Alcohol consumption increases breast cancer risk in postmenopausal women in a dose-dependent manner. The objective of the present study was to determine if the effect of alcohol on mammary cancer is modified by body weight and exogenous estrogen. Ovariectomized mice of various body weights, receiving estrogen or placebo supplementation, and consuming water or alcohol were injected with mammary cancer cells. Alcohol intake resulted in insulin sensitivity and increased tumor growth in obese mice. Exogenous estrogen alone inhibited tumor growth. The combination of estrogen and alcohol overcame the inhibitory effects of estrogen on tumor growth in obese mice. Alcohol consumption increased the circulating estrogen and leptin levels. In conclusion, alcohol and estrogen treatment can modify mammary tumor growth, possibly through the regulation of estrogen and leptin, especially in obese mice.

Breast cancer is the most prevalent cancer among American women and leads to the second highest number of cancer-related deaths after lung cancer (1). The American Cancer Society projected that about 180,000 women would be diagnosed with malignant breast cancer by the end of 2009; of those, 40,000 would succumb to the disease (1). Numerous epidemiological studies have established a positive association between alcohol consumption and breast cancer risk (2). In developed countries, approximately 4% of breast cancer cases are estimated to be associated with alcohol consumption (3). Alcohol consumption increases breast cancer risk in women in a dose-dependent manner; the risk increases by approximately 10% for every drink consumed per day, 20% for two drinks, 30% for three, and 50% for four or more drinks per day (4). Even though alcohol consumption is harmful in relation to breast cancer, it has a beneficial effect on type 2 diabetes in that it decreases insulin resistance. Alcohol improves the responsiveness of tissues to the physiological actions of insulin (5). In fact, alcohol consumption can reduce the risk of developing type 2 diabetes by as much as 30% (5). In contrast, obesity is coupled to increased insulin resistance (6). Interestingly, alcohol can overcome obesity-dependent insulin resistance, as obese alcohol drinkers had lower homeostasis model assessment (HOMA) values (a measure of insulin resistance) than obese abstainers (7). Moreover, diabetes risk diminished, secondary to increased insulin sensitivity, in extremely obese men who consumed alcohol (8). Obese postmenopausal women also showed insulin sensitivity after alcohol consumption (7). It is unclear exactly how the effects of alcohol on insulin sensitivity relate to breast cancer development.

In addition to increasing breast cancer risk, alcohol consumption, obesity and exogenous estrogen also influence the hormonal status in postmenopausal women. Postmenopausal women who drink alcohol exhibit an increase in circulating blood estrogen levels compared to non-drinkers. Blood estrogen levels can increase by as much as 22% after alcohol consumption (9). Studies have suggested that the alcohol-mediated elevation of serum estrogen is positively associated with breast cancer (2, 10). Moreover, alcohol consumption has a greater impact on breast cancer risk in postmenopausal women who use estrogen replacement therapy (ERT) compared to those who do not. Evidence has suggested that alcohol may increase breast cancer by increasing the stability of estrogen in the blood, increasing estrogen production or increasing the sensitivity of breast cancer cells to the effects of estrogen. In one study, alcohol intake in conjunction with ERT significantly elevated blood estrogen levels to a greater extent than either alcohol or ERT alone (9). Moreover, the combination of alcohol consumption and ERT increased breast cancer risk to a higher level than either risk factor alone (11). The mechanisms of how alcohol,
obesity, and estrogen influence breast cancer development are not well understood.

The objective of the present study was to determine if the effects of alcohol on breast cancer are modified by body weight and estrogen given that obesity increases breast cancer risk, estrogen is a risk factor for breast cancer, and all three factors increase estrogen levels. There is a gap in the existing animal literature regarding the alcohol-obesity-breast cancer association. Most animal studies examining the effects of modifiable risk factors on breast cancer progression have been limited to one-tiered experiments analyzing only the effects of alcohol consumption, obesity or ERT on tumorigenesis. In the present study, the effects of alcohol, body weight, exogenous estrogen, and their combined effects on mammary tumor growth were determined.

