

Selenium Concentration and Glutathione Peroxidase Activity in Normal and Neoplastic Development of the Mouse Mammary Gland

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ABSTRACT

This study was divided into three experiments. (a) The levels of selenium and glutathione peroxidase (GSH-PX) activity were determined for various developmental stages of the mammary gland: normal (virgin, pregnant, and lactating); preneoplastic hyperplastic outgrowth lines; and neoplastic mammary tissues. Tissues from the pregnant and lactating mice had higher selenium concentrations and GSH-PX activities than did tissues from virgin mice, but the levels were similar to levels found in preneoplastic and neoplastic mammary tissues of lines C4 and D2. This suggests that the appropriate comparisons for studies determining the effect of selenium on preneoplastic mammary tissues are glands from pregnant and lactating mice. In addition, these results suggest that the refractory state of mammary tumors to selenium-mediated growth inhibition is not due to the absence of GSH-PX or the inability to incorporate selenium. (b) For the second experiment, the effect of feeding various levels of selenium on selenium and GSH-PX levels was determined in mammary and hepatic tissues of virgin mice. GSH-PX activity increased when dietary selenium increased from less than 0.02 to 0.1 ppm selenium but was not further increased when dietary selenium was above 0.1 ppm. (c) Finally, the mammary GSH-PX activity was evaluated at different concentrations of the substrates in order to document the biochemical characteristics of the mammary gland GSH-PX.

INTRODUCTION

The addition of inorganic selenium to drinking water or feed at 2 to 4 ppm inhibits virus- and chemical carcinogen-induced mammary tumorigenesis in mice (18, 19, 28) and rats (5, 8, 37). Selenium inhibits both the initiating and promoting stages of tumorigenesis in mice (18, 19, 28) and rats (5, 8, 37). Two to 4 ppm inhibits virus- and chemical carcinogen-induced mammary tumorigenesis in mice (18, 19, 28) and rats (5, 8, 37). Interestingly, inhibition of mammary tumorigenesis requires the continuous presence of selenium since, upon withdrawal of the selenium supplement, mammary tumors reappear at the same rate as tumors in the control mice (29) and rats (7). Several results suggest a decreasing sensitivity to selenium-mediated inhibition as cells progress from normal to preneoplastic to neoplastic (16, 19). For example, the induction and tumor-producing capabilities of preneoplastic mammary populations are inhibited by selenium supplementation to the diet. For preneoplastic mammary populations, 3 of 8 outgrowth lines tested were responsive to selenium-mediated inhibition; i.e., preneoplastic line C4, but not line D2, was inhibited by 4 ppm selenium in the drinking water (16, 18). In contrast, the growth of established mammary tumors was not inhibited by selenium supplementation (19, 28). However, Milner et al. (4, 20, 36) have shown that selenium administered either i.p. or s.c. can inhibit the growth of some transplantable tumors, i.e., Ehrlich ascites, L1210 leukemia, and a canine mammary tumor line.

One postulated mechanism for the effect of selenium is through the action of the antioxidant selenoenzyme, GSH-PX. GSH-PX is the only known function for selenium in mammalian tissues, and the activity of GSH-PX increases with increasing levels of dietary selenium for many tissues in many species of animals (3). Selenium may have other functions, and other selenoproteins probably exist (23, 26), but at present, the GSH-PX function is best characterized. However, very little is known about mammary selenium levels or GSH-PX activity. Furthermore, it appears that some animal species and tissues have another enzyme, glutathione S-transferase (also called non-selenium-dependent GSH-PX), that has a similar function in detoxification of peroxides but is not responsive to dietary selenium (11, 12, 25, 27). The purpose of this study was to characterize mouse mammary GSH-PX. The levels of GSH-PX and selenium in normal mammary tissues at different developmental stages (virgin, pregnant, and lactating) were compared first to the levels found in preneoplastic hyperplastic outgrowth lines and neoplastic mammary tissues. The results from this first study led to a more extensive study of virgin mammary tissue where the objectives were to determine the optimal method for measuring mammary gland GSH-PX and to determine if mammary GSH-PX activity was responsive to dietary levels of selenium. The purpose of these latter experiments was to establish that mouse mammary GSH-PX is a selenium-dependent enzyme.

MATERIALS AND METHODS

The study design was divided into 3 experiments: a comparative study (Experiment 1); a dietary study (Experiment 2); and a kinetic study (Experiment 3).

Mice. Female BALB/cMed mice were bred and maintained in a closed conventional mouse colony in the Department of Cell Biology, Baylor College of Medicine. BALB/cMed mice are free of an expressible mature mouse mammary tumor virus and have a low mammary tumor incidence in virgin and breeding female mice (24). Mice were housed 4 to 6 to a cage in temperature- and light cycle-controlled rooms.

