Prevention of Mammary Tumorigenesis by Intermittent Caloric Restriction: Does Caloric Intake During Refeeding Modulate the Response?

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Chronic caloric restriction (CCR) prevents mammary tumorigenesis in rodents, but a protective effect for intermittent caloric restriction (ICR) is less well documented. We recently reported that ICR reduced mammary tumor (MT) incidence of mouse mammary tumor virus–transforming growth factor (MMTV-TGF)-α mice to a greater extent than did CCR. Here, we repeated this protocol and obtained serum and tissue samples. *Ad libitum* (AL) MMTV-TGF-α mice were fed AIN-93M diet. Beginning at 10 weeks of age, ICR mice received isocaloric AIN-93M-mod diet (2-fold increases in protein, fat, vitamins, and minerals) at 50% of *ad libitum* for 3 weeks followed by 3 weeks refeeding with AIN-93M diet. CCR mice were pair-fed AIN-93M:AIN-93M-mod (2:1) matching intakes for restriction/refeeding cycles. Mice were sacrificed for MT size, at 79 (end of 12th restriction) or at 80 (1 week after 12th refeeding) weeks of age. AL and ICR-80 mice had heavier body weights than ICR-79 and CCR mice ($P < 0.0001$). Cumulative food intakes of ICR and CCR mice were reduced 12% and 15% versus AL mice ($P < 0.0001$). However, ICR mice consumed significantly ($P < 0.0001$) more food than did AL mice during refeeding. MT incidence was 84%, 13%, and 27% for AL, ICR, and CCR mice, respectively. MT weight ($P < 0.0011$) and number ($P < 0.01$) were higher for AL mice compared with ICR and CCR mice. AL and ICR-80 mice had similar serum IGF-I levels, but only AL values were higher than those of ICR-79 and CCR mice ($P < 0.0017$). ICR mice had more MT DNA breaks compared with AL and CCR mice, suggesting enhanced apoptosis ($P < 0.02$). AL mice had higher mammary fat pad ObR and ObRb leptin receptor mRNA expression than did ICR and CCR mice ($P < 0.001$), but there was no effect on MTs. These results confirm that ICR prevents development of MTs to a greater extent than does CCR, although "overeating" during refeeding may compromise this protection. Exp Biol Med 232:70–80, 2007

Key words: apoptosis; caloric restriction; IGF-I; leptin receptor; mammary tumors; mice; prevention; weight gain; weight loss

Introduction

It is well documented that chronic caloric restriction (CCR) in the range of 20%–40% reductions decreases the incidence of chemically induced and spontaneous mammary tumors (MTs) in rodents (1–10). In some cases as little as 10% lower caloric intake has been shown to have some protective effects (10, 11). CCR also reduces the incidence of oncogene-induced MTs in several strains of transgenic mice (12, 13). However, the consequences of multiple periods of intermittent caloric restriction (ICR) on MT development have been studied to a lesser extent and the results obtained have not produced consistent findings. For example, ICR of rats treated with chemical carcinogens and fed high-fat diets did not prevent MT development (14, 15). However, it was recently reported that ICR imposed 14 weeks after carcinogen treatment lowered MT incidence in rats, although the results were not significant (16). Other studies have shown that ICR imposed by fasting/refeeding protocols reduced the incidence of spontaneous MTs in both rats and mice (17, 18). In addition, we recently reported that ICR resulting in an approximately 20% lower cumulative caloric intake compared with *ad libitum*–fed mice resulted in a 96% reduction of oncogene-induced MT incidence in

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transgenic mouse mammary tumor virus–transforming growth factor (MMTV-TGF)-α mice, while a similar degree of CCR resulted in only a 40% decrease in MT incidence (12). In a second strain of transgenic mice, MMTV-neu, ICR reduced MT incidence by 40%, while there was no protection from moderate calorie restriction in CCR mice (19).

Here, we repeated the ICR protocol in MMTV-TGF-α mice to confirm its protective action on prevention of MTs. Also, to expand the data obtained, mice were sacrificed at the end of both the final restriction (79 weeks of age) and after 1 week of final refeeding (80 weeks of age) to obtain serum samples at both time points. In addition, when available, tumor tissue was obtained for analysis. MMTV-TGF-α mice were chosen for continued study because their hormone responsive tumors develop slowly over time and represent postmenopausal breast cancer. These mice overexpress human TGF-α (20), a component of the epidermal growth factor receptor (EGFR)/ErbB cascade known to play an important role in the development and progression of some human breast cancers (21–23). Specifically, expression of TGF-α is associated with 30%–70% of human breast tumors (24–27). Transgenic mice that overexpress TGF-α are considered to be good models to use for assessment of breast cancer disease process (28, 29). Also, the results of our earlier study (12), indicating that this specific transgenic mouse exhibited a marked protective effect in response to ICR, led us to want to verify this response. Furthermore, this mouse model was shown to respond to another diet manipulation, diet-induced obesity, with specific effects on MT development (30). Since lifestyle factors such as diet are thought to have greater effect on postmenopausal than on premenopausal breast cancer, these mice provide a good animal model to investigate long-term nutritional interventions. Measurements made in tumor tissue focused on effects of caloric restriction on MT apoptosis (31) and on leptin receptors expression because of the identification of these receptors in human breast cancer cell lines and breast tumors (32, 33). The primary aim of the present study was to confirm the effects of ICR on MT development and prevention, and the secondary aim was to obtain preliminary identification of the changes in leptin receptors expression and apoptosis signaling.

Materials and Methods

Experimental Animals. Mice were obtained by mating nontransgenic heterozygous *Lepr*$^{+}$*Lepr*^{db} female mice with heterozygous TGF-α/*Lepr*$^{+}$*Lepr*^{db} male mice as previously described (34). Nontransgenic female mice are used for breeding as the transgenic female mice do not lactate. Offspring were kept with their mothers until 4 weeks of age. They were then housed with like-sexed pups, while genotype status for TGF-α and *Lepr* was determined using DNA obtained from tail biopsies (30, 35). MMTV-TGF-α/*Lepr*$^{+}$*Lepr*^{db} female mice were assigned to one of three experimental groups: (1) *ad libitum*–fed (AL) ($n = 31$), (2) intermittent caloric restricted (ICR) ($n = 39$), and (3) chronic caloric restricted (CCR) ($n = 30$). The use of heterozygous mice was based on the decision to enroll only one genotype in a specific study, and mice of other genotypes were used in other experiments. Experimental mice were housed individually in hanging stainless steel cages. Water was provided on an *ad libitum* basis. The animal room was maintained at 22°C on a 12:12-hr light:dark cycle at a humidity level of 50%. The Hormel Institute Animal Facility is Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited. The University of Minnesota Animal Care and Use Committee approved this study.

