Influence of Dietary Fat, Caloric Restriction, and Voluntary Exercise on N-Nitrosomethylurea-induced Mammary Tumorigenesis in Rats

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ABSTRACT

The effect of dietary fat, energy restriction, and exercise on N-nitrosomethylurea (NMU)-induced mammary tumorigenesis in female F344 rats was investigated. Rats were fed the NIH-07 diet until N-nitrosomethylurea administration on Day 50 of age, when they were transferred to six treatment groups. Three sedentary groups were fed either high-fat (20%, w/w), medium-fat (10%), or low-fat (5%) diets ad libitum (HFAL, MFAL, LFAL, respectively); two sedentary groups were fed high fat and medium fat diets restricted to 75% of the food consumed by their ad libitum counterparts (HFRE, MFR); and one group was fed a HFAL diet but allowed free access to an activity wheel (HFEX). Tumor yields among the three ad libitum sedentary groups were significantly greater in the HFAL and MFAL groups when compared to the LFAL group. Dietary restriction reduced tumor yields by more than 90% of ad libitum controls regardless of fat intake. Voluntary exercise reduced tumor yields and delayed time of tumor appearance in HFEX animals to levels similar to those found in LFAL animals. Animals with voluntary access to exercise wheels averaged between 1.03 and 2.85 miles/day, consumed more food (+18%), and exhibited greater weight gain (+13%) than their sedentary counterparts. Restricted animals exhibited significantly decreased body weight gains (-15%) compared to their ad libitum counterparts, but no differences in weight gains were detected among the HFAL, MFAL, and LFAL groups, despite widely varying amounts of fat intake. Body composition studies indicated that body fat content was not influenced by the quantity of fat consumed in the diet, but was significantly reduced by caloric restriction (-20 to 26%) and exercise (-20%). While the precise mechanisms underlying the tumor-promoting effects of HFAL diets and the antipromoting effects of energy restriction and exercise remain to be elucidated, available evidence suggests that these effects are not due to alterations in energy homeostasis per se, but may instead be exerted indirectly, and perhaps independently via endocrine, paracrine, or neurohormonal mechanisms.

INTRODUCTION

Currently, there is considerable debate over the relative importance of dietary fat and total caloric intake as modulators of mammary carcinogenesis (1-5). Over the past half-century, a number of investigators have demonstrated that high-fat intake stimulates the development of experimental mammary cancer in rodents (6-9). These studies have shown that dietary fat exerts its most pronounced effects on the promotion phase and that there is a requirement for a minimum level of the EFA, linoleate (10). Moreover, the results of laboratory animal studies are consistent with a substantial body of epidemiological evidence, suggesting that risk for breast cancer is correlated with fat intake, especially in the postmenopausal age groups (11, 12). Another set of data with an equally long history indicates that tumor incidence is correlated with daily caloric intake and body weight (3, 4, 13-16). Since fat is the most calorically dense nutrient, high calorie diets are often synonymous with high fat diets. Hence, it has been difficult to determine whether fat and calories exert a common or independent effect on mammary tumorigenesis (5). Recently, using the DMBA model, Kritchevsky et al. (2, 17) and Pariza (3, 18) have reemphasized the importance of caloric intake and have suggested that fat exerts its promoting effects by virtue of its being more efficiently utilized as an energy source than either protein or carbohydrate.

The resurgence of interest in the role of reduced energy intake in cancer prevention has triggered interest in the opposite side of the energy equation, namely the role of increased energy expenditure in mammary tumorigenesis. The rationale behind such an approach is that, if decreased energy intake acts to suppress tumorigenesis, then its converse, i.e., increased energy expenditure, should exert a similar suppressive effect. Supporting this view are the early experimental studies of Rusch and Kline (19) and Moore and Tittle (20) and the more recent epidemiological studies of Frisch and coworkers (21, 22) which suggest that breast cancer incidence is lower in women athletes than nonathletes. Few laboratory animal experiments (19, 20, 23-25) have investigated the effects of exercise on induced tumor development; in each of these, forced exercise was used, thus introducing an intervening stress variable (26, 27).

There is evidence to suggest that tumorigenesis may be affected differently by nutritional factors, depending upon the nature of the initiating agent. Pollard and Luckert (28), for example, reported that caloric restriction exerted an inhibiting effect on colon tumorigenesis when the initiating agent was an indirect-acting carcinogen (requiring host activation) but not when it was a direct-acting carcinogen (NMU). Since most previous studies (2, 3, 17, 18) on caloric restriction and experimental mammary cancer have used DMBA, which requires host activation, as the initiating agent it is of importance to determine the effects of energy restriction (and exercise) using a direct-acting carcinogen such as NMU (20). Further recommending the NMU model is the fact that it more closely mimics human breast cancer both histologically (30) and in endocrine responsiveness (31, 32) than the DMBA model.

