

An Experimental Paradigm for Studying the Cellular and Molecular Mechanisms of Cancer Inhibition by Energy Restriction

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With a rapid-emergence, chemically induced animal model for breast cancer, an experiment designed to test the hypothesis that energy restriction (ER) induces the loss of carcinogen-initiated cells from the mammary gland, thereby conferring a permanent protective effect against the development of cancer, failed to support this hypothesis. Nonetheless, this experiment served to define an experimental approach and a time frame on which to focus mechanistic inquiry. With an ER and energy repletion (ER-REP) protocol as a tool for identifying potential mediators of the cancer-inhibitory activity of ER, concomitant changes in plasma corticosterone and insulin-like growth factor 1 during energy restriction and repletion were observed. The relationship of the timing of these hormonal changes to the time frame of change in the carcinogenic response during ER-REP was consistent with the role of both hormones in mediating the protective effects of ER. However, a similar pattern of change in the energy-regulated hormone leptin indicated that its role in cancer inhibition also merits consideration. © 2002 Wiley-Liss, Inc.

Key words: cell deletion; energy restriction; energy repletion; corticosterone; insulin-like growth factor 1; leptin; mammary carcinogenesis; rat

INTRODUCTION

The cancer-inhibitory activity of long-term energy restriction (ER) is well documented in many model systems, including those for breast cancer [1–7]. Despite the wealth of evidence that ER is inhibitory to the development of cancer, the mechanisms that account for protection remain poorly understood. One of the impediments to progress in this area has been the uncertainty about when during the carcinogenic process, which encompasses time frame of 4–6 mo in many experimental models, mechanistic studies would be most insightful. To address this problem, it was decided to use a rapid-emergence experimental model for breast cancer [8] and to superimpose on this system a dietary protocol in which a period of long-term ER was followed by a period of energy repletion (ER-REP). This model was used to test two questions.

While long-term ER exerts a profound inhibitory effect on mammary carcinogenesis, studies in which single periods of restriction have been followed by ad libitum feeding have produced varying results. For example Kritchevsky and co-workers showed that 25% ER for the first 16 wk of a 24-wk protocol inhibits the carcinogenic response by 60%, whereas restriction for an 8-wk period during the first, middle, or last part of the 24-wk experiment results in various reductions (20%, 10%, and 40% inhibition, respectively), and restriction for only the first 4 wk has

no effect [9]. On the other hand, Sylvester and co-workers reported that ER at twice the level used by Kritchevsky et al. [9] and of similar duration to the period of ER for 1 wk before and 2 wk after carcinogen administration reduces mammary cancer incidence by approximately 70%. A similar duration of ER initiated 1 or 3 wk after carcinogen administration, however, has no effect on cancer incidence [10]. Thus, while some evidence indicates that a sustainable protective effect exists following ER, other data imply that the effect is transient. At other organ sites, with different model systems and alternative ER protocols, the data also vary. In models of liver cancer, however, a sustainable protective effect of a brief period of ER has been reported to be associated with the induction of apoptosis and the deletion of premalignant cell populations. Given that induction of apoptosis in response to ER has been reported by several labs, and by our own, in the model system for breast cancer used in this study, we decided to use

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Abbreviations: ER, energy restriction; ER-REP, energy repletion; IGF-1, insulin-like growth factor 1.

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this new experimental paradigm to test the hypothesis that ER induces the deletion of carcinogen-initiated cells (cell-deletion hypothesis), presumably by inducing apoptosis.

Based on the results of the experiment designed to investigate the cell-deletion hypothesis, a second protocol was conceived for investigating molecular mechanisms that narrowed the critical window of observation to a 7-d period after the initiation of ER-REP. Several laboratories have reported that animals subjected to ER experience an increase in plasma levels of corticosterone [11,12] that is reflected by an increase in excretion of immunoreactive corticosteroid metabolites in the urine [13]. Other laboratories have observed a decrease in circulating levels of insulin-like growth factor 1 (IGF-1) [14–16]. In both cases it has been hypothesized that the changes in circulating levels of one or the other of these hormones account for the potent cancer-inhibitory activity of ER. However, there have been no reports in which concomitant changes in these hormones brought about by ER have been investigated. Given evidence that both hormones can modulate the processes of cell proliferation and apoptosis in mammary tissue, the effect of ER on plasma levels of corticosterone and IGF-1 also were studied. In addition, plasma levels of leptin were measured not only because of leptin's potential role as an energy-related marker for cancer risk [17] but also because emerging evidence implies the independent role of leptin in accounting for the association between the host's energy status and its risk for cancer [18,19].