Feeding Regimens. From 7 to 9 weeks of age, all mice were fed a purified diet based on AIN-93M recommendations for long-term maintenance of rodents (12, 36). AL mice were maintained on the AIN-93M diet throughout the experiment. Beginning at 10 weeks of age, ICR mice were fed a modified version of the AIN-93M diet designated as AIN-93M-mod for 3-week intervals at 50% of *ad libitum* intake (12). AIN-93M-mod had 2-fold increases in protein, vitamin, mineral, and fat contents and was formulated to be isocaloric with AIN-93M diet. Following each restriction period, ICR mice were provided AIN-93M diet for a 3-week interval of *ad libitum* feeding. CCR mice were pair-fed to ICR mice by providing a mixture of 2:1 AIN-93M:AIN-93M-mod at the average daily intake for the corresponding age-matched 6-week food restriction/refeeding interval of ICR mice. Food was provided in small glass cups placed inside larger glass cups to collect spillage. Food intakes were determined daily.

Animal Procedures. Body weights were determined weekly, at which time mice were palpated to assess if MTs were present. Once MTs were detected, tumor growth was monitored with calipers, and tumor-bearing mice were sacrificed when MT size exceeded 20 mm in length. AL mice alive at 80 weeks of age were sacrificed. For ICR mice, half were sacrificed at 79 weeks of age (end of the 12th food-restriction period) and the remaining half at 80 weeks of age (1 week of refeeding in the 12th refeeding period). These mice were designated as ICR-79 and ICR-80, respectively. One-half of the CCR mice were also sacrificed at each time point, but since there were no apparent differences related to the 1-week age difference, all their results have been combined. One AL mouse was found dead, and her MT status was not able to be determined. One AL mouse was sacrificed for weight loss of 25%, and she was found to have myeloproliferative disorder, and four additional mice were sacrificed for combinations of skin sores and weight loss ~10%. Two ICR mice were sacrificed owing to lack of weight regain at 38 and 56 weeks of age, and two CCR mice with unhealed skin sores were sacrificed at 62 and 74 weeks of age. Five ICR and four CCR mice were sacrificed to correspond to ages when AL mice were sacrificed due to the presence of MTs. All mice were euthanized by CO_2 overdose.

Tissue Sample Collection and Histopathologic Analysis. At sacrificing, a blood sample was obtained and serum prepared for determination of IGF-I concentrations (IGF-I RIA kit No. DSL-2900 from Diagnostic Systems Laboratory, Webster, TX). Liver, kidneys, heart, spleen, ovaries, lungs, MTs, and other abnormal growths/tumors were removed and weighed. Samples from organs and tissues were placed in 10% neutral buffered formalin for 24 to 48 hrs and then embedded in paraffin. Sections, 5 micrometers thick, were prepared, deparaffinized in xylene (2 × 5 min), rehydrated in a graded series of ethanol solutions, and rinsed in tap water. Sections were stained with hematoxylin and eosin. Histopathologic analysis was conducted in a blinded fashion without prior knowledge of group assignment. Retroperitoneal and parametrial fat pads also were removed and weighed. Parametrial and retroperitoneal fat pads were removed and weighed and, in addition, mammary fat pad tissue was removed and frozen.

Tumor Apoptosis. To obtain information on the effects of these two caloric interventions on metabolic changes in the tumors that did develop, several factors previously identified to be involved in tumorigenesis processor were determined. To evaluate apoptosis, caspase-3 activity was measured in frozen MT tissue. Total protein was obtained as per the manufacturer's instructions (Total Protein Extraction Kit, Chemicon International Inc, Temecula, CA). The amount of protein was quantified and standardized, and caspase-3 activity was assayed as per the manufacturer's instructions (Caspase-3 Colorimetric Activity Assay Kit, Chemicon International). Also MT sections were analyzed for double-stranded DNA breaks by counting five fields per sample (ApopTag Peroxidase In Situ Olig Ligation Apopotosis Detection Kit, Chemicon International).

Western Blot Analysis. We also examined two other proteins related to leptin signaling, phosphorylated STAT-3 (pSTAT-3) and phosphorylated Akt1 (pAkt1). Western analyses were performed to determine pSTAT-3 and pAkt1 using the same extracts as for caspase-3 determination. Sixty micrograms of protein were run and separated in each gradient gel well. Then, the protein was transferred to polyvinylidene difluoride (PVDF) membrane and checked for uniformity using Poceau S (data not shown). The blots were probed with primary antibodies (Chemicon International) against pSTAT-3 or pAkt1. A secondary antibody conjugated to IRDye 800 (Rockland Inc, Gilbertsville, PA) was used to visualize the samples using an Odyssey Infrared Imaging System (LI-COR Biosciences Corp, Lincoln, NE).