The overall purpose of this study, therefore, was to analyze the influence of dietary fat level, energy restriction, and increased energy expenditure on the promotion phase of NMU-induced mammary tumorigenesis.

MATERIALS AND METHODS

Experimental Procedures. Female inbred F344 rats were purchased from Charles River Breeding Laboratories (Kingston, NY) at 28 days of age, fed the NIH-07 diet, and housed 3 to a cage in polyethylene metabolism cages (19 in x 10 in x 8 in) containing hardwood shavings and covered with filter tops. The animal room was controlled for temperature (24 ± 2°C) and humidity, and illuminated for 14 hours daily. Food and water were available ad libitum. Animals were killed when tumors were palpable but before they reached 1 cm in diameter. The animals were then euthanized with a pentobarbital overdose, and the mammary glands were excised and fixed in 10% formalin for histological evaluation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: EFA, essential fatty acid; ANOVA, analysis of variance; DMBA, 7,12-dimethylbenz(a)anthracene; HF, high fat; HFAL, high fat ad libitum; LFAL, low fat ad libitum; MF, medium fat; MFAL, medium fat ad libitum, sedentary; MFR, medium fat sedentary restricted; NMU, N-nitrosomethylurea; HFEX, high fat, ad libitum exercise, sedentary; MFR, medium fat sedentary restricted; NMU, N-nitrosomethylurea; HFEX, high fat, ad libitum exercise, sedentary.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: EFA, essential fatty acid; ANOVA, analysis of variance; DMBA, 7,12-dimethylbenz(a)anthracene; HF, high fat; HFAL, high fat ad libitum; LFAL, low fat ad libitum; MF, medium fat; MFAL, medium fat ad libitum, sedentary; MFR, medium fat sedentary restricted; NMU, N-nitrosomethylurea; HFEX, high fat, ad libitum exercise, sedentary.

4 Animals were maintained according to the revised "Guide for the Care and Use of Laboratory Animals, Department of Health, Education, and Welfare Publication NIH 85-23, revised 1985."
FAT, CALORIE RESTRICTION, EXERCISE, AND MAMMARY CANCER

± 2°C, SD), light (12-h cycle), and humidity (50%). On Day 50 of age, all animals received a single dose of NMU (37.5 mg/kg of body weight) by tail injection. The NMU (Ash Stevens, Inc., Detroit, MI) was dissolved in 5 to 10 drops of 3% acetic acid and brought up to volume in distilled H₂O yielding a stock solution of 10 mg/ml administered within 3 h of formulation.

Two days after NMU administration, rats were allocated randomly, by weight, to six experimental groups. Three sedentary groups (LFAL, Group 1; MFAL, Group 2; HFAL, Group 3) of 36 animals each were fed 20%, 10%, and 5% fat diets ad libitum (Table 1). Two other sedentary groups (MFR, Group 4; HFR, Group 5) of 36 animals each were fed 26.6% and 13.3% fat diets restricted to 75% of their respective ad libitum groups, HFAL and MFAL. One active group (HFEX, Group 6) of 30 rats was housed in wheel-cage units and fed the HF diet ad libitum.

Animals in the HFAL, MFAL, and LFAL groups were housed 3 to a cage with the exception that 6 animals each from the HFAL and MFAL were housed singly to assess average daily food consumption. The 2 restricted groups were fed 75% by weight of the mean daily dietary intake of their ad libitum counterparts (calculated over a 3-day period). The diets fed the 2 restricted groups were adjusted to ensure that they consumed an amount of fat equal to their ad libitum counterparts (calculated over a 3-day period). The animals drank ad libitum water. The rats were weighed on alternate days. On the third day, their food intake was recorded. The rats were killed by CO₂ asphyxiation. Palpable and nonpalpable (small lesions seen only at necropsy) tumors were excised, fixed in buffered formalin, blocked in paraffin, sectioned, and stained with hematoxylin and eosin. Histological diagnosis of mammary tumors (by C. X. W.) was based on criteria outlined by Young and Hallows (41).

