

# Identification of the Apoptosis Activation Cascade Induced in Mammary Carcinomas by Energy Restriction

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## ABSTRACT

Energy restriction (ER) inhibits mammary carcinogenesis and results in a marked reduction in tumor size, effects likely to be explained by ER-mediated induction of apoptosis. The goal of this study was to investigate the molecular mechanism(s) accounting for apoptosis induction. To do this, chemically induced mammary carcinomas were evaluated from rats that were *ad libitum* fed (control), 40% ER, or 40% ER but energy repleted for 7 days before study termination (ER-REP); the ER-REP group permitted the determination of the reversibility of ER-mediated effects. Cleaved products of poly(ADP-ribose) polymerase 1 were elevated by ER ( $P < 0.025$ ) providing biochemical evidence of apoptosis induction. cDNA microarray analysis identified the Bcl-2, CARD, and IAP functional gene groupings as being involved in apoptosis induction. Consistent with the microarray data, the activities of caspases 9 and 3 were observed to be ~2-fold higher in carcinomas from ER rats ( $P \leq 0.01$ ), whereas caspase 8 activity was similar in carcinomas from all three of the groups. This evidence that ER-induced apoptosis mediated by the mitochondrial pathway was additionally supported by the finding that levels of Bcl-2, Bcl-xl, and XIAP protein were significantly lower ( $P < 0.01$ ), and levels of Bax and Apaf-1 were elevated ( $P < 0.02$ ) in ER carcinomas *versus* those carcinomas from control or ER-REP rats. Additional studies revealed that Akt phosphorylation (activation) was reduced in mammary carcinomas from ER rats. Thus, it appears that ER induces apoptosis in mammary carcinomas via a cell survival factor-dependent pathway.

## INTRODUCTION

Energy restriction (ER), a potent inhibitor of experimentally induced mammary carcinogenesis, has been shown to inhibit cell proliferation and to concomitantly induce apoptosis (1). These effects are likely, at least in part, to account for the cancer-inhibitory activity of ER. We have reported recently on the mechanisms that underlie the effects of ER on cell cycle progression (2). In the experiments reported here, the mechanism(s) by which ER induces apoptosis was investigated.

Apoptosis is an essential component of the cellular regulation of tissue size homeostasis (3). The occurrence of a tumor represents a failure of tissue size regulation, and mounting evidence indicates that misregulation of apoptosis plays a causal role in the development of many tumors (4). Reciprocally, induction of apoptosis is a viable mechanism to block tumor occurrence as well as to reduce the growth rate of tumors that do emerge (5). ER profoundly reduces the occurrence of mammary carcinomas, and the carcinomas that do emerge have markedly smaller size (6). Therefore, we reasoned that apoptosis would be operative in carcinomas arising in ER rats, and that investigation of these carcinomas would provide insights about the mechanisms underlying the induction of apoptosis. This investigation also took advantage of a recently developed paradigm for studying the effects of ER on the carcinogenic process in which the cancer-inhibitory effects of ER are reversed within 7 days of refeeding at *ad*

*libitum* levels (7). Using this paradigm, mechanisms can be investigated by comparing the responses observed in control and ER-treated rats to those observed in ER rats that are energy repleted for 7 days.

Initially, experiments were conducted to determine whether biochemical evidence could be obtained to substantiate our previous report, using a morphological approach, that ER induces apoptosis in mammary carcinomas (1). The generation of cleavage products of PARP-1 was used for this purpose (8). These experiments were followed by the investigation of global effects of ER on the expression of genes involved in apoptosis induction pathways using cDNA microarrays. Candidate mechanisms were additionally evaluated by assessing caspase activation with the specific goal of determining whether ER induced apoptosis via the activation of caspase 8 or 9. On the basis of those results, the mitochondrial pathway of apoptosis induction was examined in greater detail, followed by experiments to explore the signaling pathway(s) involved in the apoptotic response.

## MATERIALS AND METHODS

**Chemicals.** The following materials were purchased from commercial sources: anti-poly(ADP-ribose) polymerase (PARP), anti-Akt, and anti-phospho-Akt antibodies (Cell Signaling Technology, Beverly, MA); anti-Apaf-1 (Upstate Biotechnology, Lake Placid, NY); anti-Bcl-2 and anti-Bax (PharMingen, San Diego, CA); anti-hILP/XIAP (H62120; Transduction Laboratory, Lexington, KY); anti- $\beta$ -actin antibody (Sigma Chemical Co., St. Louis, MO); anti-IGF-IR, anti-Bcl-xl, and goat antimouse immunoglobulin- and goat anti-rabbit immunoglobulin-horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Corp., Santa Cruz, CA); and ECL detection system (Amersham Life Science Inc., Arlington Heights, IL).

