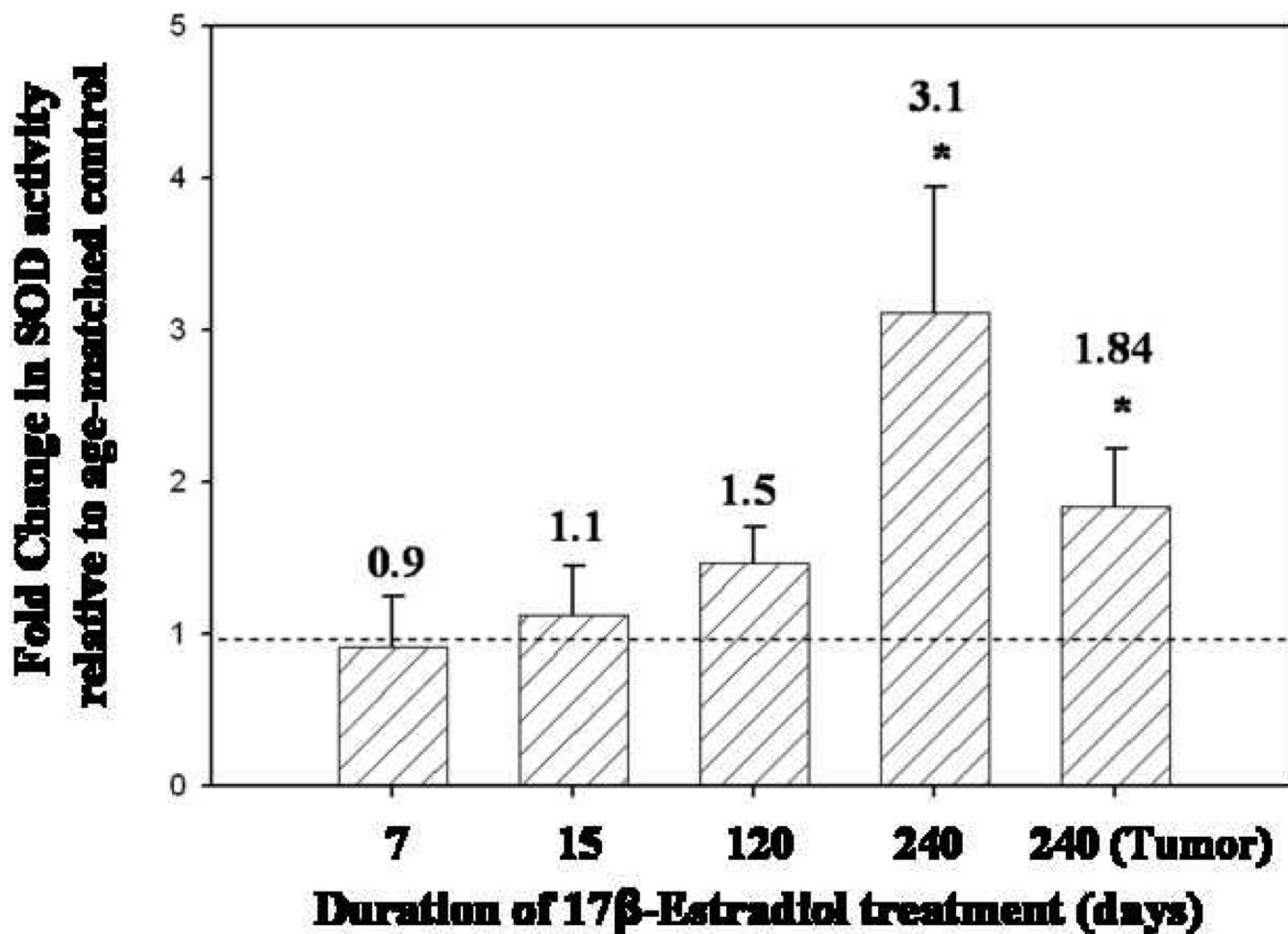


Figure 5. SOD activity is elevated in the mammary tissue of E₂-treated rats compared to age-matched controls

Female ACI rats were treated with E₂ as described in the methods section. The activity of the antioxidant enzyme SOD was measured in mammary tissue from control and E₂-treated rats from 7, 15, 120 and 240 day treatment groups. SOD activity was assessed in mammary tumor tissue as well. Fold changes were calculated for E₂-treated animals relative to age-matched controls. Fold change data are reported as an average of values obtained for at least 7 different animals ± SE. The absolute values of SOD activity (units per mg protein ± SE) in mammary tissue from control rats in the 7, 15, 120 and 240 day groups were 571 ± 220, 990 ± 240, 969 ± 150 and 561 ± 70, respectively. * indicates a p value < 0.05 compared to respective age-matched controls.