Table 1 Composition of experimental diets (g/100 g)

<table>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>34.46</td>
<td>34.23</td>
<td>20.44</td>
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<td>10.27</td>
<td>6.14</td>
<td>10.34</td>
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<td>0.32</td>
<td>0.35</td>
<td>0.43</td>
<td>0.47</td>
<td>0.35</td>
</tr>
<tr>
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<td>10.0</td>
<td>20</td>
<td>13.3</td>
<td>26.6</td>
<td>20</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.0</td>
<td>5.29</td>
<td>5.87</td>
<td>7.04</td>
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<td>5.87</td>
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<td>Minerals</td>
<td>3.5</td>
<td>3.77</td>
<td>4.54</td>
<td>4.92</td>
<td>4.3</td>
<td>4.53</td>
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<td>Vitamins</td>
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<td>1.06</td>
<td>1.17</td>
<td>1.41</td>
<td>1.56</td>
<td>1.17</td>
</tr>
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<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.21</td>
<td>0.23</td>
<td>0.28</td>
<td>0.31</td>
<td>0.23</td>
</tr>
<tr>
<td>kcal/g</td>
<td>3.85</td>
<td>4.08</td>
<td>4.53</td>
<td>4.10</td>
<td>4.71</td>
<td>4.53</td>
</tr>
<tr>
<td>% of kcal CHO</td>
<td>67.5</td>
<td>57.3</td>
<td>39.6</td>
<td>43.4</td>
<td>22.7</td>
<td>39.6</td>
</tr>
<tr>
<td>% of kcal protein</td>
<td>20.8</td>
<td>20.7</td>
<td>20.7</td>
<td>27.4</td>
<td>26.5</td>
<td>20.7</td>
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<td>% of kcal fat</td>
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<td>29.7</td>
<td>29.2</td>
<td>50.8</td>
<td>39.7</td>
</tr>
</tbody>
</table>

* AIN 76A mixture.

To determine whether tumor incidences varied as a direct function of fat intake, differences in tumor incidences among the HFAL, MFAL, and LFAL groups were compared by Bartholomew's test for ordered alternatives (44). When the null hypothesis of no difference in tumor incidence among all 6 treatment groups was rejected, pairwise comparisons were conducted using the Fisher exact test with Bonferroni's adjustment to the α-value for multiple comparisons.

The significance of differences in tumor multiplicity among the 6 treatment groups was assessed by the following 4 methods. Differences in total tumor burden/group were assessed for significance by the pairwise χ² test. Tests for trends were conducted by Bartholomew's test (44). Multiplicity defined as the number of tumors/tumor-bearing animal or the number of tumors/total number of animals at risk, was assessed by Student's t test; multiplicity, assessed in terms of the frequency distribution of tumors per animal, was tested for significance by the χ² test for homogeneity.

Total tumor volume was determined by measuring each tumor in 3
dimensions and using the formula for an ellipse ($\frac{a}{2} \times b^2$) to calculate tumor volumes. Since tumor volume data were not normally distributed, statistical comparisons were conducted by the nonparametric Mann-Whitney U test.

Animal weight gains were analyzed using a program for ANOVA with repeated measures (45). This program tests the main effects of time, diet, and activity status and their joint effects (interactions) on weight gain.

**RESULTS**

Food Consumption. On average, the LFAL group consumed 12.4, the MFAL 11.7, and the HFAL group 9 g of food/day under ad libitum sedentary conditions. The restricted groups consumed 8.8 (MFR) and 6.75 (HFR) g/day, and the HFEX group consumed 11.7 g/day. Based on the calculated energy densities of the different diets (Table 1), the HFR and MFR groups consumed 32 and 36 kcal/day compared to 41 and 48 kcal/day for their respective ad libitum counterparts (HFR, MFAL). HFEX animals consumed considerably more calories than their sedentary HFAL counterparts (53 versus 41 kcal/day).

Tumor Histopathology. With the exception of mammary tumors, no gross changes in major organs were seen. Tumors were either adenocarcinomas, adenomas, or fibroadenomas, the latter two classes being very rare [3 and 4, respectively, of a total of 192 mammary tumors (Table 2)]. There were 4, 1, 1, 2, 0, and 1 unscheduled terminations in Groups 1 to 6, respectively, due to necrotizing tumors. No animals died of extraneous causes.