Experiment 1. In Experiment 1, the mice were fed Wayne Lab Blox ad libitum. The level of selenium in the feed was 0.15 ppm (information supplied by Allied Mills, Inc., Chicago, Ill.) and in the water supply was below the limits of detection by fluorometric assay.

Mammary tissues were excised from female mice of various ages; rinsed in ice-cold, calcium- and magnesium-free, phosphate-buffered saline [NaCl (8.0 g/liter)-KCl (0.2 g/liter)-Na2HPO4 (2.0 g/liter)-KH2PO4 (0.2 g/liter)]; blotted dry; pooled, and frozen at -20°C until assayed.

* The abbreviation used is: GSH-PX, glutathione peroxidase.

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Mammary tissues from the following groups were assayed for GSH-PX activity and selenium content: 12-week-old virgin female mice; 12-week-old virgin female mice whose epithelial glands had been removed at 3 weeks of age to leave just the mammary fat pad (inguinal glands only); 17-day-pregnant (primiparous) mice; 1- to 5-day lactating (primiparous) mice; inguinal mammary glands from 12-week-old mice bearing nontumor outgrowth lines D2 and C4; and tumors from 12-week-old mice bearing D2 and C4 tumors transplanted s.c. The nontumor outgrowths and tumor tissues were transplanted as described previously (15). Ten-week-old female mice were used for timed pregnancies to provide the 17-day-pregnant and early lactating mice.

**Experiment 2.** In Experiment 2, mice were fed a semipurified diet consisting of 20% casein, 15% cornstarch, and 50% sucrose with fiber, AIN vitamin mix, dl-methionine, and choline bitartrate (2). The AIN mineral mix was prepared, but no selenium was added. The mineral mix was added to the diet. The diet was prepared in our laboratory in one batch and frozen until use. Forty female mice, 4 weeks old, were divided into 4 equal groups with 10 mice per group and fed diets with 4 different levels of added selenium as sodium selenite (0.0, 0.02, 0.1, and 0.5 ppm). The basal level of selenium in the unsupplemented diet was 0.03 μg selenium per g as measured by the fluorometric assay (21, 35). The total selenium content was measured in all diets. The body weight and food intake were measured weekly. At the end of 30 days, the total mammary glands and liver were collected from each mouse. The mammary glands from 2.5 mice were pooled to form one sample; thus, 4 samples per group were assayed for GSH-PX activity and selenium concentration. The liver was rinsed twice in cold 0.9% NaCl solution and blotted dry. All tissues were frozen under nitrogen at −20°C.

**Experiment 3.** The enzyme kinetics of GSH-PX was evaluated using 2 substrates, glutathione and peroxide, under varying conditions. GSH-PX activity was evaluated at 0.02 and 0.10 mM H2O2 and between 0.1 and 4.0 mM glutathione. The breast tissue was taken from virgin mice fed the semipurified diet, 0.5 ppm selenium. Plots of GSH-PX activity with the various levels of hydrogen peroxide and glutathione concentrations were completed in order to estimate the apparent Km for glutathione (3, 25, 34).

**GSH-PX Analysis (EC 1.11.1.9).** The tissue was removed, rinsed twice in cold 0.9% NaCl solution, blotted dry to remove blood, and then homogenized in 0.02 m phosphate buffer, pH 7.0, with a Model SDT Tissue Homogenizer (17). The homogenate was spun at 1,000 × g for 10 min to remove cellular debris. The supernatant was spun at 15,000 × g in a high-speed centrifuge for 20 min and analyzed for GSH-PX and protein. A fraction of the supernatant was removed and diluted with 0.02 m potassium phosphate buffer (pH 7.0) for GSH-PX activity determination. The GSH-PX activity was measured by coupling of peroxide with NADPH via glutathione reductase (17, 22), and the change in absorbance was measured by a spectrophotometer. Selenium-dependent GSH-PX activity was determined using hydrogen peroxide, whereas total GSH-PX activity (selenium-dependent GSH-PX and glutathione S-transferase) was determined using cumene hydroperoxide (11). Enzyme activity was expressed as μmol NADPH2 oxidized per g protein. For the kinetic experiment, glutathione and peroxide concentrations were varied. Using the couple assay, a very high level of glutathione may drive reaction alone. In order to avoid this problem, care was taken to make sure there was no change in absorbance before peroxide was added. Only the linear change in absorbance of NADPH2 was used to calculate the GSH-PX activity. The blank values were calculated from change in absorbance when the incubation medium contained either no supernatant or peroxidase. Protein Levels. Protein levels were measured by the method of Lowry et al. (14).