MATERIALS AND METHODS

Carcinogen Administration and Diets

Female Sprague-Dawley rats were obtained from Taconic Farms, Germantown, N.Y., at 20 d of age. At 21 d of age the animals were injected intraperitoneally with 50 mg 1-methyl-1-nitrosourea/kg body weight, as previously described [20]. Rats 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. Animal rooms were maintained at $22 \pm 1^\circ\text{C}$, with 50% relative humidity and a 12-h light/12-h dark cycle. Beginning at 21 d after carcinogen administration, all rats were weighed and palpated daily for detection of mammary tumors. All detectable mammary lesions were excised as reported by Thompson et al. [8] and subsequently processed for histologic classification.

Experiment 1

This experiment was designed to test the cell-deletion hypothesis by determining if ER would render a permanent protective effect against mammary carcinogenesis in the absence of continued

restriction. Sixty-six rats were randomized into two groups (33 rats/group): ad libitum fed (control) and 40% ER (starting from 6 d after carcinogen). Six weeks after carcinogen injection, the 40% ER rats were switched to the same diet as the control group and were fed ad libitum for an additional 7 wk. A modified AIN-93G diet and feeding protocol were used as previously described [13]. The diet fed to 40% ER animals was formulated to ensure an intake of all nutrients equivalent to the control group while limiting total dietary calories by reducing carbohydrate. 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 d per wk, to avoid possible confounding due to intergroup variation of meal timing, meal number, and duration of fasting between meals. Rats in the groups fed ad libitum were allowed access to an unlimited amount of diet at each meal, while rats in calorie-restricted groups were given a restricted amount of the diet at each meal.

Experiment 2

The objective of this experiment was to determine plasma levels of corticosterone, IGF-1, and leptin during ER and at intervals following ER-REP. One hundred and eight rats were randomized into three groups: ad libitum fed (control, 36 rats), 40% ER (40% ER, 36 rats) continuously (from 6 d to 49 d after carcinogen), and 40% ER for 6 wk (from 6 d to 42 d after carcinogen) but ad libitum fed from 42 d after carcinogen until killed, 49 d after carcinogen (ER-REP, 36 rats). Six rats from each group were killed at 42, 43, 44, 45, 46, and 49 d after administration of carcinogen, that is, at 0, 24, 48, 72, 96, and 168 h following the initiation of ER-REP. Other aspects of the experimental design were identical to those described under Experiment 1.

Assessment of Corticosterone, IGF-1, and Leptin in Plasma

Blood samples were collected at the midpoint of the experiment (3 wk after carcinogen administration) and at necropsy. For the samples obtained at 3 wk, blood was collected between 9:00 and 11:00 A.M., as previously described [21]. For the second collection, obtained during necropsy between 9:00 and 11:00 A.M. (after the rats lost consciousness following inhalation of gaseous carbon dioxide), blood was obtained directly from the retroorbital sinus and gravity-fed through heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) into EDTA-coated tubes (Becton Dickinson, Franklin Lakes, NJ). Plasma was isolated by centrifugation at $1000 \times g$ for 10 min at room temperature. Corticosterone in plasma was determined with antisera specific for corticosterone via a direct radioimmunoassay procedure (ICN Biomedicals, Inc., Costa Mesa, CA), as described previously [13]. IGF-1 and leptin in plasma were determined with enzyme immunoassay and