**Tissue Used for Analyses.** Histopathologically confirmed mammary carcinomas used for the experiments reported herein were obtained from a previously described investigation (7). Briefly, in that study female Sprague Dawley rats were obtained from Taconic Farms (Germantown, NY) at 20 days of age. At 21 days of age animals were injected with 50 mg methylnitrosourea/kg body weight (i.p.) as described previously (9). 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 days postcarcinogen, all of the rats were weighed and palpated daily for detection of mammary tumors. All of the detectable mammary lesions were excised as reported and were subsequently processed for histological classification (10). In this study, not more than 1 carcinoma per rat was used, and carcinomas from the control group were aged matched (duration between the day when the tumor was palpated and the day the study was terminated) to the carcinomas analyzed in the ER group.

**Experiment Design.** One hundred and eight rats were randomized into three groups, *ad libitum* fed (control, 36 rats), 40% energy-restricted (ER, 36 rats) continuously, and 40% energy restricted for 6 weeks but *ad libitum* fed until euthanized [energy repletion (ER-REP), 36 rats]. Six rats from each group were sacrificed at 0, 24, 48, 72, 96, and 168 h after the initiation of energy repletion. In this study only rats from the 168-h time point were evaluated, and there were only 5 animals with carcinomas of sufficient size for analysis. A modified AIN-93G diet and feeding protocol were used as described previously (6). The diets fed to ER animals were formulated to insure an intake of all of the nutrients equivalent to the control group, while limiting total dietary calories by reducing carbohydrate. All of the 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/week to reduce

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possible confounding due to intergroup variation of meal timing, meal number, and duration of fasting between meals.

**Caspase Activity Assay.** Mammary carcinomas were homogenized in cell lysis buffer (Biosource International, Camarillo, CA) using a Polytron tissue homogenizer (Brinkman Instruments). The lysates were clarified by centrifugation in an Eppendorf centrifuge for 20 min at 4°C. Caspase activity in the supernatant was determined as described previously (11). Briefly, 100 µg of total protein, as determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA), was incubated with 200 µM substrates Ac-DEVD-pNA (caspase-3), Ac-IETD-pNA (caspase-8), or Ac-LEHD-pNA (caspase-9; Biosource International) in 50 µl of caspase assay buffer (Biosource International). After incubation at 37°C for 2 h, the release of pNA was measured at 405 nm using SPECTRA MAX PLUS Microplate Spectrophotometer System (Molecular Devices, Sunnyvale, CA). One unit of enzymatic activity of caspases was defined as the release of 1 nmol of pNA/h at 37°C (Biosource International).

**Expression of Apoptosis Regulatory Molecules by Western Blotting.** Mammary carcinomas were homogenized in lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP40, and 0.2 unit/ml aprotinin] using Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY). The lysate was collected by centrifugation for 15 min in an Eppendorf centrifuge at 4°C, and protein concentration in the clear supernatant was determined by the Bio-Rad protein assay. For Western blotting of apoptosis regulatory molecules, 40 µg of protein lysate per sample was denatured with SDS-PAGE sample buffer [63 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.0025% bromophenol blue, and 5% 2-mercaptoethanol], subjected to SDS-PAGE on a 8% or 12% gel, and the protein bands blotted onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA). The levels of PARP-1, Bcl-2, Bcl-xl, Bax, Apaf-1, XIAP, insulin-like growth factor (IGF) -1R, total Akt, phosphorylated Akt, and β-actin were determined using the specific primary antibodies designated above, followed by treatment with the appropriate peroxidase-conjugated secondary antibody and visualized by the ECL detection system. Signals were quantitated by scanning the film with ScanJet (Hewlett Packard, Palo Alto, CA), and the intensity of the bands was analyzed by using "Image-Pro Plus" software (Media Cybernetics, Silver Spring, MD).