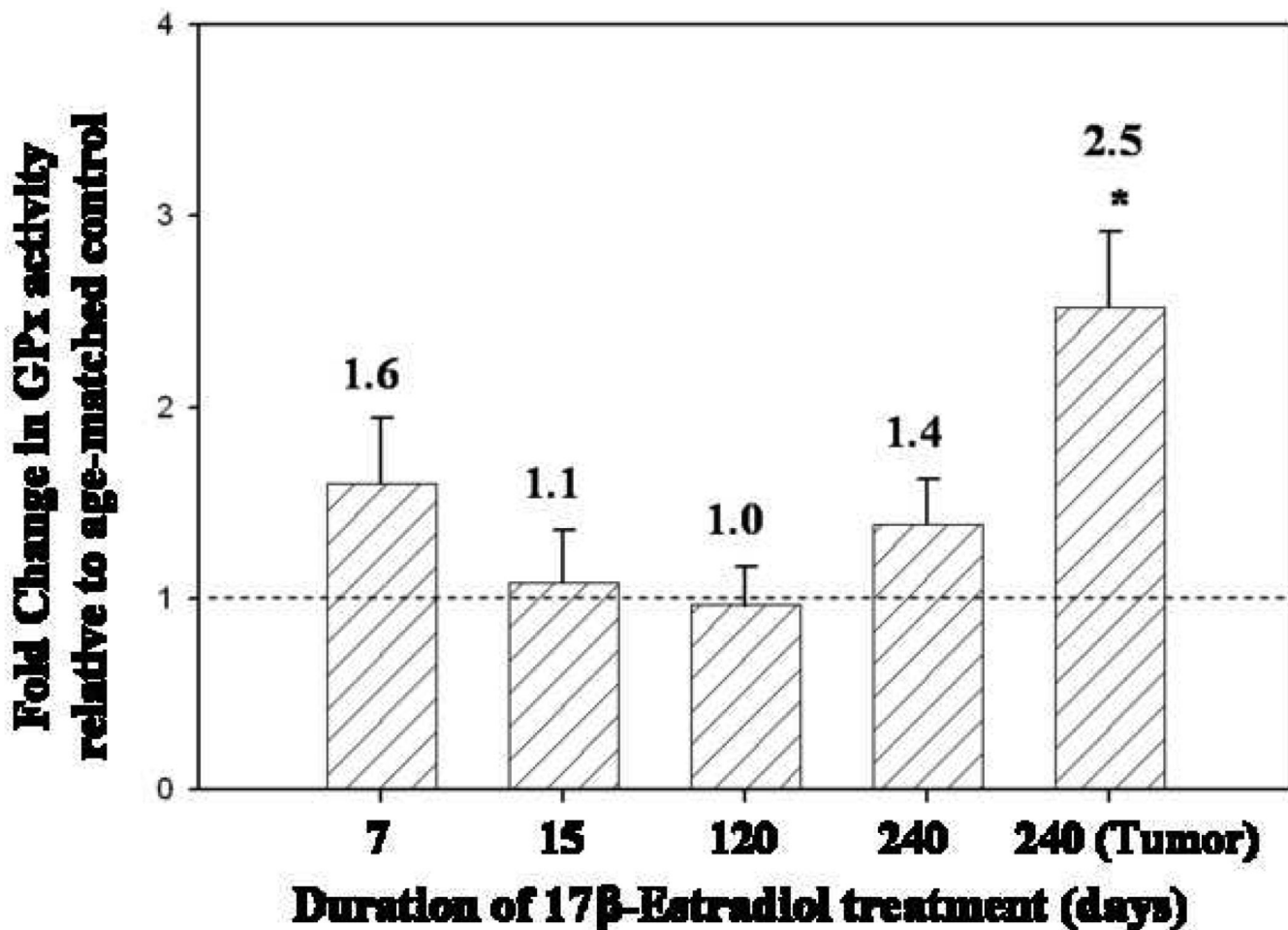


Figure 6. GPx activity levels are increased in mammary tumors compared to adjacent normal mammary tissue

Female ACI rats were treated with E_2 as described in the methods section. The activity of the antioxidant enzyme GPx was measured in mammary tissue from control and E_2 -treated rats from 7, 15, 120 and 240 day treatment groups. GPx activity was assessed in mammary tumor tissue as well. Fold changes were calculated for E_2 -treated animals relative to age-matched controls. Fold change data are reported as an average of values obtained for at least 7 different animals \pm SE. The absolute values of GPx activity (nmol per minute per mg protein \pm SE) in mammary tissue from control rats in the 7, 15, 120 and 240 day groups were 49 ± 9 , 65 ± 10 , 94 ± 10 and 92 ± 9 , respectively. * indicates a p value < 0.05 compared to respective age-matched controls.

Table 1

Activities of antioxidant enzymes in 240 day old female ACI rats

Tissue	SOD (U/mg protein)	CAT (nmol/min/mg protein)	GPx (nmol/min/mg protein)
Control Mammary	561 ± 70	88 ± 9	92 ± 9
E2-Treated Mammary	1747 ± 460*	104 ± 12	128 ± 22
Mammary Tumor	1031 ± 211*	65 ± 5	232 ± 37*
Control Liver	169 ± 11	1290 ± 170	601 ± 88
E2-Treated Liver	152 ± 13	1270 ± 350	1170 ± 81*

Female ACI rats were treated with an E₂ pellet (s.c., 3 mg E₂ + 17 mg cholesterol) for 240 days. Control rats were implanted with a pellet containing 17 mg cholesterol only. The activities of the antioxidant enzymes SOD, GPx and CAT were measured in mammary and liver tissue from control and E₂-treated rats from the 240 day treatment group. Enzyme activity was assessed in mammary tumor tissue as well. The data are reported as an average of values obtained for at least 7 different animals ± SE.

* Indicates a p value <0.05 compared to control mammary or liver compared to respective age-matched controls.