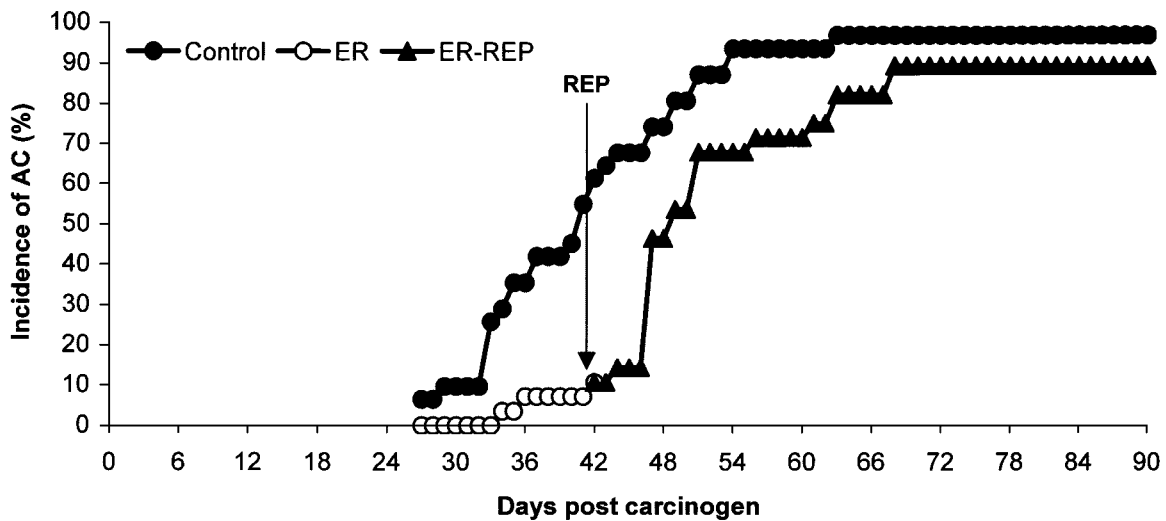
enzyme-linked immunosorbent assay kits, respectively (Diagnostic Systems Laboratories, Inc., Webster, TX).

RESULTS AND DISCUSSION

Given the varying results reported in response to episodic ER, the primary objective of Experiment 1 was to determine whether ER would result in the elimination of MNU-transformed mammary epithelial cells. These cells are the progenitors of the mammary carcinomas that develop in this model system.

The prediction was that cell deletion of clonal expansion would be manifest as a lower incidence and multiplicity of mammary cancers in animals following ER-REP compared with animals fed ad libitum throughout the study. As shown in Figure 1A, 40% ER significantly inhibited the cumulative incidence of palpable mammary adenocarcinomas during the 42-d period after carcinogen administration over which ER was imposed (61% vs. 11%; $P < 0.001$ via chi-square analysis [22]), a finding consistent with that of a previous report [13].

A.



B.

