Effect of caloric restriction on pre-malignant and malignant stages of mammary carcinogenesis

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Caloric restriction has documented beneficial effects on numerous diseases including cancer, yet the mechanism(s) that accounts for these wide ranging benefits is unknown. Part of the difficulty in defining mechanisms has been the long-term nature of experimental protocols in which these beneficial effects have been observed and the inherent difficulty of investigating mechanisms in such studies. The experiments reported were designed: (1) to determine if caloric restriction would inhibit mammary carcinogenesis in a model for this disease process that is 35 days in duration; (2) to determine if progression from pre-malignant to malignant stages of mammary carcinogenesis was affected by caloric restriction; and (3) to explore whether the effects of caloric restriction were associated with changes in adrenal function. Mammary carcinogenesis was induced in female Sprague-Dawley rats by the i.p. administration of 1-methyl-1-nitrosourea (50 mg/kg body weight) at 21 days of age. Rats were randomized to one of four dietary treatment groups: ad libitum fed, or restriction of food intake to 90, 80 or 60% of the ad libitum intake. Rats were palpated for detection of mammary tumors and all mammary lesions excised at necropsy were histologically classified. Twenty-four-hour collections of urine were obtained at weekly intervals throughout the 35-day experiment. Urine was assayed for corticosterone by direct radioimmunoassay. Caloric restriction resulted in both a dose dependent prolongation of latency to palpable carcinomas \(P<0.01\) and a reduction in final incidence of mammary cancer; the dose response was linear \(P<0.05\). The percentage of pre-malignant mammary lesions in a group increased with increasing degree of caloric restriction, whereas the percentage of carcinomas decreased \(P<0.05\). The level of cortical steroid increased linearly with increasing caloric restriction \(P<0.01\) an effect that was not attenuated over time. Poisson regression analyses with the number of cancers per rat as the dependent variable, level of caloric restriction as the independent variable and urinary cortical steroid excretion as a co-variate were performed. These analyses indicated that the variation in cancers per rat, irrespective of the treatment group to which an animal was assigned, could be accounted for by urinary cortical steroid excretion \(P<0.05\); i.e. urinary cortical steroid excretion was an independent predictor of an animal’s carcinogenic response. The data reported in this study support the use of a short term model to study the mechanism(s) by which caloric restriction inhibits mammary carcinogenesis and point to both a stage in the disease process, the conversion of pre-malignant to malignant cells, and a target tissue (adrenal gland) and chemical species (adrenal cortical steroid) that may be involved in mediating the protective effects of energy restriction. These data indicate the feasibility of identifying a chemical basis for the protective effect of caloric restriction that is independent of energy restriction \(per se\) and this, in turn, indicates that it may be possible to circumvent the practical problem of implementing a program of chronic energy restriction in human populations, yet still achieve the wide-ranging health benefits of such a program.

Introduction

Decades of investigation have clearly established that dietary restriction, in all animal species tested thus far, increases life span and reduces the occurrence of many age-associated degenerative diseases including cancer (1–4). Recent studies have convincingly demonstrated that the inhibitory effect of dietary restriction on carcinogenesis is due specifically to a reduction in the intake of calories and that inhibition of carcinogenesis is not due to a reduction in the intake of other nutrients (5–7). Moreover, in several studies protection against carcinogenesis by calorie restriction has been shown to be directly proportional to the degree of calorie restriction imposed (8,9). Such findings have led to hypotheses that the effect(s) of calorie restriction is non-specific and is linked to a limitation of energy available for cancer cells to grow. In fact, this and related hypotheses dominated the early decades of research in this area (10,11). However, an accumulating amount of evidence points to a specific effect of caloric restriction on various growth factors, oncogenes and tumor suppressor genes that are involved in the carcinogenic process (12–16). Moreover, data recently reported by our laboratory fails to support that energy restriction \(per se\) is sufficient for protection against carcinogenesis to be manifest (17). Specifically, it was observed that calorie restriction plus exercise failed to inhibit carcinogenesis, despite the fact that body weight gain, carcass energy and carcass fat of exercised and calorie-restricted animals were reduced to a greater extent than by calorie restriction or exercise alone. These unexpected findings underscore the importance of understanding the basis for the cancer inhibitory activity of agents, such as calorie restriction or exercise, that modulate energy metabolism.