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) for Leptin Receptor mRNA Expression. We also measured mRNA expression levels of leptin receptors, ObR and ObRb, in MTs and MFPs using RT-PCR method. In brief, total RNA was extracted using RNeasy mini kit (Qiagen Inc, Valencia, CA) as per the manufacturer's protocol. Equal amounts of tissue samples were taken from the animals from each group. Isolated total RNA was quantified, and equal amounts of RNA from each animal were used for RT-PCR. Total RNA was reverse transcribed using random hexamers, RNase inhibitor, reverse transcriptase (Superscript II), and oligo(dT) primers (GibcoBRL, Grand Island, NY) to synthesize first-strand cDNA from the mRNA. PCR primers were designed using nucleotide sequence for a mouse ObR (U42467 Genbank accession number) and ObRb (U58861 Genbank accession number). The following primers were used: for ObR, forward 5′-CAGATTCGATATGGCTTAAGT-3′ and reverse 5′-GTTAAAATTCACAAGGGAAGC-3′; and for ObRb, forward 5′-ACACTGTTAATTTCACACCAGAG-3′ and reverse 5′-TGGATAAACCCTTGCTCTTCA-3′. These primers gave the final product sizes of 475 and 446 base pair (bp) for ObR and ObRb, respectively. The PCR was performed under the following conditions: 94°C for 4 min for denaturing, 35 cycles of 94°C for 30 secs, 62°C for 30 secs, 72°C for 1 min, and final extension at 72°C for 10 min. The reactions were carried out in a final volume of 25 μl in thermocycler in the presence of Taq DNA polymerase. Water was used as negative control and mouse β-actin primers that produce an approximately 600 bp product were used as internal controls. The PCR products were separated on 1% agarose gel and stained with ethidium bromide. The product size was determined by concurrently separating 100 bp DNA ladder on the same gel. The DNA gel was scanned, the intensity of bands quantified by densitometry using the program called UN-SCAN-IT gel (Silk Scientific, Orem, UT), and results expressed as ratio of intensity of the gene of interest to that of β-actin in samples from individual animals. Values from the AL mice were taken as 100%, and then the results from the ICR and CCR mice were calculated according to AL values in percentage. The DNA obtained from tissue samples was run on agarose gel and subsequently extracted from the gel and sent to the Advanced Genetic Analysis Center, University of Minnesota, St. Paul, Minnesota, for sequencing to confirm that the bands analyzed the genes of interest, ObR and ObRb.

Statistical Analysis. Statistical analysis was done with consultation with the Biostatistics Core University of Minnesota Cancer Center. Comparisons of body weight curves were done by one-way analysis of variance (ANOVA) for repeated measures. Comparisons among dietary groups in Table 1 were made by ANOVA followed by Newman-Keuls test to determine statistical differences between specific groups. MT incidence data were calculated by chi-square analysis, while age of MT detection and MT number and weight were analyzed by Kruskal-Wallis test. Data in Table 3 were analyzed by Fisher exact test. Density results from RT-PCR analysis were done by one-way ANOVA with Bonferroni's multiple comparison test. Significance was set at $P < 0.05$ and values are presented as mean ± SEM.

Table 1. Final Body and Fat Pad Weights, Fat Pad:Carcass Ratio, and Serum IGF-I Levels of MMTV-TGF-α-*Lepr*$^{+}$*Lepr*^{db} Mice in Intermittent Restriction/Refeeding Study[a]

	Final body weight (g)	Fat pad weight (g)[b]	Fat pad:carcass ratio[b]	Serum IGF-I (ng/ml)
Ad libitum–fed ($n = 30$)	31.2 ± 0.8[a]	1.616 ± 0.190[a,b]	0.054 ± 0.005[a,b]	493 ± 27[a] ($n = 29$)
Intermittent restricted/refed-79 ($n = 16$)	25.0 ± 0.7[b]	1.224 ± 0.313[b]	0.040 ± 0.007[b]	369 ± 28[b] ($n = 14$)
Intermittent restricted/refed-80 ($n = 16$)	32.5 ± 0.6[a]	2.052 ± 0.135[a]	0.069 ± 0.004[a]	457 ± 22[a,b] ($n = 16$)
Chronic calorie restricted ($n = 30$)	26.2 ± 0.5[b]	1.420 ± 0.135[b]	0.054 ± 0.004[a,b]	383 ± 20[b] ($n = 24$)
ANOVA	$P < 0.0001$	$P < 0.001$	$P < 0.0075$	$P < 0.0017$

[a] Values within a column with different superscript letters are significantly different.
[b] Fat pad weight, combined right and left parametrial and retroperitoneal fat pads; Carcass, final body weight minus organ (heart, lung, liver, kidneys, and spleen) and tumor weights.

Results

Food Intakes and Body and Fat Pad Weights of the Animals. As expected, ICR (1660 ± 34 g) and CCR (1604 ± 54 g) mice had significantly reduced cumulative food intakes over the course of the experiment in comparison with AL mice (1863 ± 92 g) (ANOVA $P = 0.0001$, both ICR and CCR $P < 0.001$ versus AL). The decreases in food intake were modest (i.e., 12% for ICR and 15% for CCR mice, respectively). However, this result is somewhat distorted by the earlier age of death for AL mice. Further examination of food intake by restriction and refeeding periods indicated that ICR mice consumed the least amount of food during restriction periods followed by CCR mice, while AL mice had the highest intake (not shown). This finding is as expected from the nature of the experimental design. However, during refeeding periods, ICR mice (1144 ± 19 g) consumed significantly more food than did AL mice (995 ± 26 g), while CCR mice (792 ± 8.2 g) consumed less than the two former groups (ANOVA $P < 0.0001$; $P < 0.001$ among all groups). ICR mice consumed more total calories than was originally anticipated from the results of earlier studies using this protocol (12, 19, 37, 38).

There was a significant difference in body weight curves among the groups (Fig. 1). AL and CCR mice exhibited slow steady weight gains followed by body weight plateaus at 40 and 50 weeks of age, respectively. In contrast, ICR mice demonstrated a pattern of weight loss during each caloric restriction period followed by rapid regain of their lost weight during the first week of refeeding and then maintenance of their body weight in the range of age-matched AL mice for the remaining 2 weeks of each refeeding period. Final body weights of AL mice were similar to those of ICR-80 mice (i.e., ICR mice sacrificed at 80 weeks of age after 1 week of refeeding) (Table 1). Body weights of CCR mice and ICR-79 mice (i.e., ICR mice sacrificed after the final restriction interval) were similar and significantly lower than the two other groups (Table 1).

Combined parametrial and retroperitoneal fat pad weights exhibited a slightly different statistical pattern than did body weights (Table 1). ICR-80 and AL mice had the heaviest fat pads, although those of AL mice were not significantly different from CCR mice. Fat pad weights of ICR-79 mice were significantly reduced compared with all other groups. When fat pad weights were calculated relative to carcass weight (body weight minus tumors and organs) the values were not significantly different among ICR-80, CCR, and AL groups or between ICR-79, CCR, and AL groups. Serum IGF-I levels for AL mice were significantly higher than those of ICR-79 and CCR mice (Table 1), while IGF-I levels of ICR-80 mice were not significantly different in comparison with any other groups.