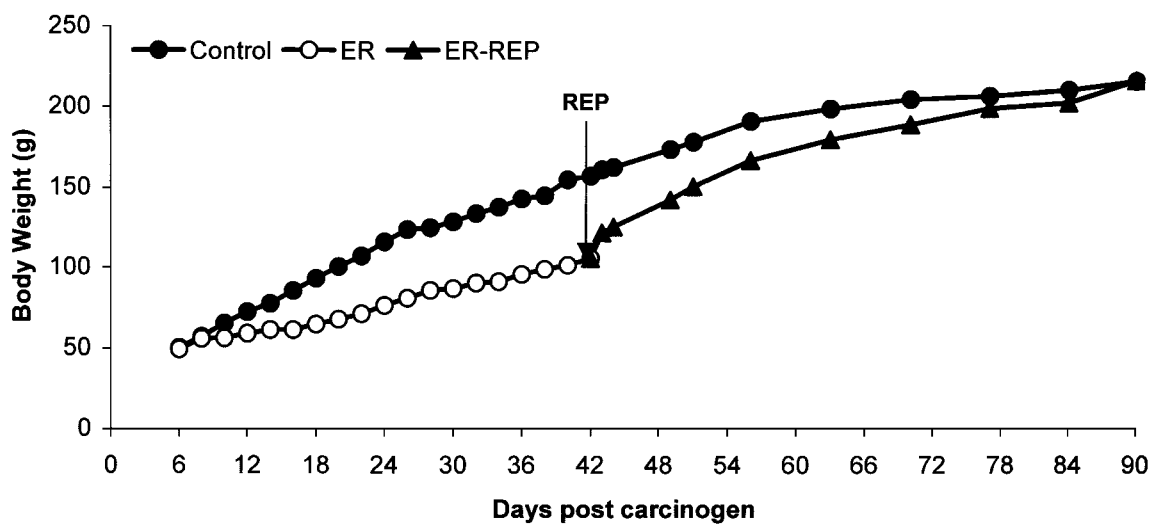


Figure 1. Results from Experiment 1. The cumulative incidence (A) of palpable mammary adenocarcinomas (AC) and the body weight (B) of rats fed with either AIN-93G (control) nonrestricted diet or AIN-93G modified for use in ER experiments and fed at a level 40% less than the diet consumed by the control group. The energy-restricted diet was fed for 6 wk, followed by ER-REP, with ad libitum feeding of the control diet for 7 wk.

However, upon ER-REP, the incidence of palpable cancer increased rapidly and, within 60 d, was no different from the incidence observed in the control group. A similar pattern of change in the multiplicity of palpable mammary carcinomas also was observed (data not shown). Thus, the data shown in Figure 1A fail to support the hypothesis that ER conveys a sustainable protective effect against cancer via a cell-deletion mechanism with this experimental paradigm.

The lack of a sustainable protective effect in this model raises the question of why the results within and among other studies vary. One possible explanation may lie in the data shown in Figure 1B. These body-weight data illustrate that a common perception, that ER is always accompanied by weight loss, is incorrect. On the contrary, during the ER phase of Experiment 1, animals on restricted diets remained in positive energy balance, that is, they gained weight in the absence of weight loss, although their slower rate of growth resulted in a 32% difference in body weight between the control and ER rats by 42 d after carcinogen administration. While experiments certainly can be designed in which ER-induced weight loss occurs, it is not a requisite effect of imposing ER. Thus, it is clear that animals on ER can remain in positive energy balance and continue to gain weight and still be protected against tumor development, as shown in Figure 1A. Because deletion of preneoplastic cells has been reported in studies in which ER induced significant weight loss, perhaps weight loss is essential to the induction of cellular and molecular mechanisms that induce the deletion of premalignant cells. On the other hand, during ER in which a positive energy balance is maintained, ER may inhibit carcinogenesis via suppression of clonal expansion. This topic, which has considerable practical ramifications given current worldwide trends in the misregulation of body weight [23], clearly merits further investigation.

As noted in the introduction, a long recognized problem in the investigation of the cancer inhibitory effects of various agents, including ER, is the identification of the time points in a carcinogenesis experiment that would be most advantageous to

investigate mechanisms. Examination of the cancer incidence data shown in Figure 1A indicated that the loss of protection against mammary carcinogenesis after the initiation of ER-REP was rapid and implied that release from ER would cause an equally rapid “de-repression” of the mechanisms accounting for the ER inhibitory effect. With this in mind, Experiment 2 was designed and implemented to determine the pattern of change of two hormones implicated as mediators of ER’s cancer-inhibitory activity, that is, corticosterone and IGF-1 [11–16]. Table 1 shows that at the midpoint (21 d post carcinogen) and on the last day (42 d of carcinogen) of ER, plasma corticosterone levels were significantly elevated and plasma IGF-1 levels were markedly reduced in ER rats compared with the levels seen in controls fed ad libitum. The effects of ER on corticosterone or IGF-1 have been published, but to our knowledge, this is the first report of the concomitant effects of ER on both hormones. This observation underscores the possibility that the cancer-inhibitory effects of ER are due to concurrent changes in circulating levels of these hormones. This hypothesis has considerable merit, because plasma levels of corticosterone and IGF-1 are inversely related, tightly linked, and associated with changes in the homeostatic regulation of mammary gland tissue size homeostasis and in the development of mammary tumors [13,16].