**Selenium Concentration.** Selenium levels were determined in the following manner (9, 21, 35). After a wet digestion with nitric, perchloric, and hydrochloric acids, the digestate was heated to 60°C with 2,3'-diaminonaphthalene and extracted with cyclohexane. The fluorescence of each extraction was measured at 256 nm excitation and 606 nm emission with a spectrofluorometer. The mean value for the National Bureau of Standards bovine standard (selenium = 1.1 μg/g) was 1.0 ± 0.02 μg selenium per g.

**Statistical Methods.** The data in Experiment 2 (selenium, GSH-PX, and weight gain) were analyzed statistically using analysis of variance.

**RESULTS**

**GSH-PX and Selenium Concentration in Normal and Neoplastic Mammary Tissues (Experiment 1).** The mean selenium concentration in mammary tissues of virgin mice consuming laboratory chow containing over 0.1 ppm selenium was 0.04 μg selenium per g wet weight (Table 1). Pregnant mice consuming the same diet had a mean of 0.3 ± 0.06 μg selenium per g, while the lactating mice had mean levels from 0.11 to 0.17 μg selenium per g. Primary tumors from nontumor lines C4 and D2 had selenium levels (0.47 to 0.53 μg selenium per g) that were 10 times greater than virgin tissues. The nontumor hyperplastic outgrowth lines (D2 and C4) had selenium levels (0.3 and 0.1 μg selenium per g, respectively) similar to levels found in breast tissue of pregnant and lactating mice. Mammary fat pads from which the glandular epithelial component had been removed from 3-week-old virgin mice (14) had a selenium concentration of less than 0.05 μg selenium per g of wet weight.

The virgin mice had lower GSH-PX activity (46 units/g protein) than did pregnant (83 units/g protein) and lactating (143 to 153 units/g protein) mice. The D2 and C4 hyperplastic outgrowth lines and D2 and C4 tumor tissues had mean GSH-PX activities in the same range as the levels found in pregnant and lactating tissues (Table 1).

**Table 1.** Selenium concentration and GSH-PX activity in normal, nontumor outgrowth, and neoplastic mammary tissues of BALB/c mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>GSH-PX activity (μmol/g protein)</th>
<th>Selenium Concentration (μg/g, wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin</td>
<td>2</td>
<td>46.0 ± 0.02</td>
</tr>
<tr>
<td>Pregnant</td>
<td>18</td>
<td>83.5 ± 11.0</td>
</tr>
<tr>
<td>Lactation</td>
<td>4</td>
<td>152.6 ± 32.0</td>
</tr>
<tr>
<td>(1-2 days)</td>
<td>4</td>
<td>105.6 ± 23.0</td>
</tr>
<tr>
<td>Lactation</td>
<td>5</td>
<td>143.8 ± 35.5</td>
</tr>
<tr>
<td>(3-5 days)</td>
<td>5</td>
<td>160.3 ± 30.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Selenium Concentration (μg/g, wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin</td>
<td>0.0414</td>
</tr>
<tr>
<td>Pregnant</td>
<td>0.323 ± 0.055</td>
</tr>
<tr>
<td>Lactation</td>
<td>0.109 ± 0.026</td>
</tr>
<tr>
<td>(1-2 days)</td>
<td>0.165 ± 0.043</td>
</tr>
<tr>
<td>Lactation</td>
<td>0.320 ± 0.171</td>
</tr>
<tr>
<td>(3-5 days)</td>
<td>0.131 ± 0.022</td>
</tr>
</tbody>
</table>

The two different pools of mammary gland taken from 5 virgin mice, each of which was 10 to 12 weeks old.

Mean ± S.E.

HOG, nontumor hyperplastic outgrowth line.
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Chart 1. The effect of various levels of dietary selenium on selenium-dependent and total GSH-PX activity and selenium concentration in mammary tissues. Units, \( \mu \text{mol NADPH oxidized per min} \). Increased levels of dietary selenium resulted in significant increases in selenium concentration and GSH-PX activity when analyzed by analysis of variance. The basal diet contained 0.03 ppm selenium, so the diets contained 0.03, 0.06, 0.13, and 0.53 ppm total selenium. Diets analyzed by fluorometric assay.

Chart 2. The effect of various levels of dietary selenium on selenium-dependent and total GSH-PX activity and selenium concentration in liver tissue. Increased levels of dietary selenium resulted in significant increases in selenium concentration and GSH-PX activity when analyzed by analysis of variance. The basal diet contained 0.03 ppm selenium, so the diets provided 0.03, 0.06, 0.13, and 0.53 ppm total selenium. Diets analyzed by fluorometric assay.