Survival Analysis. Analysis of survival curves (Fig. 1) indicates that mammary tumors appeared in the order HFAL > MFAL > HFEX > LFAL > MFR > HFR. Tests for overall trend were highly significant ($P < 0.01$) by Cox’s analysis for adjusted trends and the generalized Kruskal-Wallis test ($P < 0.01$). Pairwise comparisons revealed that tumors appeared more rapidly in the HFAL than the LFAL groups ($P < 0.01$). No difference was observed when the HFAL group was compared with the MFAL group. Tumor appearance was dramatically delayed in the two restricted groups compared to their ad libitum counterparts ($P < 0.01$). Tumor appearance was also significantly delayed in the active group versus its sedentary control ($P < 0.05$). When viewed over time (Fig. 1), it can be seen that all ad libitum groups, including the active group, exhibited a similar time to initial tumor (63 to 70 days). However, the subsequent rate of tumor appearance was increased in proportion to the amount of fat in the diet of sedentary rats; voluntary exercise, in contrast, reduced the subsequent rate to initial tumor appearance, and the subsequent rate of tumor appearance in restricted animals was drastically delayed with, in most rats, a complete absence of tumors at termination of the experiment (85 days, MFR; 150 days, HFR).

Tumor latency, when assessed in terms of mean (or median) days until first tumor, also indicated that tumor appearance was delayed by both energy restriction and increased energy expenditure. Mean (median) days to first tumor, in order of earliest appearance, were: MFAL, 102 (106); HFAL, 109 (113); HFEX, 125 (146); LFAL, 128 (150); MFR, 147 (150); and HFR, 150 (150).

Tumor Incidence. Tumor incidence data were analyzed in terms of total adenocarcinomas, nonpalpable adenocarcinomas, and total palpable adenocarcinomas (Table 3). Tests for overall negative trend were significant for the HFAL, MFAL, and LFAL groups when assessed in terms of either total adenocarcinomas ($P < 0.05$) or total palpable adenocarcinomas ($P < 0.05$) but not nonpalpable adenocarcinomas. Pairwise comparisons indicated a significant difference between the HFAL and LFAL groups ($P < 0.05$) and the MFAL and LFAL groups ($P < 0.05$) but not between the HFAL and MFAL groups. Pairwise comparisons between HFAL and MFAL group and their calorie-restricted counterparts (HFR and MFR) revealed a striking inhibition in tumor incidence in the energy-restricted groups (>90%). The difference in incidence of palpable adenocarcinoma between the active (HFEX) and sedentary (HFAL) groups was statistically significant ($P < 0.05$) (Table 3). It is noteworthy that the frequency of nonpalpable (microscopic) adenocarcinoma was reduced significantly in the two restricted groups but not in the LFAL or exercised ad libitum groups when compared to their respective controls (Table 3).

Tumor Multiplicity. When assessed in terms of total tumor burden/group (Table 2), an overall negative trend ($P < 0.05$) was found among the three ad libitum sedentary groups (HFAL > MFAL > LFAL). Pairwise comparisons revealed that the LFAL group exhibited a significantly reduced tumor yield in comparison to its MFAL and HFAL counterparts ($P < 0.05$). HFEX animals exhibited a reduced total tumor burden compared to their sedentary controls, but the difference did not achieve statistical significance. Both calorie-restricted groups exhibited dramatically reduced tumor yields in comparison to their ad libitum-fed counterparts.

Examination of the patterns of the frequency distribution of tumors/animal in the various groups revealed that the high incidence group HFAL exhibited a pattern characterized by a higher frequency of animals bearing one or more tumors, whereas the low incidence groups (LFAL, MFR, HFR, HFEX) exhibited a pattern in which the distribution as a whole was shifted to the left (Table 4). The MFAL group exhibited an intermediate pattern. Tests for trend indicated a significant negative trend for the 3 ad libitum sedentary groups [Group HFAL > MFAL > LFAL ($P < 0.05$)]. Pairwise comparisons of tumor frequency data (including animals with 0 tumors), by the $\chi^2$ test for homogeneity, between the HFAL and HFEX groups indicated a significant decrease in tumor multiplicity in

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**Table 2. Total mammary tumors by histopathological classification**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (sedentary)</th>
<th>% of fat</th>
<th>No. of effective rats</th>
<th>Adenoma</th>
<th>Fibroadenoma</th>
<th>Adenocarcinoma</th>
<th>Comedo carcinoma</th>
<th>Papillary adenocarcinoma</th>
<th>Cribriform adenocarcinoma</th>
<th>Total mammary tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ad libitum (sedentary)</td>
<td>5</td>
<td>36</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>25</td>
<td>9</td>
<td>38*</td>
</tr>
<tr>
<td>2</td>
<td>Ad libitum (sedentary)</td>
<td>10</td>
<td>36</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>43</td>
<td>5</td>
<td>50</td>
</tr>
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<td>Ad libitum (sedentary)</td>
<td>20</td>
<td>36</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>41</td>
<td>10</td>
<td>59</td>
<td>9</td>
</tr>
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<td>4</td>
<td>Restrict (sedentary)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
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</tr>
<tr>
<td>5</td>
<td>Restrict (sedentary)</td>
<td>26</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Ad libitum (active)</td>
<td>20</td>
<td>30</td>
<td>1</td>
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<td>2</td>
<td>0</td>
<td>24</td>
<td>10</td>
<td>38</td>
</tr>
</tbody>
</table>