Figure 2B–D shows the plasma hormone levels from the ER-REP phase of Experiment 2. In addition, the cumulative occurrence of newly detected mammary carcinomas following ER-REP during the same time frame of Experiment 1 is shown (Figure 2A). These were compared to facilitate the inspection of the time frame of changes in plasma hormone levels of Experiment 2 and changes in the rate of new tumor occurrence after ER-REP as observed in Experiment 1. After a 4-d delay following initiation of ER-REP, a marked increase in the number of new mammary carcinomas was detected in the ER group. As shown in Figure 2B and C, rapid and reciprocal changes in plasma corticosterone and IGF-1 levels were noted, and they preceded the change in the rate of mammary tumor occurrence. Plasma corticosterone, which was elevated during the ER phase of the

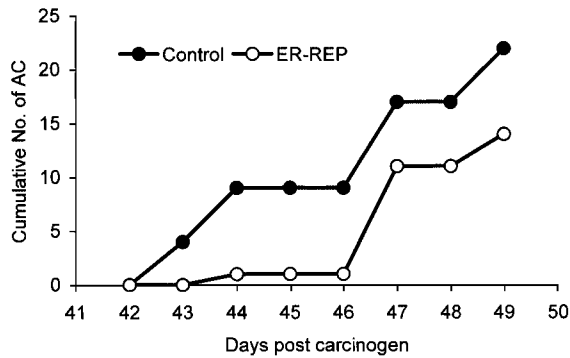
Table 1. Effect of ER on Levels of Plasma Corticosterone, IGF-1, and Leptin*

Plasma levels (ng/mL)	Midpoint		Last day of ER	
	Control	40% ER	Control	40% ER
Corticosterone	188 ± 30	350 ± 36 [†]	268 ± 73	564 ± 58 [†]
IGF-1	381 ± 21	160 ± 16 [†]	625 ± 56	349 ± 26 [†]
Leptin	1.35 ± 0.10	0.54 ± 0.13 [†]	1.75 ± 0.14	0.90 ± 0.07 [†]

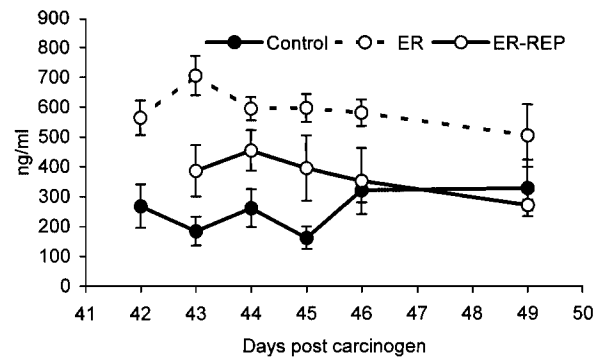
*The animals were from Experiment 2, described in Materials and Methods. Results are expressed as the mean ± SE. Midpoint: 21 d post carcinogen; last day of ER: 42 d post carcinogen.

[†]Significantly different from corresponding control value, $P < 0.001$ by analysis of variance.