Mammary Tumors. MT incidence for AL mice was 84% in comparison with 15% and 27% for ICR and CCR mice, respectively (Table 2). All groups were significantly different from each other. There was a significant difference in the average number of MT per tumor-bearing mice

Table 2. Mammary Tumor Incidence, Age of Tumor Detection, and Tumor Burden for MMTV-TGF-α *Lepr*$^{+}$*Lepr*^{db} Female Mice in Intermittent Restriction/Refeeding Study

	Mammary tumor incidence	Age of mammary tumor detection (weeks)	Mammary tumor burden	
			Number	Weight (g)
Ad libitum–fed ($n = 26/31$)	84[a]	67.9 ± 2.5[b]	3.2 ± 0.3[c]	1.059 ± 0.142[d]
Intermittent restricted/refed ($n = 6/39$)	15	79.4 ± 0.3	1.6 ± 0.4	0.480 ± 0.247
Chronic calorie restricted ($n = 11/30$)	27	74.5 ± 2.6	1.8 ± 0.4	0.242 ± 0.087

[a] Mammary tumor incidence of the three groups significantly different from each other by chi-square analysis, $P < 0.001$.
[b] Kruskal-Wallis Test = not significant.
[c] Kruskal-Wallis Test = $P < 0.01$.
[d] Kruskal-Wallis Test = $P < 0.0011$.

Table 3. Summary of Mammary Tumor Histopathology from Transgenic MMTV-TGF-α *Lepr*$^{+}$*Lepr*^{db} Female Mice in Caloric Restriction Study[a]

	Total samples	High-grade (%)	Low-grade (%)	Atypical hyperplasia (%)	Benign (%)
Ad libitum–fed	75	2 (3)	51 (66)	15 (19)	7 (9)
Intermittent restricted/refed	14	0 (0)	8 (57)	0 (0)	6 (43)
Chronic calorie restricted	20	1 (5)	12 (60)	3 (15)	4 (20)

[a] Fisher exact test $P < 0.044$ significance for the association between diet group and histopathological classification.

among the groups with AL mice having twice the number as did ICR and CCR mice ($P < 0.02$) (Table 2). Also AL mice had the highest MT weight ($P < 0.006$), followed by an intermediate tumor weight for ICR mice; CCR mice had the lightest tumor weight. The average age at which MTs were detected was 12 weeks younger for AL mice compared with ICR mice and 7 weeks younger compared with CCR mice; however, this did not reach statistical significance (Table 2).

A summary of mammary tumor pathology is presented in Table 3. A total of 75 samples were removed from AL mice and examined as possible MTs. Of these, two were high-grade and 51 were low-grade adenocarcinomas. Of the remaining samples from AL mice, 15 exhibited signs of atypical hyperplasia and seven were benign tissue. For ICR mice 14 samples were examined. No high-grade adenocarcinomas or atypical hyperplasia samples were identified from ICR mice, while eight low-grade adenocarcinomas were found and six samples were benign tissue. Twenty samples were analyzed from CCR mice and, of these, 1 was a high-grade adenocarcinoma, 12 were low-grade adenocarcinomas, 3 exhibited atypical hyperplasia, and 4 were benign. Overall there was a significant difference among the groups.

Effect of Calorie Restriction on Mammary Tumor Apoptosis and Leptin Receptor Expression. MTs obtained from ICR mice had caspase-3 activity 70% higher than did MTs from the other two groups (Fig. 2A). Because of the small sample size available from the ICR group, the results did not reach statistical significance by ANOVA; however, when ICR and AL values were compared by Student's *t* test, there was a significant difference ($P = 0.04$). A second assay used to determine the degree of apoptosis was done on fixed MT samples to

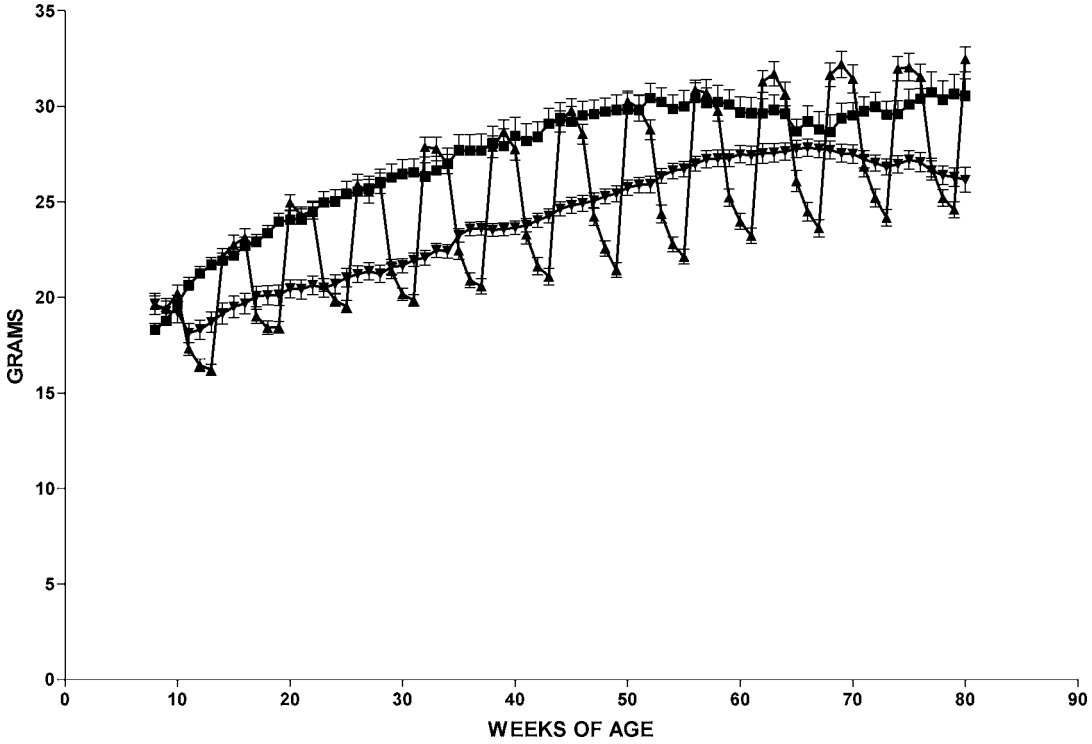

Figure 1. Body weight curves for mice in intermittent restriction study. *Ad libitum*–fed (AL) mice (■) ($n = 15$–31 depending upon age); intermittent calorie restricted mice (ICR) (▲) ($n = 32$–36 depending upon age—except at 80 weeks of age, $n = 16$ mice due to sacrificing of half of the live mice at 79 weeks of age); and chronic calorie restricted (CCR) mice (▼) ($n = 24$–30 depending upon age—except at 80 weeks of age, $n = 11$ mice due to sacrificing of half of the live mice at 79 weeks of age). ANOVA $P < 0.0001$, ICR and CCR groups significantly different from *ad libitum*–fed mice but not from each other. Values are means ± SEM.