Materials and Methods

Mouse husbandry and diet. A total of 240 specific pathogen-free ovariectomized Friend Virus B (FVB) female mice were purchased from the Jackson Laboratory (Bar Harbor, MA, USA) at 6 weeks of age. They were housed according to National Institutes of Health (NIH) guidelines and animal procedures were performed following Institutional Animal Care and Use Committee approval. Animals were maintained on a 12-hour light/12-hour dark cycle at 24°C. Following two weeks of acclimatization, the mice were randomized into placebo and estrogen pellet groups; within these groups, the mice were subdivided into water and alcohol consuming LF and HF groups. The estrogen pellets were manufactured to deliver estrogen (17 β-estradiol ELISA (IBL-America, Minneapolis, MN, USA) and IGF-1 immunoassay kits (R&D System, Minneapolis, MN, USA)). Serum leptin (pg/ml) and insulin (pg/ml) levels were measured using a mouse bone panel Lincoplex kit (Line Research, St Louis, MO, USA). Blood alcohol (mg/dl) levels were determined using a Nicotinamide adenine dinucleotide (NAD)/reduced form of NAD (NADH) kit (Sigma, St Louis, MO, USA). The procedures recommended by the manufacturer of the kits were followed.

Body composition and uterine weight. Body weight, food consumption and liquid consumption were measured weekly.

Pellet implantation. Estrogen or placebo pellets were implanted into the dorsal area between the neck and shoulder of mice at week 19 after the start of the alcohol and diets. The estrogen pellets were manufactured to deliver estrogen (17 β-estradiol) for 90 days at a rate of 0.72mg per day (Innovative Research of America, Sarasota, FL, USA).

Glucose tolerance test and insulin tolerance test. To evaluate insulin sensitivity, the glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed on ten animals per group as previously described (12).

Cancer cell line. Invasive, estrogen receptor negative Met-1 mouse mammary cancer cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were grown at 37°C with 5% CO2 and harvested when they were 70-80% confluent. The mice were injected subcutaneously with 5×104 cells/50 μl of serum-free DMEM 4 weeks after the start of the estrogen treatment. After cancer cell injection, tumor size was measured every two or three days with calipers.

Statistical analysis. Body composition (body weight, body fat, BMD), uterine weight, tumor volume, and serum analysis (estrogen, IGF-1, insulin, and leptin) were analyzed by multivariate analysis of variance and post hoc comparison of the means using Tukey’s honestly significant difference. All the results are presented as the mean±standard error of the mean (SEM). SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all the statistical comparisons. P-values ≤0.05 were considered statistically significant.

Results

Effect of body weight, alcohol consumption, and estrogen on body composition. The baseline body weight and body fat levels were similar between all the groups at the start of the experiment (p>0.05). Figure 1 portrays the effect on body weight of feeding the CR, LF, and HF diets. The CR mice had the lowest body weight, LF mice had an intermediate body weight and HF mice had the highest body weight. The water and alcohol consuming LF and HF groups (p<0.01), but not the CR group (p>0.05), underwent a significant reduction in body weight after estrogen treatment. As shown in Table I, food consumption in both the water and alcohol consuming mice was similar (p>0.05), however, the mice on alcohol consumed additional calories from alcohol which provided 1.42kcal/g. Even though the alcohol consuming the LF and HF mice consumed more calories their body weights were not statistically different from LF and HF mice consuming water (p>0.05). The only statistically significant body weight difference was observed between the CR water and alcohol groups of mice (p<0.05). The blood alcohol concentrations of the mice consuming 20% alcohol ranged from 40-52 mg/dl regardless of the experimental group (Table II). Figure 2 shows the effect of diet on body fat levels. No differences in body weight and body fat levels were observed between the water and alcohol consuming mice (p>0.05). After 18 weeks on the study, the body weight
and body fat levels in the water and alcohol plus placebo groups were similar to those found in the mice of the water and alcohol plus estrogen groups (p>0.05); however, after estrogen treatment a dramatic decrease in body weight and fat levels (p<0.05) compared to placebo was noted. Also a significant increase in BMD and uterine weight (p<0.05) was seen in the mice receiving exogenous estrogen (Table I).