PX activity (selenium-dependent plus glutathione S-transferase) in breast and liver tissues of mice fed increasing levels of dietary selenium from the basal diet (0.02 ppm selenium) to the diets containing added selenium levels of 0.02 and 0.10 ppm selenium (Charts 1 and 2). However, there was no increase in total GSH-PX or selenium-dependent GSH-PX in breast or liver tissues of mice fed diets between 0.10 and 0.50 ppm added selenium. In contrast to the GSH-PX activity, the concentration of selenium in breast and liver tissues continued to increase with increasing levels of dietary selenium (Charts 1 and 2).

GSH-PX Enzyme Kinetics (Experiment 3). Hepatic GSH-PX has been characterized as following the ping-pong BiBi model (34), so a Lineweaver-Burk plot can give only an estimate of the zone of first-order kinetics or an “apparent \( K_m \)” for glutathione with a glutathione concentration up to 2 mM. For the 0.02 mM H\(_2\)O\(_2\), GSH-PX activity did not increase when glutathione increased from 2 to 4 mM, while there was a nonlinear increase when the 0.10 mM H\(_2\)O\(_2\) was used.

DISCUSSION

Two general conclusions can be derived from the results of these experiments. (a) Virgin mammary tissue contains a selenium-dependent GSH-PX, the enzymatic characteristics of which are similar to that reported in other tissues (3). (b) There was no obvious correlation between GSH-PX activity or selenium concentration and the general developmental state of the mammary gland. In fact, the tissues from pregnant and lactating mice had GSH-PX activity and selenium concentrations similar to levels found in preneoplastic and neoplastic tissues. These results suggest that the morphological and biochemical states of the mammary epithelium in pregnant and lactating mice represent more appropriate controls than the mammary epithelium in virgin mice for comparing the effects of selenium in normal and neoplastic mammary tissues.

For kinetic studies (Experiment 3), the data clearly demonstrated that the methods for measuring GSH-PX activity were similar to that for hepatic tissues (3, 9, 14, 32).

Mammary and hepatic GSH-PX activity (Charts 1 and 2) increased with increasing dietary selenium, thus demonstrating a selenium-dependent GSH-PX activity. However, neither mammary nor hepatic GSH-PX activity increased when dietary selenium increased from 0.1 to 0.5 ppm, yet selenium concentrations in these 2 tissues continued to increase. When dietary selenium is below 0.4 ppm, it is well known that rodent hepatic selenium levels increase proportionately to dietary selenium (3, 31). In contrast, rat hepatic GSH-PX activity peaks around 0.1 ppm selenium (10). Ip and Sinha (8) found no increase in mammary gland GSH-PX activity when rats were fed 0.1 or 2.5 ppm selenium with saturated fat diets (1.0% corn oil and 24% hydrogenated oil). Thus, the data of Ip and Sinha (8) as well as our data suggest that mammary GSH-PX is increased when dietary selenium is below 0.1 ppm and when the level of unsaturated fat source is between 1 and 5%.

There was no obvious correlation between GSH-PX activity and selenium concentration in the preneoplastic lines D2 and C4 or the tumors derived from these lines. Tumor tissues had the highest selenium concentration, suggesting that these tissues may be concentrating selenium. In contrast, the tumor tissues had low selenium concentrations similar to that of preneoplastic and normal mammary tissues. Increased selenium levels in the drinking water inhibited the tumor-producing capability of the C4 preneoplastic outgrowth line but not line D2 (18). Additionally, the growth of transplanted C4 and D2 tumors was not inhibited by increased selenium levels in the drinking water (18). Thus, the refractory state of the D2 preneoplastic outgrowth line and D2 and C4 tumors to selenium-mediated inhibition of growth cannot be explained by the absence of GSH-PX activity or the level of selenium in these cell populations.

The results demonstrating greater selenium and GSH-PX levels found in mammary tissues from pregnant and lactating mice than in tissues from virgin mice illustrate the problem of identifying proper controls for selenium studies with preneoplastic and
neoplastic tissues. Several variables are changing during the process of normal mammary gland differentiation, namely, the ratio of epithelial to stromal cells and the differentiative state of the mammary cells. At this time, we have not determined which is the more significant factor relative to cellular selenium concentration and GSH-PX levels. The contribution of adipose cells to total tissue selenium is negligible, since mammary gland free fat pads have the same level of selenium as do intact mammary fat pads from 10- to 12-week-old virgin mice. These results suggest that the appropriate controls for selenium metabolism in dysplastic and neoplastic tissues are the presence of milk in the tissue. Although we do not know the level of selenium in mouse milk, selenium levels in human milk are known to be 1-2 ppb in breast milk and formula fed infants. Am. J. Clin. Nutr., 35: 521-526, 1982.

In summary, the results of these experiments document the level of GSH-PX and selenium for the various developmental states of the mammary gland.

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REFERENCES

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