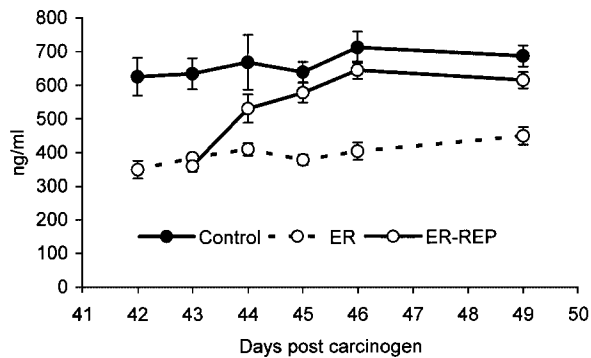
A. Cumulative number of new tumors



B. Plasma corticosterone



C. Plasma IGF-I



D. Plasma leptin

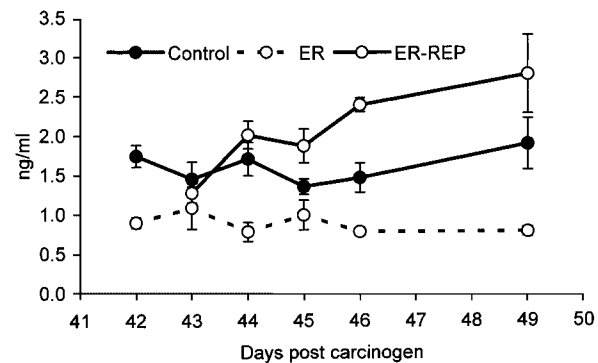


Figure 2. Results from Experiments 1 and 2. (A) The number of new palpable mammary adenocarcinomas (AC) detected only during the period of ER-REP in Experiment 1. (B–D) Plasma corticosterone (B), IGF-1 (C), and leptin (D) in rats from Experiment 2 fed with either nonrestricted AIN-93G (control) diet or AIN-93G modified for use in ER experiments and fed at a level 40% less than the diet consumed by the control group. The energy-restricted diet was fed for 6 wk, followed by ER-REP, with ad libitum feeding of the control diet for

7 d, that is, from 42 d to 49 d after carcinogen. Plasma hormone data were evaluated by analysis of variance (ANOVA), with treatment and time after ER as independent variables [22]. For treatment the ANOVA result was statistically significant ($P < 0.01$) for each of the hormones evaluated, whereas for time after ER the ANOVA result was significant for IGF-1 and leptin ($P < 0.01$) but not for corticosterone.

experiment, decreased within 24 h of ER-REP and remained intermediate to plasma levels seen in the control or ER groups. Plasma IGF-1, which was suppressed by ER, rebounded rapidly following the initiation of ER-REP, and plasma levels were comparable to those of control levels within 72 h. Thereafter, plasma IGF-1 levels remained comparable to levels in the control animals.

The association noted between changes in plasma hormone levels and the rate of mammary tumor occurrence are consistent with a cause-effect relationship and suggest a rapid "de-repression" of the cellular and molecular mechanisms regulated by corticosterone and IGF-1. Thus, we propose that key changes in the mechanisms that account for the suppression of mammary carcinogenesis by ER probably occur within the first 72 h after release from ER. With the three-group experimental design outlined in Experiment 2, it should be possible to elucidate these critical events and thus permit delineation of the mechanisms that account for inhibition of mammary carcinogenesis by ER.

Based on the data shown in Figure 2, it is tempting to speculate that the changes in corticosterone and IGF-1 account for the cancer-inhibitory activity of ER. However, the plasma leptin data shown in Table 1 and Figure 2D provide an example of one of the many other changes that also take place in response to ER. Similarly to the pattern of change observed in plasma IGF-1 levels, at all time points assessed, the levels of plasma leptin decreased significantly in rats subjected to ER compared with control rats. Upon ER-REP, plasma leptin levels rebounded rapidly. The possibility exists that such changes, which have been reported in other model systems [24], account in part for ER's cancer-inhibitory activity. In this regard, leptin may not serve simply as a marker for the energy status of an organism [25]; emerging evidence indicates that it also may reflect cancer risk [17]. Some of the recently reported activities of leptin, apart from its role in satiety, indicate that it could exert independent effects on mechanisms that can modulate mammary carcinogenesis [18,19,26]. It is notable that of the

three hormones assessed, leptin was the only one that overshot the level observed in plasma of the control group, and this effect persisted for the 7-d period during which the effects of ER-REP were investigated.

In conclusion, experiments designed to test the cell-deletion hypothesis of cancer prevention by ER failed to support this hypothesis. Nonetheless, these experiments served to define an experimental approach and a time frame of observation within a rapid-emergence model for breast cancer on which to focus mechanistic inquiry. Evidence is presented showing concomitant changes in plasma levels of corticosterone and IGF-1 that imply that they play a role in mediating the protective effects of ER. However, other data suggest that it is advisable to exercise caution in assuming that changes in IGF-1 and corticosterone alone account for ER's cancer-preventive activity. The potential role of an energy-regulated hormone, leptin, merits additional consideration.

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