**Table I. Final body composition and calorie consumption in the different alcohol, diet and estrogen groups.**

<table>
<thead>
<tr>
<th></th>
<th>Placebo pellet</th>
<th></th>
<th></th>
<th>Estrogen pellet</th>
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</thead>
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<td></td>
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<td>Water</td>
<td>Alcohol</td>
<td>Water</td>
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</tr>
<tr>
<td>Diet (kcal)</td>
<td>7.8±0.1</td>
<td>9.7±0.0</td>
<td>7.8±0.1</td>
<td>10.6±0.0</td>
<td>7.8±0.1</td>
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<tr>
<td>Alcohol (kcal)</td>
<td>4.4±0.2</td>
<td>3.4±0.1</td>
<td>2.8±0.1</td>
<td>4.1±0.2</td>
<td>3.3±0.2</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>Total energy intake (kcal)</td>
<td>7.8±0.1</td>
<td>11.0±0.0</td>
<td>12.3±0.0</td>
<td>11.6±0.0</td>
<td>11.9±0.2</td>
<td>12.6±0.2</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>0.046±</td>
<td>0.049±</td>
<td>0.049±</td>
<td>0.046±</td>
<td>0.055±</td>
<td>0.058±</td>
</tr>
<tr>
<td></td>
<td>0.0007±</td>
<td>0.0005±</td>
<td>0.0007±</td>
<td>0.0006±</td>
<td>0.0007</td>
<td>0.0014</td>
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<tr>
<td>Uterine weight (mg)</td>
<td>8.3±1.0</td>
<td>7.8±0.8</td>
<td>6.4±0.8</td>
<td>8.6±1.4</td>
<td>7.5±0.8</td>
<td>32.9±3.3</td>
</tr>
<tr>
<td></td>
<td>6.9±0.8</td>
<td>4.4±0.5</td>
<td>4.4±0.5</td>
<td>42.3±1.9</td>
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<td>36±2.2</td>
</tr>
</tbody>
</table>

Values are expressed as the mean±SEM for eighteen mice per group. *Significantly different from estrogen group (within same diet and liquid categories).

**Figure 1. Effect of diet, alcohol consumption and estrogen treatment on body weight. HF: High fat, LF: low fat, CR: calorie restricted. Closed arrows indicate the time of pellet implantation at week 19. Open arrows represent the time of Met-1 cell injection at week 23. The body weights were measured once a week as indicated in the Materials and Methods, however, the graph depicts measurements recorded every 5 weeks.**

**Effect of body weight, alcohol consumption, and estrogen on insulin sensitivity.** The GTT showed that the CR mice were glucose tolerant as depicted in Figure 3A, that is, they cleared the injected glucose more rapidly, resulting in lower blood glucose levels compared to the LF and HF groups of mice (p<0.05). Also obesity (HF fed mice) resulted in slower glucose clearance, indicative of insulin resistance.
However, the alcohol-consuming HF mice cleared blood glucose faster than the water-consuming HF mice. Estrogen treatment resulted in a trend toward improved glucose clearance. The ITT showed that obesity (HF diet) induced insulin resistance as seen in Figure 3B ($p<0.05$). Nonetheless, the alcohol consuming HF mice also cleared blood glucose much faster than the water consuming mice in response to exogenous insulin ($p<0.05$). Estrogen supplementation induced insulin sensitivity in the LF and HF groups of mice ($p<0.01$), but alcohol consumption did not modify this effect ($p>0.05$).

**Effect of body weight, alcohol consumption, and estrogen on tumor growth.** Figure 4 shows that tumor growth was affected by body weight. The CR mice developed the smallest tumors, while the obese mice (HF diet) developed the largest tumors. The CR and HF mice consuming alcohol tended to develop significantly larger tumors than the water consuming mice ($p<0.05$); however, the LF mice consuming alcohol and water developed similar sized tumors ($p>0.05$). Estrogen treatment dramatically inhibited the tumor growth rate in all of the water-consuming groups regardless of body weight (CR, LF, HF; $p<0.05$). On the other hand, the combination of alcohol, obesity and estrogen treatment clearly affected tumor growth rate. The CR, LF and HF mice receiving estrogen treatment and consuming alcohol had varying tumor growth rates: slow, intermediate, and rapid, respectively.

**Hormones: insulin, IGF-1 and leptin.** The mice receiving estrogen supplementation exhibited an increase in circulating blood estrogen (17 β-estradiol) levels compared to the placebo groups (Table II). Moreover, the serum leptin levels increased proportionately to the body fat levels. However, alcohol consumption significantly enhanced the leptin levels without affecting body fat levels in the placebo group ($p<0.05$). After estrogen treatment, the leptin levels drastically decreased in all the body weight groups in conjunction with body fat levels when compared to the placebo group ($p<0.05$); but the leptin levels were still consistently higher for the mice on alcohol. The serum insulin and IGF-1 levels were elevated with increasing body fat levels for all the groups ($p<0.05$). The estrogen treated mice on water and alcohol displayed reduced insulin and IGF-1 levels in contrast to the placebo group ($p<0.05$).