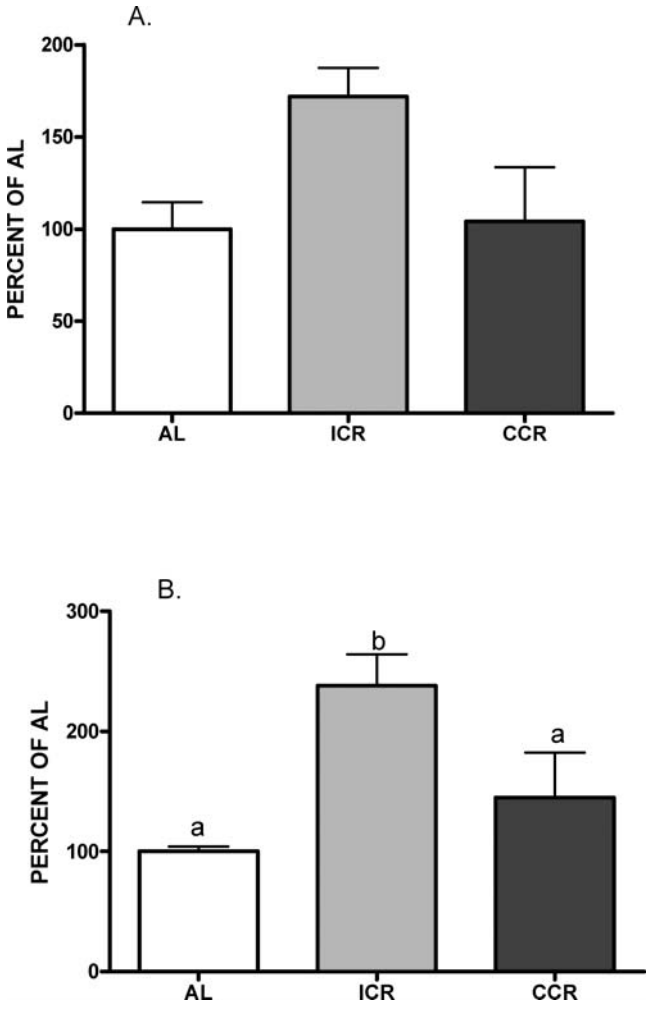

Figure 2. (A) Caspase-3 activity of MTs from *ad libitum*–fed (AL) mice ($n = 5$); intermittent calorie restricted (ICR) mice ($n = 2$, both 79 weeks of age); and chronic calorie restricted (CCR) mice ($n = 3$). ANOVA, $P = 0.128$. Results presented as percentage of AL value. (B) DNA breakage of MTs from *ad libitum*–fed (AL) mice ($n = 3$); intermittent calorie restricted (ICR) mice ($n = 3$); and chronic calorie restricted (CCR) mice ($n = 3$; two at 79 weeks of age and one at 80 weeks of age). ANOVA, $P = 0.02$, columns with different letter superscripts are significantly different from each other. Results presented as percentage of AL value.

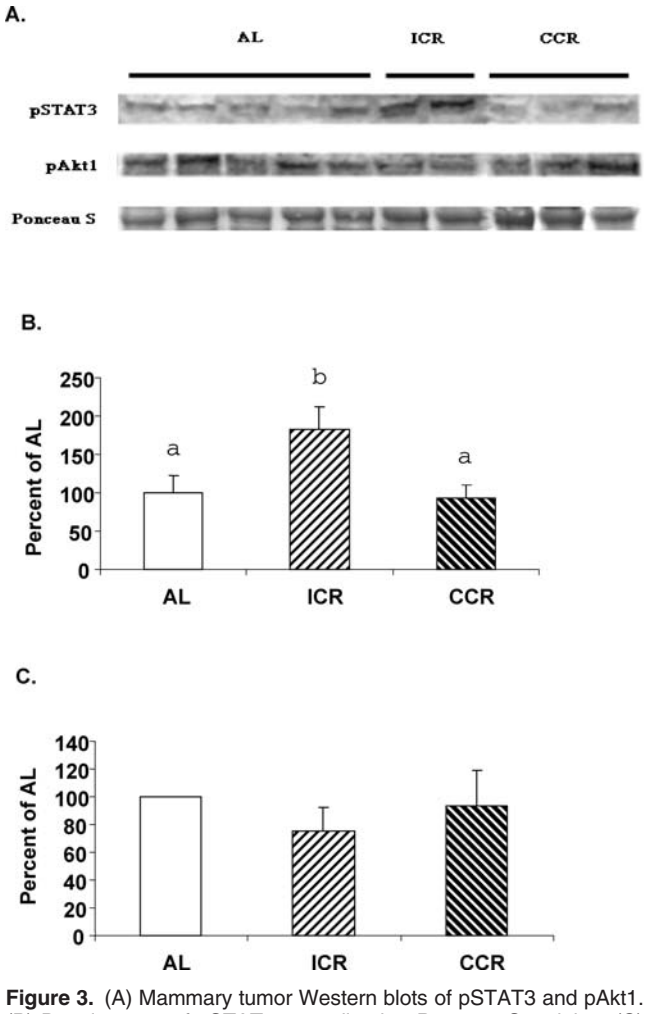

Figure 3. (A) Mammary tumor Western blots of pSTAT3 and pAkt1. (B) Densitometry of pSTAT3 normalized to Ponceau S staining. (C) Densitometry of pAkt normalized to Ponceau S staining. *Ad libitum*–fed (AL) mice ($n = 5$); intermittent calorie restricted (ICR) mice ($n = 2$, both 79 weeks of age); and chronic calorie restricted (CCR) mice ($n = 3$). Significant differences among groups indicated with different superscripts, pSTAT3 expression ANOVA = 0.0129.

detect double-stranded DNA breaks. There were significantly more DNA breaks in MTs obtained from ICR mice compared with those from either AL or ICR mice (Fig. 2B). Figure 3A indicates that pSTAT3 expression was significantly higher in MTs from ICR mice compared with the other two groups ($P < 0.02$ for ICR vs. AL and $P < 0.014$ for ICR vs CCR), while there were no differences in pAkt1 (Fig. 3B).