* Test for overall negative trend: Group 3 > Group 2 > Group 1 ($P < 0.05$) [Bartholomew’s test (44)]. Pairwise tests: Group 4 or 5 versus Groups 1, 2, 3, and 6 ($P < 0.0001$) and Group 3 versus Group 1 ($P < 0.05$); Group 2 versus Group 1 (not significant), Group 3 versus Group 6 (not significant) by $\chi^2$ test.

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*Adenoma, Fibroadenoma, Adenocarcinoma, Comedo carcinoma, Papillary adenocarcinoma, Cribriform adenocarcinoma.*
the active versus the sedentary (P < 0.01). When multiplicity was expressed in terms of mean number of tumors/tumor-bearing animal, no significant differences were found between any of the groups. When expressed in terms of mean number of tumors/total animals at risk, the MFR and HFR exhibited significantly decreased multiplicity; all other comparisons were nonsignificant due to the highly variable and skewed nature of the tumor multiplicity data.

Analysis of tumor volume data revealed a positive linear relationship between increased tumor volume and increased body fat intake among the 3 sedentary ad libitum groups (HFAL > MFAL > LFAL) (Table 5). However, no differences in mean tumor volumes could be found between the HFAL and HFEX groups.

Activity Profiles. The activity profiles are compared for the most and least active of 30 animals in the activity group; all others fell between these two extremes (Fig. 2). Note that interest in the running wheel reached a peak at 1 mo and slowly waned over time, reaching a plateau around 3 mo. The number of revolutions/day ranged from a high of 25,000 to a low of 400 with the average number of revolutions/day ranging from 2000 to 4000 over the duration of the experiment. On average, the animals ran 1.8 mile/day. The maximum number of miles run per day by an individual rat was 14, and the minimum was 0 miles. In order to determine whether an association existed between the degree of activity and tumor incidence, animals in the active group were segregated into tertiles based on their relative degree of activity (Fig. 3). To our surprise, it was found that the lowest tertile (least active rats) exhibited the greatest reduction in tumor incidence. This relationship held true whether total activity, the early (most active phase), or the later (least active) phase was used as the basis for tertile calculations. Comparison of mean animal weights by activity tertile revealed no differences in weight at termination between groups despite considerable differences in activity. This lack of effect on animal weight gain persisted regardless of how exercise tertiles were calculated (data not shown).

Weight Gain Data. Animal weight gain curves for Groups 1, 2, and 3 were statistically indistinguishable, indicating that, despite wide differences in the caloric density of their respective diets, the animals in the HFAL, MFAL, and LFAL groups consumed food approximately in an isocaloric manner (Fig. 4). Profile analysis based on body weight measures revealed, as expected, that all groups gained weight as a function of time (P < 0.05), and that there was a significant interaction between time and calorie restriction (i.e., a depression in the weight gain profile) and time and exercise (i.e., an elevation in the weight gain profile) (P < 0.0001 in both cases). At termination, the mean (±SD) body weights for the LFAL, MFAL, and HFAL groups were 191 ± 8.5, 193 ± 10, 197 ± 11 g, respectively. Mean body weights for the MFR and HFR groups were 164 ± 6.5 and 165 ± 5.4, respectively. Active animals (HFEX) exhibited the highest mean body weight (217 ± 17 g) with a greater degree of individual variation than the sedentary groups. At termination, energy-restricted groups exhibited a 15% decrease, and active animals, a 13% increase in body weight when compared to their respective ad libitum sedentary controls.

Body Composition. Deposition of fat in adipose tissue, expressed as a percentage of body fat, was similar in all three sedentary, ad libitum-fed groups, indicating that the percentage of body fat was not dependent on fat intake (Table 6). Animals on energy-restricted diets and active animals exhibited significantly lower percentages of body fat compared to their ad libitum sedentary counterparts. In general, water content varied inversely with fat content; protein and ash were similar in all six groups.