The experiments reported in this study were conducted using a short term model for mammary carcinogenesis recently published by our laboratory (18). This model offers significant advantages compared to other related models. Morphologically identifiable intermediate stages in the disease process comparable to those that occur in the human disease can be studied and the disease process is compressed into a 5-week versus a 6-month time period. The experiments reported were designed:

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During two food access periods or meals per day. Caloric restriction was then plotted in panels B–D. Panel B shows the pattern of daily food intake, the cumulative weight gain to food intake ratio and body weight gain. The data ratified that ad libitum-fed groups were essentially identical. The rats had continuous access to food. For the next 3 days rats were meal fed, ad libitum as described in a subsequent paragraph. the relationship between cancers per rat, level of caloric restriction, and initiated. All rats were meal fed and given two meals per day (6:00–9:00 a.m. and 2:00–5:00 p.m.), 7 days per week. The meal feeding protocol was used so that the same pattern of meal eating would be imposed on calorie-restricted and on ad libitum fed rats. Using this design, possible confounding due to intergroup variation of meal timing, meal number, and duration of fasting that has not generally been controlled in caloric restriction experiments was avoided. Rats in the ad libitum-fed groups were allowed access to an unlimited amount of diet each meal, while rats in calorie restricted groups were given a restricted amount of the diet each meal as designated in the protocol. Uneaten or spilled diet was carefully collected to permit calculation of the actual amounts of diet consumed.

### Materials and methods

#### Animals

A total of 147 weanling female Sprague–Dawley rats were obtained from Taconic Farms Germantown, NY, and were housed individually in stainless steel metabolic cages with wire mesh bottoms. The cages were equipped with adjustable width external tunnel feeders that permitted accurate quantification of food intake. The animal facility in which the rats were housed is AAALAC accredited. Rooms were maintained at 22 ± 1°C with 50% relative humidity and a 12-h light/12-h dark cycle. The work reported was reviewed and approved by AMC’s Institutional Animal Care and Use Committee (IACUC) and conducted according to IACUC guidelines.

#### Experimental design

The sequence of events that comprised the experimental protocol is shown in Table I. Because a limited number of metabolic cages equipped with tunnel feeders were available, two experiments were conducted. In experiment 1, 75 rats were administered 50 mg MNU/kg body weight (i.p.) at 21 days of age as previously described (18). Following carcinogen treatment rats were randomly divided into three groups: ad libitum fed, restricted-fed 90% of ad libitum intake, or restricted-fed 80% of ad libitum intake. Twenty-five rats were assigned to each group. In experiment 2, 72 rats were injected with either saline in which MNU was dissolved (24 rats) or 50 mg MNU/kg body weight (48 rats). One half of the rats injected with the saline solvent or with MNU were randomized to one of two dietary treatment groups: ad libitum fed or restricted-fed to 60% of ad libitum intake. Rats in all groups were meal fed as described in a subsequent paragraph.

#### Diets

A modification of AIN-93G diet was used (21). The diets fed to caloric-restricted animals were formulated to ensure an equivalent intake of all nutrients, while limiting total dietary calories. The composition of the diets provided to ad libitum-fed and caloric-restricted rats is presented in Table II.

#### Feeding protocol

After administrating carcinogen, all rats were acclimated to their environmental conditions and fed modified AIN-93G diet for 8 days. For the first 5 days rats had continuous access to food. For the next 3 days rats were meal fed, meaning that they could consume an unlimited quantity of food, but only during two food access periods or meals per day. Caloric restriction was then

### Table I. Sequence of events that comprised the experimental design

<table>
<thead>
<tr>
<th>DOA*</th>
<th>21</th>
<th>25</th>
<th>27</th>
<th>30</th>
<th>32</th>
<th>39</th>
<th>42</th>
<th>46</th>
<th>53</th>
<th>56</th>
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<tbody>
<tr>
<td>DPC*</td>
<td>0</td>
<td>4</td>
<td>6</td>
<td>9</td>
<td>11</td>
<td>18</td>
<td>21</td>
<td>25</td>
<td>32</td>
<td>35</td>
</tr>
</tbody>
</table>

| MNUI-injectionb | X  |
| Calorie restriction | ←|←|←|←|←|←|←|←|←|←|
| Tumor palpation | 1  |
| Urine collection | 2  | 3  |
| Termination | ←|←|←|←|←|←|←|←|←|←|

*Days of age, DOA; days post-MNU, DPC.
*bDetails of the procedures listed are provided in the Materials and methods section.