Expression levels of leptin receptors were measured in MT and mammary fat pad samples using RT-PCR techniques. In MTs, there were no significant differences among the groups in terms of either ObR or ObRb mRNA expressions ($P > 0.05$) (Fig. 4A and B). On the other hand, mammary fat pad samples from AL mice had significantly higher levels of both ObR (~40 %) and ObRb (~32 %) mRNA expressions compared with mammary fat pad samples from ICR or CCR mice (Fig. 5A and B). There were no apparent differences between samples obtained at 79 weeks of age after restriction or at 80 weeks of age after 1 week of refeeding in terms of either ObR or ObRb mRNA expression in either MT or mammary fat pad samples (data not shown).

Discussion

The findings in the present study confirm that multiple cycles of intermittent caloric restriction/refeeding accompanied by body weight fluctuations protects MMTV-TGF-α mice from developing MTs. Significantly decreased MT incidence and tumor weight were documented. In the present study, the benefit of ICR versus CCR on extension of MT latency was not as great as previously reported, and the MT incidence rate was not quite as low (12). However, results from both studies clearly indicate that ICR provides a

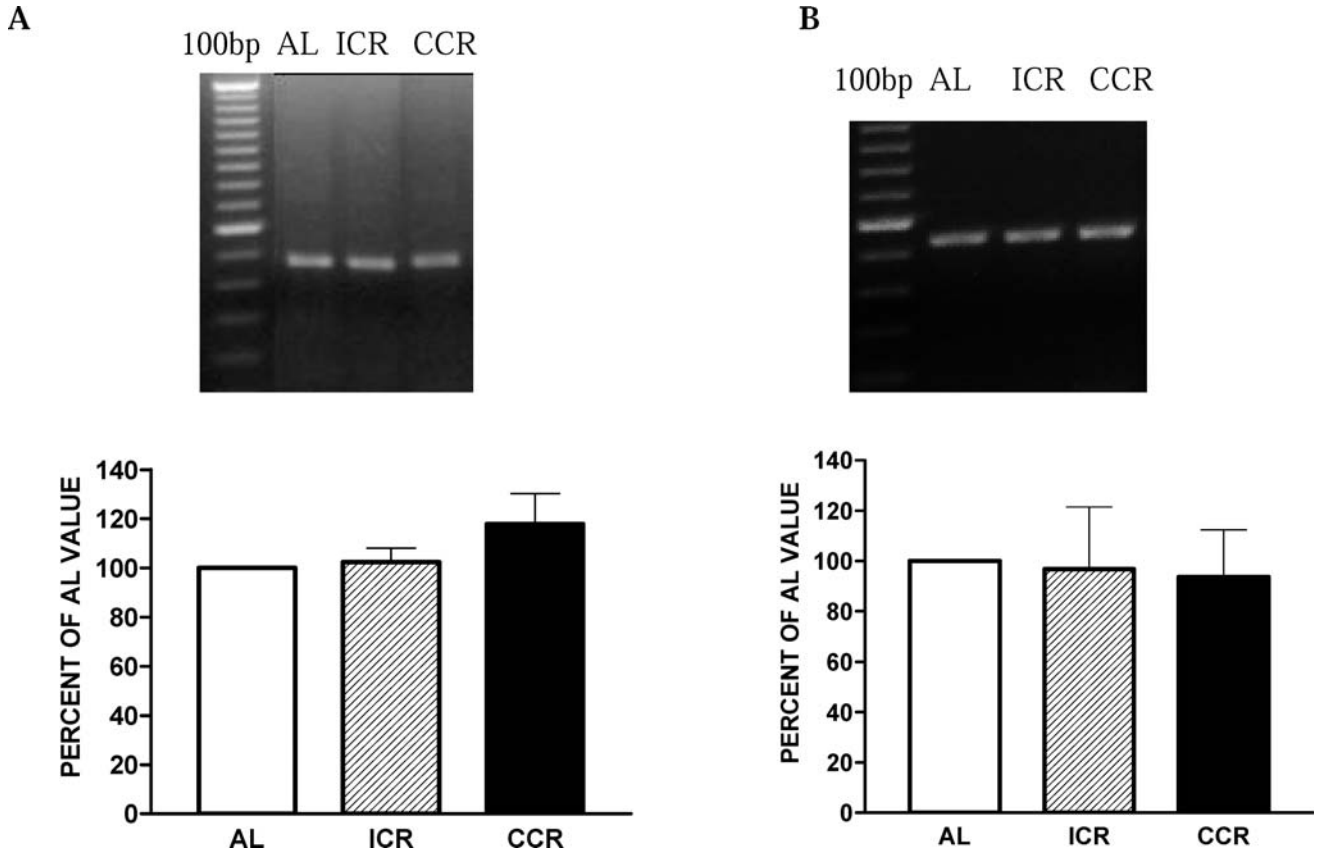

Figure 4. RT-PCR for mRNA expression of total leptin receptor, ObR (A) and long form of the leptin receptor, ObRb (B) in MTs of mice from *ad libitum*–fed (AL) ($n = 5$); intermittent calorie restricted (ICR) ($n = 3$, two at 79 weeks of age and one at 80 weeks of age); and chronic calorie restricted (CCR) ($n = 5$) groups. The data shown are representative of three to five mice from each group. Results are presented as percentage of AL value. There were no significant differences among the groups.

greater protective effect on oncogene-induced mammary tumorigenesis than does the same degree of restriction implemented in a chronic fashion. In addition, this protective effect of ICR occurs with only a moderate overall caloric deficit. Thus, it is evident that the manner in which calorie restriction is implemented affects MT development and is independent of the degree of restriction. Of interest, this finding contrasts several earlier studies that suggested the degree of caloric restriction determines its level of protection (11, 39).

