Dietary energy restriction does not inhibit pancreatic carcinogenesis by N-nitrosobis-2-(oxopropyl)amine in the Syrian hamster

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Dietary energy restriction was previously shown to be effective in preventing a wide range of experimentally induced cancers. Studies were conducted to assess the influence on pancreatic carcinogenesis of dietary energy restrictions (reduced fat and carbohydrate) of 10%, 20%, or 40% in comparison with control in Syrian hamsters treated with N-nitrosobis(2-oxopropyl)amine (BOP). Two carcinogenesis studies were conducted. One used a single treatment with 20 mg BOP/kg body weight and followed hamsters for 102 weeks following treatment, and the other used three weekly treatments of 20 mg BOP/kg body weight and followed hamsters for 45 weeks after treatment. Hamsters were fed control or energy restricted diet beginning the week following the last BOP treatment. Pancreatic carcinomas were induced in 9–18% of the hamsters in the first experiment and in 59–66% of the animals in the second. Dietary energy restriction did not influence carcinoma incidence in either study, and in the second experiment the multiplicity of tumors was higher in the 40% energy restriction (ER) group than in control hamsters. Plasma corticosterone was suppressed by BOP treatment, particularly in the 20% and 40% ER hamsters in the second experiment, and diet or BOP treatment did not significantly alter plasma cortisol. Pancreatic protein kinase Cζ measured by Western blot was highest in the cytosol and particulate fractions of the 40% ER hamsters in the first experiment. These results indicate that dietary energy restriction is not effective in the prevention of BOP induced pancreatic carcinogenesis in the Syrian hamster.

Introduction

Dietary energy restriction has been shown to be a potent and reproducible inhibitor of numerous models of rodent cancer (1–5). In addition, studies of human cancer risk suggest that obesity and physical inactivity are potential factors in the risk of cancer of the breast, colon, prostate and endometrium (6). Previous studies in our laboratory aimed at assessing the role of dietary fat in pancreas cancer using the Syrian hamster model suggested that a slight dietary restriction of hamsters resulted in a delay in the onset of pancreatic cancer in hamsters (7). This study initially used meal-fed control diet groups in comparison with freely fed control diet animals. The meal-fed hamsters were allowed free access to food when it was apparent that they had learned to consume less than the hamsters that had never been restricted (7).

The studies in the present report were designed to determine if dietary energy restriction of hamsters following treatment with the pancreatic carcinogen N-nitrosobis(2-oxopropyl)amine (BOP*) would inhibit pancreatic ductular carcinogenesis. Two carcinogenesis studies were conducted, one with a single dose of the pancreatic carcinogen BOP, which resulted in a low incidence and multiplicity of pancreatic carcinomas, and the other with multiple doses of carcinogen, which resulted in a much higher incidence and number of pancreatic carcinoma. In addition, in the first of these studies we assessed protein kinase C (PKCζ) at the end of the experiment in a portion of the pancreas of control animals because of the importance of this enzyme in cell proliferation and because in our previous studies the expression of this kinase in the duct epithelial cells (8) was reduced in energy restricted hamsters (9). In the second experiment we assessed circulating glucocorticoid hormones in plasma of hamsters in each of the treatment groups. Previous studies indicated that elevations in glucocorticoid hormone may mediate some of the cancer prevention by dietary energy restriction in the skin (10) and lung (11). Furthermore, topical (12) and systemic (13) glucocorticoid treatment inhibited tumorigenesis in mouse skin.

Materials and methods

Male Syrian golden hamsters were obtained from the Eppley Institute breeding colony (Onei[SYR]) at 6 weeks of age for all experiments. They were housed at a temperature of 20 ± 2°C, relative humidity of 40 ± 5% and a 12-h dark–light cycle in controlled facilities. All procedures on live animals were approved by the University of Nebraska Medical Center Institutional Animal Review Committee. Hamsters were randomized into the experimental groups and fed control hamster diet from 6 weeks of age until 1 week following the final BOP treatment. They were then fed the assigned experimental diet or the control diet (Table I) until the end of the experiment. Diet formulations

Table I. Experimental diets for control and dietary energy restricted (ER) hamsters