![Fig. 1. Kaplan-Meier life table curves for cumulative mammary tumor incidence for Groups 1 to 6. Life table data include all palpable tumors (adenocarcinoma and fibroadenoma). Data points represent total palpable tumors including those that repressed during the experiment. Ordinate, proportion of animals surviving per unit of time without a tumor (1.0 represents 100% tumor-free animals). Abscissa, days past NMU treatment. Test for overall trend: Cox's test for adjusted trends (P < 0.01) and generalized Kruskal-Wallis analysis (P < 0.01). Pairwise comparisons: Cox's test: HFAL versus LFAL (P < 0.001); MFAL versus LFAL (P < 0.05); HFAL versus MFAL (not significant); HFAL versus HFR (P < 0.001); MFAL versus MFR (P < 0.001); HFAL versus HFEX (P < 0.001).](image-url)

Table 3 Mammary tumor incidence

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>% of fat</th>
<th>No. of rats</th>
<th>Nonpalpable adenocarcinoma</th>
<th>Palpable adenocarcinoma</th>
<th>Total adenocarcinoma</th>
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<td>1</td>
<td>Ad libitum (sedentary)</td>
<td>5</td>
<td>36</td>
<td>13</td>
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<td>Ad libitum (sedentary)</td>
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<tr>
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<td>30</td>
<td>12</td>
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<td>14</td>
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</table>

*a Number of animals with one or more nonpalpable adenocarcinomas.
*b Number of animals with one or more palpable adenocarcinomas.
*c Number of animals with one or more palpable and/or nonpalpable tumors.
*d Test for overall negative trend: Group 3 > Group 2 > Group 1 (P < 0.05) (Bartlomelom's test). Pairwise tests: Group 3 versus Group 1 (P < 0.05); Group 2 versus Group 1 (P < 0.05); Group 3 versus Group 6 (P < 0.05) by Fisher's exact test (palpable adenocarcinoma). Other comparisons (nonpalpable and total adenocarcinoma) not significant.
**Table 4 Tumor frequency distribution**

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<td>5</td>
<td>35</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>9</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* n = 36 (Groups 1 to 5), n = 30 (Group 6).

Overall test for significance (comparing all 6 groups) by χ² test for homogeneity (P < 0.05). Pairwise comparisons: Group 1 versus Group 3 (P < 0.05); Group 1 versus Group 2 (P < 0.05); Group 3 versus Group 6 (P < 0.01); all group versus Group 5 (P < 0.001); Group 2 versus Group 3 and Group 4 versus Group 5, not significant, by χ² test.

**Table 5 Tumor volume (mm³)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>% of fat rats</th>
<th>Mean ± SEM</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ad libitum (sedentary)</td>
<td>5</td>
<td>36</td>
<td>504 ± 30 (2-&gt;8,424)</td>
</tr>
<tr>
<td>2</td>
<td>Ad libitum (sedentary)</td>
<td>10</td>
<td>36</td>
<td>682 ± 316 (2-&gt;14,236)</td>
</tr>
<tr>
<td>3</td>
<td>Ad libitum (sedentary)</td>
<td>20</td>
<td>36</td>
<td>1,212 ± 333 (4-&gt;17,740)</td>
</tr>
<tr>
<td>4</td>
<td>Restrict</td>
<td>13</td>
<td>36</td>
<td>354 ± 140 (99-&gt;611)</td>
</tr>
<tr>
<td>5</td>
<td>Restrict</td>
<td>26</td>
<td>36</td>
<td>63 ± 36 (26-&gt;299)</td>
</tr>
<tr>
<td>6</td>
<td>Ad libitum (exercise)</td>
<td>20</td>
<td>30</td>
<td>1,554 ± 535 (2-&gt;12,130)</td>
</tr>
</tbody>
</table>

* Mean ± SEM.

**DISCUSSION**

As expected, animals fed LFAL diets exhibited significantly lower tumor yields than animals fed HFAL diets (Tables 2 to 5). However, the results of feeding the MF diet were less clear. For example, when assessed in terms of either tumor incidence or multiplicity, the MFAL group exhibited a significantly higher tumor yield than the LF group (Tables 3 and 4), suggesting that HFAL and MFAL diets exert similar promoting effects. When assessed in terms of total mammary tumors/group, however, the difference between MFAL and LFAL groups did not achieve statistical significance (Table 2); tests for trend indicated an ordered sequence (HFAL > MFAL > LFAL) in all but one case [total adenocarcinoma (Table 3)], suggesting that the MFAL group occupied an intermediate position between the HFAL and LFAL groups. In contrast, previous studies conducted in our laboratory using a lower dose of NMU (25 mg/kg) indicated that animals fed a MFAL diet exhibited tumor yields significantly lower than those fed a...
HFAL diet (46). The reasons for these incongruities remain to be determined, but they may be due to (a) limitations in the resolving power inherent in the model used or (b) differences in the dose of NMU administered.