A possible explanation for the minor differences between the present and our earlier study is that the overall degree of caloric restriction, 12%, in the present study was not as great as 20% obtained previously (12). In the present study, ICR mice consistently overate during refeeding periods relative to AL mice, which narrowed the caloric deficit between these two groups. Of interest, we previously noted using a different transgenic mouse model MMTV-neu, that overeating during the refeeding periods occurred and may have compromised the protective effect of this regimen in those mice (19). This issue is now being addressed in ongoing studies with controlled caloric intake during refeeding.

In addition to our studies using transgenic mice with oncogene-induced MTs, several publications have reported that ICR/refeeding interventions prevent spontaneous MT development in rodents. For example, mice fed restricted amounts of either high fat or high carbohydrate diets twice a week (in essence fasted and refed) exhibited a very low incidence of MTs; and the mice that developed MTs had an extended latency (18). In a second study, rats fasted every other day had an 80% lower incidence rate of spontaneous MTs compared with *ad libitum*–fed rats (17). MT latency and tumor burden also were significantly affected by fasting every other day. Additional studies have reported extended survival resulting from caloric restriction/refeeding interventions. For example, F1(New Zealand Black [NZB] × New Zealand White [NZW]) female mice that experienced repeated cycles of fasting (4 days) and refeeding (8 days) exhibited increased longevity (40). Also fasting/refeeding in rats extended life-span (41–43). Of particular note is the report that 1 day of fasting a week with controlled feeding for the rest of each week, resulting in a 16% decrease in weekly caloric intake, prevented lymphoma metastasis in adult $p53^{+/-}$ mice almost to the same degree as 40% decrease in caloric intake implemented by CCR (44).

On the other hand, when caloric restriction refeeding was implemented in rats treated with chemical carcinogens,

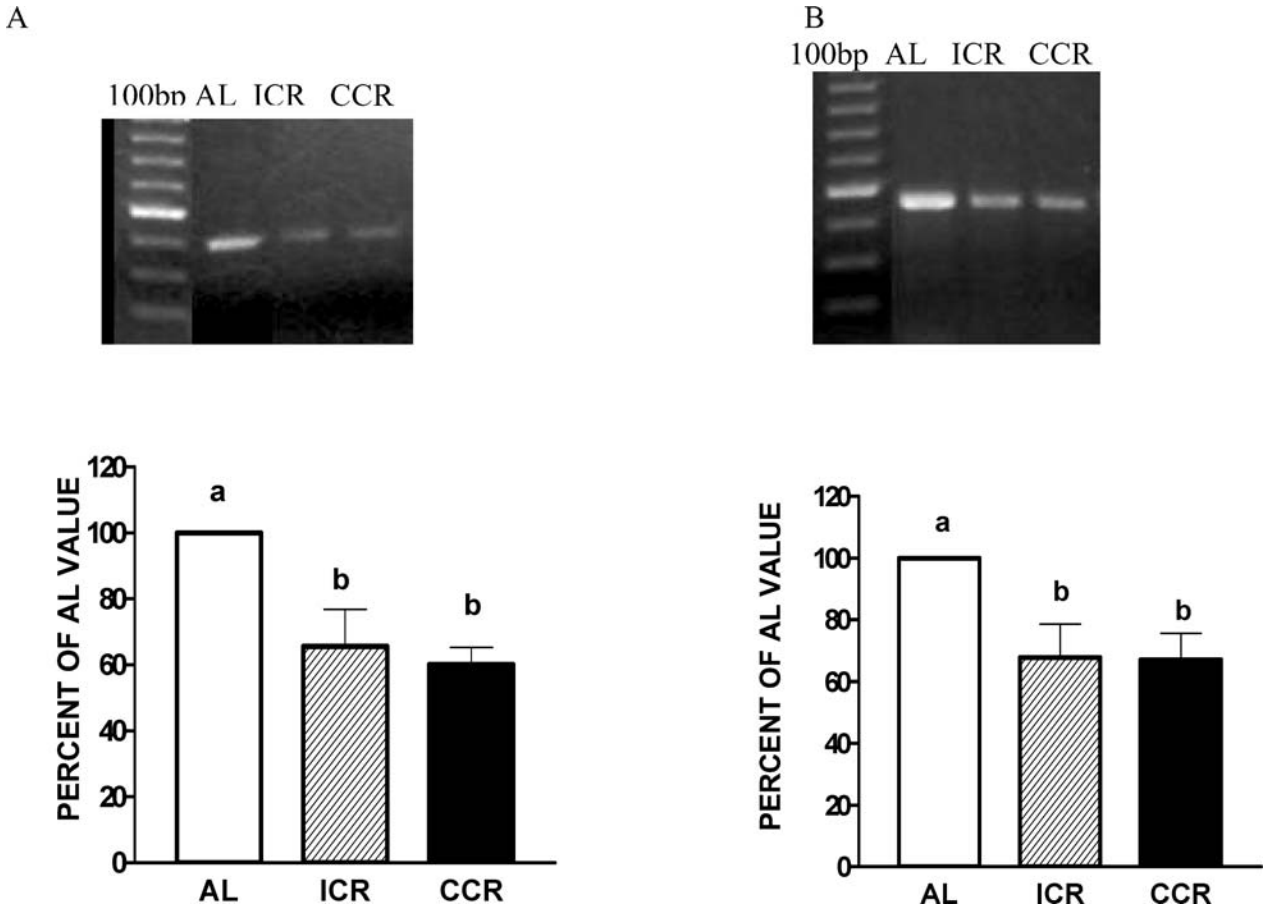

Figure 5. RT-PCR for mRNA expression of total leptin receptor, ObR (A) and long form of the leptin receptor, ObRb (B) in mammary fat pad samples from *ad libitum*–fed (AL) ($n = 5$); intermittent calorie restricted (ICR) ($n = 5$, two at 79 weeks of age and three at 80 weeks of age); and chronic calorie restricted (CCR) ($n = 5$) groups. Results presented as percentage of AL value. There was significantly ($P < 0.001$) less mRNA expression of ObR and ObRb in mammary fat pad samples of the mice from ICR and CCR groups compared with AL group.