#### Table II. Composition of diets

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Control</th>
<th>90% RF</th>
<th>80% RF</th>
<th>60% RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>32.50</td>
<td>30.56</td>
<td>28.12</td>
<td>20.84</td>
</tr>
<tr>
<td>Cerealose</td>
<td>32.50</td>
<td>30.56</td>
<td>28.12</td>
<td>20.84</td>
</tr>
<tr>
<td>Solka-Floc</td>
<td>5.00</td>
<td>5.56</td>
<td>6.24</td>
<td>8.33</td>
</tr>
<tr>
<td>Casein</td>
<td>20.00</td>
<td>22.22</td>
<td>25.00</td>
<td>33.33</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.30</td>
<td>0.33</td>
<td>0.38</td>
<td>0.50</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.00</td>
<td>5.56</td>
<td>6.26</td>
<td>8.33</td>
</tr>
<tr>
<td>AIN-93 G vitamin mixb</td>
<td>1.00</td>
<td>1.11</td>
<td>1.25</td>
<td>1.67</td>
</tr>
<tr>
<td>AIN-93 G mineral mix</td>
<td>3.50</td>
<td>3.88</td>
<td>4.38</td>
<td>5.83</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.20</td>
<td>0.22</td>
<td>0.25</td>
<td>0.33</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Energy (kcal/g)</td>
<td>3.94</td>
<td>3.93</td>
<td>3.93</td>
<td>3.90</td>
</tr>
<tr>
<td>Protein/total kcal</td>
<td>0.21</td>
<td>0.23</td>
<td>0.26</td>
<td>0.35</td>
</tr>
<tr>
<td>Carbohydrates/total kcal</td>
<td>0.68</td>
<td>0.64</td>
<td>0.60</td>
<td>0.46</td>
</tr>
<tr>
<td>Fat/total kcal</td>
<td>0.11</td>
<td>0.13</td>
<td>0.14</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*Given in g/100 g; restricted fed (RF).
*bThe composition of the vitamin and mineral mixes is given in reference (21).

#### Results

**Food intake, energy utilization ratio and body weight gain**

As shown in Figure 1, panel A the food intake, the cumulative weight gain to food intake ratio and growth curves of animals in both control groups were essentially identical. The data from both groups were pooled into a single control group as plotted in panels B–D. Panel B shows the pattern of daily

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**References**

1. Z. Zhu, A.D. Haegele and H.J. Thompson
Caloric restriction and mammary carcinogenesis

Fig. 1. Effect of caloric restriction on dietary intake, body weight gain and the ratio of cumulative body weight/cumulative diet intake. Panel A. Diet intake (DI), cumulative ratio of body weight gain (BWG) to diet intake and group mean body weights of rats fed ad libitum (control group) in Experiment 1 and Experiment 2. Values were not statistically different. The data from both control groups were pooled for presentation in Panels B–D. Diet intake, cumulative ratio of body weight gain to diet intake and body weight of control and calorie restricted rats are shown in panels B, C and D, respectively. Values are mean ± SEM for each point. Differences among groups were analysed by ANOVA.

Food intake throughout the course of the experiment. Food intake in all groups increased steadily and the amounts of food consumed confirmed that the intended levels of caloric restriction were achieved. Panel C shows the ratio of cumulative weight gain to cumulative diet intake. This ratio provided an indication of the partition in the use of ingested dietary energy for growth relative to maintenance functions. This ratio was lower with increasing level of caloric restriction, an observation that was consistent with the expectation that when an animal’s available energy for maintenance and growth is restricted, a greater proportion of that energy is utilized for maintenance versus growth. The rate of body weight increase is shown in panel D. Two points are noteworthy. First, all rats were in positive energy balance, i.e. they were growing. Secondly, as a percentage of the control group, animals fed 90, 80 or 60% of the amount eaten by the ad libitum fed rats (control group) had final group mean body weights that were 87, 81 and 69% of that observed in the control group, respectively.