<table>
<thead>
<tr>
<th>Component</th>
<th>Control (%)</th>
<th>10% ER (%)</th>
<th>20% ER (%)</th>
<th>40% ER (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>19.0</td>
<td>20.4</td>
<td>22.1</td>
<td>26.6</td>
</tr>
<tr>
<td>Dextrin</td>
<td>45.0</td>
<td>43.2</td>
<td>41.0</td>
<td>35.5</td>
</tr>
<tr>
<td>Dextrose</td>
<td>15.4</td>
<td>14.8</td>
<td>14.1</td>
<td>12.2</td>
</tr>
<tr>
<td>Fiber (non-nutritive)</td>
<td>10.0</td>
<td>10.8</td>
<td>11.7</td>
<td>14.0</td>
</tr>
<tr>
<td>Mineral mixa</td>
<td>4.3</td>
<td>4.6</td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Vitamin mixa</td>
<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
<td>4.8</td>
<td>4.6</td>
<td>3.9</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Grams fed/g consumed by control</td>
<td>1.0</td>
<td>0.93</td>
<td>0.86</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*aAbbreviations: BOP, N-nitrosobis(2-oxopropyl)amine; ER, energy restriction; PKC, protein kinase C.

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were based on previous hamster diets (7) for the macronutrients and vitamin mix and updated mineral mix (9). Energy restricted diets were formulated by removing energy from fat and carbohydrate and increasing the percentage composition of all other components to keep the absolute intake of other dietary components in each restricted group equivalent to the average intake in control hamsters. Control diets were freely fed and restricted diets were fed daily in the amounts indicated in Table I. Fresh diet was provided and diet intake of control hamsters was recorded weekly. Hamsters were weighed weekly.

BOP was synthesized and the purity assayed by published methods (14). In the single dose BOP experiment hamsters were treated with a single injection of 20 mg BOP/kg body weight i.p. at 8 weeks of age and maintained on the assigned experimental or control diet until 102 weeks after BOP. Fifty hamsters were assigned to each of the four diet groups: Control, 10% energy restriction (ER), 20% ER and 40% ER. Forty of these hamsters were treated with BOP and 10 with saline. In the multiple dose experiment hamsters were treated with three weekly doses of 20 mg BOP/kg body weight s.c. at 8, 9 and 10 weeks of age and maintained for 42–44 weeks after the last BOP treatment. Forty hamsters were assigned to each of the three diet groups: control, 20% ER and 40% ER. Thirty of these hamsters were treated with BOP and 10 with amonc. Multiple dosing of BOP was associated with one death in control-fed hamsters, one in 20% ER animals and four in the 40% ER group. The experiments were terminated by pentobarbital overdose in the single-dose study and by cardiac puncture in the multiple-dose study. Following complete necropsy, pancreas tissue was weighed (multiple-dose experiment only) and the pancreas was placed in Bouin's fixative overnight, transferred to 70% ethanol, processed for histology, cut in serial slides (2–4/block) and stained with hematoxylin-eosin. The pancreas tissue of non-BOP-treated hamsters was flash frozen at the end of the single-dose experiment for analysis of PKCζ protein by Western blot using a polyclonal anti-peptide antibody (GibCo-BRL) as previously reported (8,9).

Blood was collected by cardiac puncture following pentobarbital anesthesia in the hamsters on the multiple-dose experiment. Cortisol and corticosterone, the predominant glucocorticoid hormones in hamsters were assayed by radioimmunoassay (15). Body weight was analyzed statistically by ANOVA. Tumor multiplicity and plasma corticosterone and cortisol were analyzed by Student's t-tests to assess differences between means. PKCζ protein was analyzed by calculating the relative density of the experimental (10, 20 and 40% ER) PKCζ band on the Western blot in comparison with the control band on the same blot. The influence of diet was assessed using linear regression techniques and a t-test was used to assess difference from the control.

**Results**

Body weight of hamsters was not influenced by BOP treatment and Figure 1 shows body weights of BOP-treated hamsters in the single-carcinogen-dose study. At 50 and 93 weeks of the experiment significant differences between all dietary groups were observed ($P < 0.0001$). Hamster body weights were reduced after 5 weeks with BOP by dietary energy restriction, and the greatest reductions were in the 40%-ER group. In the 20%-ER groups some of the weight was recovered over the next 10–15 weeks, and at the end of the study the body weights were in the order of control > 10% ER > 20% ER > 40% ER. Survival of these hamsters is shown in Figure 2. While statistically significant differences were not observed between dietary groups, hamsters in the control diet group had the shortest survival in both the BOP-treated and saline-treated (data not shown) hamsters. Dietary energy restriction did not influence the morphology, incidence or time of death with pancreatic cancer in this experiment (Table II).