MT development was either unaffected (14) or slightly enhanced (15). These latter two studies used high fat diets with corn oil as the major source of dietary fat, and caloric restriction periods were characterized by weight maintenance as opposed to weight loss, making comparisons among the different studies difficult. However, a recent study reported that when the restriction protocol was implemented 4 months after carcinogen administration, a protective effect of this intervention was found (16). Thus, it is possible that when chemical carcinogens are used to induce MTs that caloric restriction at the time of administration affects tumor development. This possibility is supported by several earlier studies. Specifically for MTs Sesca *et al.* (45) reported that latency was shortened and tumor incidence was 100% in rats fasted for 3 days, 1 week after DMBA was administered. Other results of chemically induced hepatic and colon malignancies also indicate that this type of intervention (i.e., fasting followed by refeeding at or near the time of carcinogen administration may enhance tumorigenesis; Refs. 46–50).

In addition to timing of the intervention, the response to ICR/refeeding on mammary carcinogenesis may be dependent upon tumor etiology. For example, transplanted and spontaneous MTs had different cell kinetics when examined during refeeding after fasting (51). Also, when we used the feeding protocol described here with MMTV-neu mice that are estrogen receptor negative (52), their response was not as robust as that described for MMTV-TGF-α mice (19). Further evidence for tumor etiology playing a role in the response to nutrition intervention is that MMTV-neu mice obtained no protective effect of CCR on MT development (19) and, additionally, dietary obesity had no effect on mammary tumorigenesis in MMTV-neu mice, while in MMTV-TGF-α mice dietary obesity resulted in significantly shortened MT latency (30, 53). Although the number of studies is limited, it appears that the ICR/refeeding protocol has the greatest effect on MTs that develop slowly (i.e., spontaneously and/or as a result of specific oncogenes).

The effect of these caloric restriction interventions on metabolic pathways in MTs also was evaluated, although the study was limited by the few MTs available from calorie-restricted mice. Apoptosis was chosen as it was assessed in earlier studies of MT prevention by caloric restriction (31, 54). We determined that caspase-3 activity, a

protein reflecting apoptosis rate, was higher in MTs obtained from ICR mice in comparison with MTs from both AL and CCR mice. A second detection method, determination of double-stranded DNA breaks, produced similar results. Previously, assessment of apoptosis by 5'bromo-2′deoxyuridine labeling indicated no effect in adenocarcinomas from CCR rats with 10% and 20% reduced intakes, although at 40% there appeared to be a slight increase (54). In another study from the same laboratory, a decrease in Bcl-2 detected by gene array was found in MTs from 40% restricted rats, while refeeding increased it relative to the restricted samples, but the value was still less than that obtained from tumors of *ad libitum*–fed rats (31). This rat model is substantially different from the mouse model used in the present study with respect to animal age and MT etiology and latency, so it is difficult to make direct comparisons of the results.

The presence of proteins involved in leptin signaling were assessed in MTs as well as in mammary fat pads, as earlier *in vitro* studies using human breast cancer cell lines had indicated leptin enhanced cell proliferation (32, 33, 55). The involvement of the leptin axis in mammary tumorigenesis is also supported by *in vivo* studies indicating that MMTV-TGF-α mice that are either leptin deficient or lack functioning leptin receptors do not develop MTs (34, 35). STAT3 is a well recognized component of leptin signaling (56, 57). We found that pSTAT3 was higher in the MTs from ICR mice compared with MTs from the other two groups, while pAkt1 another leptin signaling protein was not significantly affected. However, there were no significant differences among the groups in terms of the presence of total leptin receptor, ObR, or the signaling form of the leptin receptor, ObRb, mRNA expressions in MTs. In contrast, ObR and ObRb mRNA expression was higher in mammary fat pad tissue from AL mice compared with ICR and CCR mice. This result suggests that leptin receptors may have a role in the early phase of MT development. These findings are preliminary being based on the limited availability of tumor tissue, but they provide identification of pathways and proteins to be examined in greater detail in future studies.

IGF-I has been implicated as a metabolic mediator of caloric restriction in prevention of aging and tumorigenesis. As expected, CCR was associated with reduced serum IGF-I levels in CCR compared with AL mice, and ICR-79 mice had reduced IGF-I levels following 3 weeks of restriction. Refeeding following caloric restriction resulted in a rapid restoration of IGF-I levels of ICR mice into the range of AL mice. This observation is in agreement with results from other restriction/refeeding studies (12, 19, 44, 58). It is interesting to note that although IGF-I levels of restricted/refed rodents are not significantly different from *ad libitum*–fed controls, the concentrations consistently are found to be slightly reduced. Whether this has a physiological effect mediated through IGF-I binding proteins or receptors is currently being assessed.

Epidemiologic studies implicate IGF-I in the development of human breast cancer (59–63). However, whether lowering IGF-I levels results in breast cancer prevention is not known. Also, whether decreased caloric intake affects breast cancer development in humans is unclear. Based on assessment of age-cohorts, it was speculated that decreased caloric intake of adolescents during World War II in Norway contributed to decreased breast cancer incidence in adulthood (64), but in another study, individuals exposed to reduced food intake in the Netherlands during similar time periods did not exhibit this effect (65). Protection resulting from caloric restriction during adolescence and young adulthood on breast cancer development was identified from follow-up of women with anorexia nervosa (66). This study also may suggest a protective effect of caloric restriction/refeeding as these women were treated for their eating disorder and presumably experienced more than one period of restriction/refeeding. Only one study has directly assessed the effect of ICR on breast cancer development and no effect, either positive or negative was reported (67). Whether ICR/refeeding and/or the resulting weight-cycling have negative health consequences has been the subject of debate, but, in general, there have not been supporting experimental results. However, a recent study indicated that weight-cycling in obese postmenopausal women may compromise their immune status (68).

The limited number of animal and human studies makes it difficult to know the extent of the effect of ICR/refeeding intervention has on the prevention of breast/mammary tumors. However, understanding the mechanism(s) of this protective effect should provide valuable insights into tumor prevention strategies. This protective action in rodents occurs with only moderate reduction in cumulative caloric intake and without significant long-term effects on body weight and fat pad weights after refeeding. Identification of signal transduction, cell cycle and/or other pathway(s) affected, as well as other factors such as age when intervention is initiated, body weight (normal weight vs. obesity) status, and tumor etiology all need to be evaluated. Given that many women decrease caloric intake and lose weight, but with refeeding regain the lost weight, these findings may provide some assurance that this phenomenon is not detrimental with respect to breast cancer development, and in fact may be protective.

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