With regard to caloric reduction, our results unambiguously show that restricting energy intake to 75% of ad libitum controls dramatically reduces tumor yields, regardless of total fat intake. These results are consistent with those reported by Lavik and Baumann (47), Kritchevsky (2), Pariza (3), and Thompson (48), but they contradict the earlier studies of Tannenbaum (13, 14) who observed that the influence of fat intake was observable even in calorie-restricted animals. The reasons for this discrepancy are unclear, but could lie in differences in dietary constituents, degree and type of calorie restriction, or in the models used (Tannenbaum used the spontaneous mouse mammary tumor model, whereas the others used chemically induced rat mammary tumor models).

It is noteworthy that the degree of tumor inhibition due to caloric restriction was considerably greater in this study than that reported in previous studies (2, 3, 17, 18). The reason for this is unclear at present but may be related to the fact that the inbred F344 strain was used in this study, whereas the outbred Sprague-Dawley rat was used in the other studies. Also, in the latter, the lipid-soluble procarcinogen DMBA (which requires host activation) served as the initiating agent in other reported studies, while in the present study we used the direct-acting, water-soluble carcinogen NMU (which does not require host activation). Hence, despite the fact that diet restriction was implemented 2 days after carcinogen administration, differences in the pharmacokinetics and tissue disposition of the two carcinogens may have had a bearing on the observed results.

To our knowledge, this is the first report showing that voluntury exercise can effectively reduce tumor yields and delay time of tumor appearance in an experimental tumor model. Several investigators have reported the results of forced exercise in the form of a motor-driven wheel cage, or treadmill, on tumorigenesis (19, 20, 23–25). Rusch and Kline (19), in an early study, using a motor-driven rotating cage, reported a decrease in the growth of a transplantable fibrosarcoma and a reduction in weight gain in active versus sedentary ABC mice. Later, Moore and Tittle (20), using motorized wheel-running, reported a complete absence of DMBA-induced mammary tumors in exercised animals, a reduction in body weight, and a decreased body fat content. More recently, three conflicting reports (23–25) appeared in abstract form using the DMBA model and varying amounts of forced treadmill running as the form of exercise. Bennink et al. (23) reported that exercise caused a modest reduction in tumor incidence (~16%), a greater one in tumor size (~23%), and a 40% reduction in fat stores compared to sedentary controls. Yednak et al. (24) reported that activity reduced tumor incidence under LFAL, but stimulated it under HFAL conditions; and in marked contrast to our results, Thompson et al. (24) found that exercised animals exhibited greater DMBA-induced mammary tumor incidence than sedentary controls (100% versus 79%).

As the above indicates, the results of exercise studies to date are as inconsistent as the exercise protocols used were varied. The study of Thompson et al. (24) is perhaps the most instructive, since it underlines what may prove to be a key variable in activity studies, namely, the intensity of exercise. When our data were grouped into tertiles based on level of activity (Fig. 3), it was found that the least active tertile exhibited the lowest tumor incidence; that is, the antipromoting effect of activity was inversely proportional to the amount of exercise. The possibility therefore may be considered that intense, forced exercise, typical of treadmill running, may enhance tumor development (as seen in the Thompson study and in our highest exercise tertile), while less intense exercise may act to inhibit tumor development.

A second reason for the inconsistencies found in previous studies may be chronobiological in nature. Under voluntary wheel-running conditions, animals are active during the evening hours in keeping with the nocturnal habit of rodents. Treadmill protocols, however, are, for practical reasons, often conducted during daylight hours when rodents are relatively inactive. Hence, differences in physiological responses to low and high intensity exercise (49, 50) and to disturbances in circadian light/dark rhythms (51) may underlie the variable tumor responses noted above.

The quantity and pattern of wheel-running reported here were consistent with those reported by others (52–57). For example, Tokuyama and Okuda (52) reported that female Wistar rats ran an average of 11,000 revolutions/day. Goodrick (53) reported an average of 9,000 revolutions/day. Moreover, Russell et al. (54), Shyu et al., and Goodrick (53) all reported exercise patterns similar to ours, namely, an early peak followed by a gradual decline. Compensatory hyperphagia, resulting from voluntary wheel activity, has also been observed by others (56, 57). While a decrease in body fat content was consistently noted in the above studies, variable results were obtained with regard to body weight gain. In one study, body weight gains were negligible (57), while a decrease (53) and an increase (56) were observed in the other 2 studies. It may be noted that the weight differential (HFEX versus HFAL) was manifested over the last 10 wk of the experiment when activity levelled off (Fig. 2), suggesting that high caloric intake continued despite a lower level of energy expenditure during this period. Our finding of reduced body fat content in HFEX and MFR and HFR restricted animals (Table 5) indicates that both experimental conditions act to alter energy homeostasis leading to mobilization of lipid from adipose tissue to meet energy needs. In contrast to Boissonneault et al. (18), however, we did not see a
compensatory increase in lean body mass in either the restricted or active groups. Our results indicate that, while the fat:lean body mass ratio may serve as an accurate index of tumor incidence under certain conditions, the correlation cannot be generalized, since the LFAL group which had the highest ratio of fat to protein exhibited reduced, rather than enhanced, tumor yields (Tables 2 and 3).