Body weights in the multiple carcinogen dose tumor study are shown in Figure 3. Statistical analysis at the end of the experiment revealed highly significant differences in body weight ($P < 0.0001$) and the pattern of weight loss followed by maintenance proportional to the extent of dietary energy restriction was also observed in this study. Pancreas weights at necropsy were highest for the BOP-treated control hamsters ($0.75 \pm 0.11$ g/100 g body weight (±SE)) and lower for all other groups (0.33 ± 0.01 for the 40%-saline group to 0.45 ± 0.02 for the 20%-ER BOP group). Since this study was of much shorter duration than the single-carcinogen-dose study described above, we did not observe differences in the survival of hamsters and 25–29 hamsters were alive at 42 weeks after BOP treatment. The incidence and multiplicity of pancreatic cancer in this study is shown in Table III. There was no influence of dietary energy restriction on the time of appearance or on the overall incidence of pancreatic cancer between the three dietary groups. The multiplicity of pancreatic carcinomas in the tumor-bearing animals in the 40% dietary energy restricted group was significantly greater than in the freely fed control ($P < 0.02$).

Plasma cortisol values were higher than corticosterone levels in hamsters (Figure 4). Plasma corticosterone was significantly suppressed in all BOP-treated hamsters and in the hamsters
Table II. Influence of energy restriction on pancreatic carcinoma incidence, morphology, and latency in the single-BOP-dose studya

<table>
<thead>
<tr>
<th>Diet group</th>
<th>No. of effective animalsb</th>
<th>No. of tumor-bearing hamsters</th>
<th>% Incidence in effective hamsters</th>
<th>Morphology</th>
<th>Average latency after BOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35</td>
<td>5</td>
<td>14</td>
<td>3. ductular carcinoma in situ</td>
<td>92.8 ± 10.8</td>
</tr>
<tr>
<td>10% ER</td>
<td>35</td>
<td>3</td>
<td>9</td>
<td>2. ductular adenocarcinoma</td>
<td>90.0 ± 4.0</td>
</tr>
<tr>
<td>20% ER</td>
<td>38</td>
<td>5</td>
<td>13</td>
<td>1. invasive ductular adenocarcinoma</td>
<td>80.0 ± 6.0</td>
</tr>
<tr>
<td>40% ER</td>
<td>33</td>
<td>6</td>
<td>18</td>
<td>4. ductular adenocarcinoma</td>
<td>91.8 ± 5.0</td>
</tr>
</tbody>
</table>

aHamsters were treated with 20 mg BOP/kg body weight at 8 weeks of age. Dietary treatment did not significantly influence incidence, morphology or average latency of pancreatic cancer.
bEffective animals are animals dying 26 or more weeks after BOP.

Fig. 3. Body weights of hamsters in the multiple-carcinogen-dose study. Body weight data are shown only for the BOP-treated hamsters since BOP did not significantly influence body weight. Values are shown as mean ± SEM. Statistical analysis at the end of the experiment (n = 23–27 hamsters/group) by ANOVA demonstrated significant differences between dietary groups (P < 0.0001).

Table III. Influence of energy restriction on the incidence and multiplicity of pancreatic carcinomas in hamsters in the multiple-BOP-dose studya

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>No. of effective hamstersb</th>
<th>No. of hamsters with carcinoma(s)</th>
<th>% Incidence</th>
<th>No. carcinomas/effective animal (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29</td>
<td>17</td>
<td>59</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>20% ER</td>
<td>29</td>
<td>19</td>
<td>66</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>40% ER</td>
<td>26</td>
<td>17</td>
<td>65</td>
<td>1.7 ± 0.3c</td>
</tr>
</tbody>
</table>

aHamsters were treated with 3×20 mg BOP/kg body weight weekly beginning at 8 weeks of age.
bEffective animals are animals dying 26 or more weeks after BOP.
cP < 0.02 compared with control treatment group by Student’s t-test.

discussion

In the present study, dietary energy restriction did not protect against pancreatic carcinogenesis by BOP. We conducted two carcinogenesis studies that provided no evidence of dietary energy restriction protection against pancreatic carcinogenesis by BOP in the Syrian hamster. The first experiment used a single treatment with carcinogen, followed animals for nearly 2 years and resulted in a low incidence of pancreatic cancer. This experiment was designed to have a low pancreatic cancer yield that should have been sensitive to dietary modulation. The second experiment was designed with multiple doses of

PKCζ in the saline-treated hamsters at the termination of the single-dose-carcinogenesis study are shown in Figure 5. PKCζ protein expression was higher in the cytosol (Figure 5A) and particulate (Figure 5B) factions of pancreas from hamsters fed 40% ER diet than in the control or 10% ER hamsters. The difference was significant in the particulate fraction from the 40% ER hamsters.
for control, above the bar (P < 0.02). For both cytosolic and particulate fractions n = 4 for control, n = 7 for 10%, ER n = 2 for 20% ER and n = 5 for 40% ER.