**Discussion**

This study established, for the first time, a stratified mouse model to evaluate the effect of three risk factors for breast cancer concurrently. Specifically, the effects of alcohol on mammary tumor growth were successfully shown to be modified by body weight, estrogen and a combination of the two. Alcohol consumption induced insulin sensitivity and enhanced tumor growth in overweight and obese mice. Body weight influenced mammary tumor growth: that is the mice with a lean phenotype developed the smallest tumors and the

<table>
<thead>
<tr>
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<th>Estrogen pellet</th>
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<td>Alcohol</td>
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<td>CR LF HF</td>
<td>CR LF HF</td>
<td>CR LF HF</td>
<td>CR LF HF</td>
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<tr>
<td>Insulin (pg/ml)</td>
<td>1881± 3613± 3780± 2592± 3057± 3491±</td>
<td>146 286 350</td>
<td>884± 1640± 1615± 1289± 1627± 1634±</td>
<td>71 239 100 75 170 120</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>2025± 3927± 6452± 2821± 4042± 7059±</td>
<td>198± 353± 753±</td>
<td>984± 1609± 1677± 1335± 1672± 2607±</td>
<td>48 229± 97 109 112± 207</td>
</tr>
<tr>
<td>IGF-1 (pg/ml)</td>
<td>333± 766± 909± 590± 682± 888±</td>
<td>55 52 69</td>
<td>256± 499± 535± 408± 453± 636±</td>
<td>21 47 26 49 20 55</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>1.47± 1.95± 2.06± 1.89± 2.29± 2.30±</td>
<td>0.22± 0.55± 0.64±</td>
<td>22.14± 38.36± 32.54± 34.70± 49.27± 38.40±</td>
<td>4.45 8.63 7.52 4.47 5.90 5.20</td>
</tr>
<tr>
<td>BAC (mg/dl)</td>
<td>0.0± 0.0± 0.0± 51.8± 42.8± 44.7±</td>
<td>0.0± 0.0± 0.0±</td>
<td>0.0± 0.0± 0.0± 42.3± 40.3± 41.3±</td>
<td>0.0 0.0 0.0 1.9 0.8 0.8</td>
</tr>
</tbody>
</table>

Values are expressed as the mean±SEM for eighteen mice per group. *Significantly different from CR and HF (within same liquid category). **Significantly different from CR (within same liquid category). aSignificantly different from estrogen group (within same diet and liquid categories). BAC: blood alcohol concentration.
obese mice the largest tumors. Supplemental estrogen triggered the loss of body fat and induced insulin sensitivity. Finally, tumor growth was suppressed by exogenous estrogen, but, in conjunction with alcohol consumption, the inhibitory effect of estrogen on the tumor growth rate in the overweight and obese mice was overcome.

Epidemiological studies have shown that obesity increases the risk of morbidity and mortality from breast cancer, but only in postmenopausal women (13, 14). For instance, for every one unit increase in body mass index (BMI) there is about a 3% increased risk of developing breast cancer (10). The excess body fat is also associated with insulin resistance, which consequently results in a compensatory rise in plasma insulin (15). The present mouse model effectively modeled the epidemiology data in that lean, overweight and obese mice displayed an adipose tissue dose-dependent increase in tumor growth along with decreasing insulin sensitivity. An increase in serum insulin, leptin, IGF-1 and estrogen levels was also observed in proportion to body weight. This suggested that hormones and/or insulin resistance may be responsible for the increased tumor growth in obese mice as they have been shown to influence various processes involved in breast cancer development (16).

In addition to obesity, alcohol consumption enhanced tumor development. Interestingly, alcohol also had a contradictory role and increased the ability of the body to respond to insulin, resulting in insulin sensitivity. It is unclear how alcohol can have both a deleterious and beneficial effect. Possibly alcohol increases the ability of insulin responsive tissues, such as skeletal muscle and adipose tissue, to respond to hormones (i.e., insulin, estrogen, leptin) and thereby prevent insulin resistance and eventual type-2 diabetes, however, at the same time also sensitizing mammary cancer cells to hormones and consequential...
primary tumor growth and/or a more metastatic phenotype. In fact, alcohol accelerated the mammary tumor growth rate in mice (17) and increased the metastatic potential of breast cancer cells in a dose-dependent manner in vitro (18, 19). In the present study, alcohol consumption resulted in elevated serum estrogen and leptin levels mechanistically implicating these hormones in tumor development. Alternatively, alcohol could affect adipose tissue and skeletal muscle indirectly by inducing adiponectin production (20), which stimulates insulin sensitivity in muscle and fat cells (21). On the other hand, alcohol intake could potentially stimulate insulin sensitivity by increasing the secretion of insulin (22). Experiments by Huang and Sjoholm (22) showed that alcohol promoted secretion of insulin by the pancreas, and that the increased insulin secretion was linked to increased insulin sensitivity in alcohol-consuming animals.