Carcinogenic response

All mammary tumors excised at necropsy and lesions excised from mammary whole mount preparations were histologically diagnosed and classified as intraductal proliferations (IDP), ductal carcinoma in situ (DCIS) or adenocarcinoma (AC). Mammary lesions containing areas of malignant and pre-malignant cells were scored as cancers. As expected, the administration of MNU resulted in the induction of both pre-malignant and malignant lesions. The carcinogenic response in both control groups was statistically indistinguishable; thus the data were pooled for the purposes of analysis and presentation. The effect of calorie restriction on the latency to detection of palpable mammary carcinomas is shown in Figure 2. Latency in this model system is short. Nonetheless, latency was delayed by caloric restriction (P < 0.01), and no palpable carcinomas were detected in rats restricted to 60% of ad libitum fed controls. This figure also shows the incidence of mammary carcinomas based on all lesions identified at necropsy. Caloric restriction resulted in a dose-dependent reduction in cancer incidence (P < 0.01). Table III and Figure 3 show the effects of caloric restriction on the average number of pre-malignant and malignant lesions induced in the mammary gland. The overall effect of caloric restriction on induction of IDP, DCIS and AC was evaluated simultaneously using multivariate regression analyses. The ordering of the magnitude of inhibitory activity of caloric restriction on lesion
occurrence was AC > DCIS > IDP. Interestingly, the inhibitory effect of caloric restriction on IDP was not statistically significant ($P = 0.18$). It was particularly notable that the percent of IDP and DCIS in a group increased with increasing degree of caloric restriction, whereas the percentage of AC decreased (Figure 3). Since exploratory graphical analysis of these data suggested that the protective effect of caloric restriction was increasing at an increasing rate with greater restriction, regression analyses were performed. Those analyses supported a linear rather than a curvilinear response curve ($P < 0.05$).

# Adrenal Function

The effect of caloric restriction on urinary excretion of cortical steroid (CS) is shown in Figure 4. Data were obtained weekly throughout the experiment. The level of cortical steroid increased with increasing caloric restriction ($P < 0.01$). This same relationship was observed irrespective of whether the data were expressed as $\mu$g CS per kg body weight/day, total amount of CS excreted in 24 h, $\mu$g CS excreted per mg creatinine or $\mu$g CS per mg creatinine per day. It is noteworthy that administration of carcinogen caused a transient increase in urinary cortical steroid excretion in comparison to rats not treated with carcinogen. Interestingly, the level of urinary cortical steroid was not only directly related to level of caloric restriction, but the effect was maintained throughout the period of observation. The only exception was in the MNU-treated control group in which levels decreased throughout the experiment and which were indistinguishable statistically from those of untreated animals during the last 2 weeks of the experiment.

The results of Poisson regression analyses with number of cancers per rat as the dependent variable, level of caloric restriction as the independent variable and urinary cortical steroid as a covariant revealed that when the variance in cancers per rat that was attributed to urinary cortical steroid was removed from the regression analysis, that there was no effect on cancers per rat that was attributed to level of caloric restriction. In other words, urinary cortical steroid excretion of an independent predictor of the carcinogenic response as affected by caloric restriction.

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**Table III. Effect of calorie restriction on the proportion of intraductal proliferations, ductal carcinoma in situ and carcinomas in mammary glands**

<table>
<thead>
<tr>
<th>Group</th>
<th>IDP</th>
<th>DCIS</th>
<th>AC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.08</td>
<td>0.37</td>
<td>4.55</td>
<td>6.00</td>
</tr>
<tr>
<td>90% RF</td>
<td>0.88</td>
<td>0.16</td>
<td>2.72</td>
<td>3.76</td>
</tr>
<tr>
<td>80% RF</td>
<td>1.08</td>
<td>0.04</td>
<td>1.76</td>
<td>2.88</td>
</tr>
<tr>
<td>60% RF</td>
<td>0.63</td>
<td>0.13</td>
<td>0.29</td>
<td>1.04</td>
</tr>
</tbody>
</table>

*All rats were meal fed. Animals in the control group were fed ad libitum at each meal; calorie-restricted rats (RF) were fed 90, 80 or 60%.