BOP to give a high incidence of cancer in a shortened time. This study was necessary because the low pancreatic cancer yield in the first study may have been too low to provide evidence of protection by energy restriction. It is not clear if this absence of prevention by caloric restriction is peculiar to the pancreas or to the hamster. Human observational studies have noted that pancreas cancer patients often have low body weight at the time of presentation (16). The possibility that this observation is more of a consequence of the disease than a predisposing factor was supported by a recent epidemiological study showing that body weight gain of >10 kg since the age of 30 nearly doubled the risk of pancreatic cancer in current or previous smokers (17).

It is possible that dietary energy restriction is not effective in cancer prevention in the hamster. It has been suggested that the hormonal alterations that occur in under feeding may mediate the inhibition of cancer (18). The data are strongest for a role of elevated glucocorticoid hormone as a mediator of cancer prevention in skin (10) and lung (11). This suggestion comes from the observations in adrenalectomized rodents where the inhibition of carcinogenesis is lost (10,11) and in glucocorticoid-treated rodents where cancer prevention has been observed (12). These studies were conducted in mice and rats, which have been reported to have elevated circulating glucocorticoid hormone in diet or energy restricted animals (10). Furthermore, self-imposed energy restriction in the anorexia nervosa patient was demonstrated to elevate circulating cortisol in humans (19). In contrast in our studies, hamsters did not experience elevated corticosterone or cortisol, the two primary glucocorticoid hormones in this species (15), when dietary energy restriction was imposed. Thus, it is possible that energy restriction will not inhibit other cancers in this species. We are not aware of other studies of diet or energy restriction in the inhibition of carcinogenesis in hamsters. Such studies would be interesting to assess the requirement for elevated glucocorticoid hormone for energy restriction prevention of carcinogenesis. Previous studies with hamsters demonstrated that both adrenocorticotropic treatment and acute stress increased plasma corticosterone and cortisol, and that only plasma cortisol concentrations were elevated by chronic stress in hamsters (15).

With regard to increased pancreatic cancer multiplicity in the hamsters fed the 40% ER diet it must be stressed that these data were collected on moribund hamsters. Thus it is not clear if the 40% ER diet allowed the hamsters to live longer with their disease and develop more cancers, or if the 40% ER diet provided a better environment for development of multiple tumors. Although survival in the carcinoma-bearing hamsters was similar in all diet groups, the design used in these studies does not allow distinction between these possibilities. Furthermore, there is a small possibility that the smaller pancreas size in the 40% ER/BOP-treated group allowed more tumors to be identified. However, this is unlikely since such differential diagnosis would mainly be a problem for microcarcinomas and carcinomas in situ, and these lesions were evenly distributed between dietary groups.

Another observation in the present study was the elevated PKCζ protein in the pancreas of the ER hamsters. This observation was not consistent with our earlier observations in hamsters pre-fed 10%, 20% or 40% ER diets where an inhibition of PKCζ protein expression was observed with increasing restriction (9). Our earlier work in younger hamsters indicated that PKCζ protein was observed primarily in pancreatic duct epithelial cells (8) and reductions of 48–76% in PKCζ expression were observed after 8 weeks of dietary energy restriction (10–40% reduction in comparison with controls) while 76–82% reductions were observed in the hamsters fed 10% to 40% ER diets for 15 weeks (9). In the present study, the pancreas of control, 10%, 20% and 40% ER hamsters indicated no influence of 10% dietary ER, an intermediate increase in PKCζ in the pancreas of the 20% ER hamster and a 300–400% increase above control values in the pancreas of the 40% ER hamsters (significant only in the particulate fraction). These data were obtained in the saline-treated long-term survivors, thus it is possible the differential survival contributed to the observed difference.

In summary, dietary energy restriction (10% to 40% restricted in comparison with control ad libitum intake) was not effective in the prevention of pancreatic carcinogenesis by BOP in the Syrian hamster. Furthermore, dietary energy restriction did not result in elevated glucocorticoid hormone secretion in this species.

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References
Carcinogenesis by N-nitrosobis-2-(oxopropyl)amine


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