A synergistic increase in the serum leptin levels was also observed in the obese mice consuming alcohol. Both alcohol consumption and obesity are associated with high levels of circulating leptin and are linked to breast cancer development (4, 23-25). In cell culture studies, leptin has been shown to induce the growth and invasiveness of breast cancer cells (25, 26). Leptin also has the ability to up-regulate the activity of aromatase, thereby promoting breast cancer development through increased estrogen levels in the mammary gland where the breast cancer cells are located (24).

Estrogen is a steroid hormone that is produced primarily by the ovaries in premenopausal women. However, after menopause, estrogen is produced in peripheral tissues (e.g. adipose tissue) by the aromatization of androgen to estrogen by aromatase (27). Aromatase is found in estrogen-producing cells such as white adipose tissue (28). Women with higher amounts of fat have higher levels of aromatase; thus, obese postmenopausal women have higher estrogen levels than lean postmenopausal women (10). Elevated estrogen levels are considered to be a risk factor for breast cancer (27). Interestingly, in the present study estrogen treatment actually ameliorated tumor growth in the mice consuming water, irrespective of body weight. The present data challenge much of the existing literature; however, more and more studies are beginning to elucidate the importance of estrogen for tumor suppression. Nkhata et al. found chemically induced obese mice injected with T47-D human breast cancer cells exhibited reduced tumor growth after estrogen pellet implantation compared to placebo (29). It is possible that the effects of estrogen on tumor growth depend on the timing of estrogen supplementation. An observation from the Woman’s Health Initiative indicated that women had a reduced risk of breast cancer when estrogen therapy was initiated five or more years after menopause (30). Moreover, the various stages of carcinogenesis may be affected differently by estrogen. For example, estrogen may inhibit tumor growth, but encourage invasion and metastasis. In the present study, only tumor growth was assessed, but lung metastases were observed upon necropsy (JH and NPN unpublished observation). Future experiments will analyze the effects of

\[\text{Figure 4. Effect of body weight, alcohol and estrogen on mammary tumor growth. HF: High fat, LF: low fat, CR: calorie restricted. *Significantly different from estrogen group (within same diet and liquid categories). Tumors were measured every 2-3 days as indicated in the Materials and Methods, however, the graph depicts measurements recorded every 5 days.}\]
alcohol, obesity and estrogen on breast cancer metastasis.

When alcohol was consumed by mice with different body weights receiving estrogen treatment, a body weight-dependent increase in tumor volume was noted. This suggested that the combination of estrogen and alcohol overcame the inhibitory effects of estrogen alone on tumor growth rate. Moreover, enhanced insulin sensitivity was observed, most likely the result of estrogen and alcohol together sensitizing the tissues to the effects of insulin. However, no such cooperative effect was seen on the hormones assayed, notably, estrogen and leptin. This was because the body weight and body fat levels drastically decreased after estrogen supplementation. As a result, a decrease in estrogen and leptin production was seen. This does not rule out these hormones as players in tumor development. In fact, estrogen is an ideal candidate. Postmenopausal women who consume alcohol have higher circulating estrogen levels compared to non-drinkers (2). In addition, the literature suggests alcohol may impede estrogen clearance from the blood, once again increasing estrogen levels (31, 32). After menopause, many women use ERT to prevent hypo-estrogenic problems thus, further increasing blood estrogen levels (33). Moreover, adipose tissue enhances the levels of circulating estrogen (10). Due to the high levels of estrogen (33). Moreover, adipose tissue enhances the levels of circulating estrogen (10). Due to the high levels of estrogen in obese postmenopausal women drinking alcohol, their cells are continuously surrounded and influenced by estrogen. We propose that alcohol consumption sensitizes tissues to the positive effects of hormones thereby promoting insulin sensitivity and sensitizes mammary cancer cells to the negative effects of estrogen, which results in the promotion of breast carcinogenesis.

Acknowledgements

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