*These data have a negative binomial distribution. They were statistically analysed by multivariate analysis of variance. There was a highly significant overall effect of calorie restriction on lesion occurrence ($P < 0.01$); this effect was primarily due to a reduction in AC ($P < 0.001$), and to DCIS ($P = 0.04$); the effect on IDP was not significant ($P = 0.18$).
Caloric restriction and mammary carcinoogenesis

Discussion

Overview
While the effects of caloric restriction on mammary carcinoogenesis have been investigated extensively, the data presented in this study provide new insights about the protection that is rendered by caloric restriction, and these data demonstrate the utility of a new model system for investigating the effects of caloric restriction on the process of mammary carcinoogenesis. Having stated this, an important question to address at the outset of this discussion is whether the phenomenon of caloric restriction mediated inhibition of carcinoogenesis merits further investigation. We submit that it does. While it could be argued that a sufficient rationale for this work rests on data indicating that (1) caloric restriction is the most universally protective agent against carcinoogenesis that has been identified (5–9), or (2) that caloric restriction is the best tolerated and most beneficial intervention defined for cancer prevention that also has documented beneficial effects on numerous other diseases (1–4), we promote a different rationale. Emerging evidence indicates that the protective effects of caloric restriction are unlikely to be accounted for directly by factors related to energy metabolism and that simply invoking an energy restricted state is insufficient to protect against cancer (12–17). This suggests a chemical basis for the protective effect of caloric restriction that is independent of energy restriction per se and this in turn indicates that it may be possible to circumvent the practical problem of implementing a program of chronic energy restriction in human populations, yet still achieve the wide-ranging health benefits of such a program. Thus, by understanding the basis for the protective effect of caloric restriction, new insights may be gained that point to both a genetic cascade of events that can be targeted to prevent cancer in the absence of detrimental side-effects and the chemical basis by which to regulate these events. It is with these goals in mind that we judge that the pursuit of the mechanistic basis of caloric restriction-mediated inhibition of carcinoogenesis has merit.

Carcinogenic response
As shown in Figure 2 caloric restriction resulted in both a dose-dependent prolongation of latency to palpable carcinomas and a reduction in final incidence of mammary cancer. The dose-response curve is best fit by a linear rather than a quadratic regression equation; thus these data are consistent with previous reports that the protective effect of caloric restriction in other chemically-induced models of mammary carcinoogenesis is directly proportional to the level of caloric restriction imposed (8,9). Thus, these findings support the use of what we refer to as a short-term model of mammary carcinoogenesis as described in reference (18) for the investigation of the protective effects of caloric restriction against this disease process. This is not a trivial point. The labor required to carefully conduct controlled feeding studies is considerable and the opportunity for error is great, particularly if studies must be conducted for 4–6 months. The ability to study the effects of caloric restriction on mammary carcinoogenesis in an experimental protocol of 35 days duration is therefore significant. In addition, since the various elements of carcinoogenesis are compressed from 6 months to 5 weeks, we argue that this model will facilitate the investigation of mechanisms.

One particular advantage of the short-term model (18), is that both pre-malignant and malignant mammary gland lesions can be detected and the effects of a treatment on their frequency can be investigated. As shown in Table III, caloric restriction dramatically reduced the number of mammary carcinomas detected at necropsy and the response was caloric restriction dose dependent and best fit by a linear regression model. However, it was observed that restriction did not cause a proportionate reduction of all lesion types, but as shown in Figure 3, the percentage of lesions within a treatment group that were classified as IDP or DCIS actually increased with increasing level of caloric restriction; whereas, the proportion of lesions that were classified as AC decreased. We are unaware of any previous reports of differential effects of caloric restriction on the frequency of occurrence of pre-malignant and malignant mammary gland lesions and recognize the need for cautious interpretation of such data. Nonetheless, these data imply that caloric restriction may be inhibiting a molecular cascade of events involved with progression from locally non-invasive stages of the disease to the invasive state. These data also imply a specificity in the effect(s) of caloric restriction that previously has not been reported. Clearly, such data provide a new direction on which to focus mechanistic inquiries.