Based on the work of Forbes (58) and on his own studies using the DMBA-tumor model (3), Pariza has proposed that the tumor-promoting effect of HFAL diets is a direct consequence of the host's more efficient use of fat calories compared to calories from other sources. According to this hypothesis, animals fed HFAL diets would be expected to store more fat than animals fed a LFAL diet. Supporting this view is the finding by Boissonneault et al. (18) that calorie-restricted animals exhibited low tumor yields even when fed a HFAL diet, and that LFAL animals weighed less, stored less body fat, and increased their protein content (lean body mass) compared to HFAL animals. Contrary evidence, however, can also be cited. For example, under ad libitum feeding conditions, HFAL and LFAL animals consumed roughly equal amounts of calories, but exhibited widely divergent tumor yields (Tables 2 and 3). Also, not all HFAL diets promote mammary tumorigenesis equally despite the fact that they contain equivalent high caloric densities. For example, HFAL diets containing olive (59), coconut (59), or marine oils (60) lack tumor-promoting effects. Although the reasons for this are uncertain, some evidence points to a limiting effect of dietary EFA (10). In the present study it was found, in contrast to Boissonneault's (18) report, that body weight, percentage of body fat, and lean body mass were similar in HFAL, MFAL, and LFAL groups (Table 6; Fig. 4). Our body weight results are in keeping with the majority of reports indicating that, under ad libitum conditions, weight gain curves from animals fed HFAL, MFAL, or LFAL diets are indistinguishable in short-terms studies (9).

Various mechanisms, other than caloric excess, have been proposed to explain the promoting effect of HFAL diets, including fat-induced alterations in hormone secretion, eicosanoid metabolism, intracellular communication, and immune functions (9). But none of these has been conclusively demonstrated. With regard to the tumor-inhibiting effect of energy restriction, a number of hypotheses can be considered. Energy restriction may lead, for example, to reduced availability of nutrients for the growing tumor (5). Energy restriction has also been associated with endocrine alterations (61), a decrease in circulating prolactin (62), altered EFA content of adipose tissue stores (63), and enhanced natural killer cell activity (64). Moreover, since the NMU tumor expresses an activated H-ras oncogene (65), energy restriction may suppress the expression of this gene. Dietary energy restriction may also exert an anti-promoting effect by inhibiting overall body weight gain (4). It is well known that caloric restriction results in a more aggressive and irritable animal (66), a fact noted in this study; hence neurochemical changes, associated with energy restriction, may also be associated with reduced tumor yields (67).

How increased energy expenditure may inhibit mammary tumorigenesis is only remotely understood. Most studies on the potential health benefits of exercise have focused on longevity (68), circulatory disorders, and related changes in plasma lipoproteins (69, 70). There have been some studies linking changes in circulating hormone titers (71, 72), prostanoids (73), β-endorphin levels (74, 75), and enhanced immune functions (76) to exercise but, as of the present, it is unclear precisely how these changes may be related to carcinogenesis.

It is noteworthy that our studies in a laboratory animal are consistent with the results of epidemiological studies by Frisch and coworkers (21, 22). In these studies, it was reported that the prevalence (life-time occurrence) rates of cancers of the reproductive tract (uterus, cervix, and ovary) and breast were significantly lower in former women college athletes compared to nonathletes (21). In addition, women athletes were taller and heavier and exhibited lower body fat content than nonathletes. It was also shown that the prevalence rates of benign breast disease (a risk factor for breast cancer) and benign tumors of the reproductive tract were significantly lower in women athletes compared to nonathletes (22).

In summary, the promoting effect of HFAL diets and the antipromoting effects of energy restriction—increased energy expenditure, observed in this study, did not correlate with either body weight, caloric intake, or the ratio of fat to lean body mass, suggesting that these effects may be mediated indirectly and, possibly, independently via paracrine, endocrine, or neuroendocrine mechanisms.

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Influence of Dietary Fat, Caloric Restriction, and Voluntary Exercise on N-Nitrosomethylurea-induced Mammary Tumorigenesis in Rats

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