Energy metabolism
The body weight data shown in Figure 1, panel D emphasize that both ad libitum-fed and restricted-fed animals were in positive energy balance; all rats were gaining weight in parallel, but at different rates that were proportional to the level of caloric restriction imposed. This point is important since there is frequently the perception that in studies of energy restriction that animals are starving and that they are losing weight; this clearly was not the case. Whereas the food intake data in panel B serve to verify that the targeted levels of food restriction were attained, the cumulative weight gain/diet intake ratios shown in Figure 1, panel C indicate that the manner in which total dietary energy ingested was utilized for growth versus maintenance functions was significantly impacted by caloric restriction. As noted in the Results section this ratio provides a useful index by which to monitor whether the effect of energy restriction on the relative use of energy is changing within the same treatment group over time, as well as providing an index to assess relative differences among groups at any given time. Thus, the data presented in Panel C indicate that despite the fact that a fixed level of dietary restriction was imposed over time, that a stable pattern of relative energy utilization was maintained. Without such data, it could be argued that the effective degree of energy restriction might decline over time due to metabolic compensations in energy metabolism. However the data reported are not supportive of that hypothesis.

Adrenal function
An objective of this study was to assess the potential merit of an old hypothesis, namely that a change in adrenal function, more specifically the over production of cortical steroids is associated with the cancer inhibitory activity of caloric restriction (19,20). Due to the time averaged, non-invasive nature of urine collection, cortical steroid abundance in 24-h urine collections was selected as the method of choice for assessing adrenocortical activity. Urinary cortical steroid measurement is a well established method of estimating adrenal cortical activity that largely avoids the well documented confounding issues associated with serum cortico-steroid measurement (episodic secretion, diurnal variation, and sample collection induced stress) (25–27).

The data reported in Figure 4 strongly support the hypothesis
that adrenal cortical activity increased in proportion to the level of caloric restriction imposed, and that the increase paralleled proportionately the degree of inhibition of carcinogenesis observed. While circumstantial, these data support further evaluation of the hypothesis that the adrenal gland is involved in mediating the protective effect of caloric restriction. The data presented in Figure 4 are also of interest because they show (1) that carcinogen administration was associated with an increase in the level of cortical steroid excretion, but that the increase was transient; (2) that the occurrence of mammary carcinomas in this model system was not associated with an increase in urinary cortical steroid excretion, an observation that casts doubt on the perception that animals bearing tumors become stressed in the sense of stimulating adrenal cortical activity, and (3) that the stimulation of adrenal activity detected by this assay did not diminish over time, at least over the period of observation studied. The fact that this activity was not diminished lends additional support to a causal role of the adrenal gland in the inhibition of carcinogenesis. Whether changes in this activity occur when other agents that protect against cancer are administered is a matter of speculation, but may merit evaluation.

Summary

Evidence is accumulating that indicates that caloric restriction triggers a host of physiological and biochemical changes that are associated with health benefits in many animal species, and it is becoming apparent that specific molecular events are likely to account for its cancer inhibitory activity. The data reported in this study support the use of a short-term model to study these mechanisms and point to both a stage in the disease process, the conversion of pre-malignant to malignant cells, a target tissue (the adrenal gland) and a chemical species (cortical steroids) that may be involved in mediating the protective effects of energy restriction. This knowledge should serve to facilitate the elucidation of the molecular mechanisms that underlie the cancer inhibitory activity of caloric restriction.

Acknowledgements

The authors thank John McGinley, Kim Rothhammer and Weiqin Jiang for their excellent technical assistance. This work was supported by PHS grant CA 52626 from the National Cancer Institute.

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25. Zadik,Z., De Lacerda,L., De Carmargo,L.A., Hamilton,B.P., Migeon,C.J. and Good,R.A. (1995) Dietary lipids and calorie restriction affect mammary adrenal cortical activity, and (3) that the occurrence of mammary carcinomas in this model system was not associated with an increase in urinary cortical steroid excretion, an observation that casts doubt on the perception that animals bearing tumors become stressed in the sense of stimulating adrenal cortical activity, and (3) that the stimulation of adrenal activity detected by this assay did not diminish over time, at least over the period of observation studied. The fact that this activity was not diminished lends additional support to a causal role of the adrenal gland in the inhibition of carcinogenesis. Whether changes in this activity occur when other agents that protect against cancer are administered is a matter of speculation, but may merit evaluation.

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