**cDNA Microarrays.** Total RNA was isolated from mammary tumors using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's directions as described previously (2). Total RNA was used as a template for biotinylated probe synthesis using Nonrad-GEArray Q series kit (SuperArray Inc., Bethesda, MD) as described previously (2). GEArray Q series membranes (SuperArray Inc.) were prehybridized with GEArray Hybridization Solution (SuperArray Inc.) containing denatured sheared salmon sperm DNA (100 µg DNA/ml; Life Technologies, Inc., Grand Island, NY) at 60°C for 2 h and hybridized in the Hybridization Solution (SuperArray Inc.) containing denatured cDNA probe of the samples at 60°C overnight. After washing the membrane twice with wash solution 1 (300 mM sodium chloride, 30 mM sodium citrate, and 1% SDS) and twice with wash solution 2 (15 mM sodium chloride, 1.5 mM sodium citrate, and 0.5% SDS) for 10 min each at 60°C, the membrane was blocked in GEAblocking solution Q for 40 min at room temperature (SuperArray Inc.) and incubated with diluted alkaline phosphatase-conjugated streptavidin (1:5000 dilution) in buffer F (SuperArray Inc.) for 30 min at room temperature. After the membrane was washed in a washing buffer (SuperArray Inc.) three times and rinsed in a rinsing solution (SuperArray Inc.), the membrane was incubated with chemiluminescent substrate and exposed to X-ray film. Signals were quantitated by scanning the film with ScanJet (Hewlett Packard), and the intensity of the spots was analyzed by using the Image-Pro Plus software (Media Cybernetics). β-Actin and glyceraldehyde-3-phosphate dehydrogenase were used as positive controls, and bacterial plasmid (pUC18) was used as a negative control.

**Statistical Analyses.** Data derived from caspases activity assays were analyzed by ANOVA, and post hoc comparisons were made using the Bonferroni multiple-range test (12). Data derived from cDNA microarrays were initially analyzed by multivariate ANOVA to determine whether differences in gene expression within functional gene groupings were attributable to treatment (13). For functional groups in which evidence of an effect of treatment was obtained, the effect of treatment on the expression of genes within a functional group was performed via ANOVA using the Bonferroni multiple-range tests for post hoc comparisons. Western blot analyses represent semi-quantitative estimates of the amount of a specific protein that is present in a

cell extract. This fact was taken into account in the statistical evaluation of the data. The data displayed in the graphs are reported as means ± SE of the actual scanning units derived from the densitometric analysis of each Western blot. All of the values are the means of three different experiments. However, for statistical analyses, the units of scanning density derived from the analysis of the Western blots using Image Pro Plus were first ranked. This approach is particularly suitable for semiquantitative measurements that are collected as continuously distributed data. This approach has the advantage of maintaining the relative relationships among data being compared without giving undue weight to outlying results. The ranked data were then subjected to multivariate ANOVA. Statistically, this is a robust approach that takes into account both the fact that levels and/or activities of proteins in a molecular pathway may not vary independently of one another, as well as the issues that exist when multiple comparison are being made on a particular set of data. For those variables that were found to be statistically significant in the multivariate ANOVA, the Bonferroni multiple-range test was used for post hoc comparisons.

## RESULTS

**Characteristics of Animals and Tumors.** As noted above, tissue that was evaluated in this study was obtained from the experiment reported previously (7). General characteristics of animals and tumors used in this study are summarized in Table 1. As expected, the body weights and tumor volumes of animals in the ER group were significantly lower ( $P < 0.001$ ) than in either the *ad libitum*-fed control or ER-REP group. Tumor age was matched between the control and ER group.

**Effects of ER and ER-REP on Cleavage of PARP.** The effect of ER and ER-REP on the level of intact PARP-1 and its  $M_r$  24,000 and 89,000 cleavage fragments in mammary carcinomas was investigated by Western blotting. The results are shown in Fig. 1 and Table 2. Levels of both cleavage products were elevated in mammary carcinomas of ER rats in comparison with levels observed in carcinomas from control ( $P < 0.01$ ) or ER-REP-treated rats ( $P < 0.025$ ).

**Effects of ER and ER-REP on Transcript Expression Profiles.** As shown in Table 3, cDNA filter arrays were used to identify the likely candidate pathways by which ER induced apoptosis. Levels of transcripts of 96 genes representing 11 functional groupings of genes involved in apoptosis were examined. Statistical evidence of differential regulation of gene expression by ER was found for three functional gene groupings, the *Bcl-2* family, the *CARD* family, and the *IAP* family. The effects of ER and ER-REP on the specific genes in each family that were significantly up or down-regulated are shown in Fig. 2.

**Effects of ER and ER-REP on Caspases Activities.** To identify the caspases involved in apoptosis induction by ER, activities of two initiators and one executioner caspase were determined. As shown in Table 4, the activities of caspases 9 and 3 were increased significantly ( $P = 0.01$  and  $P < 0.001$ ) in mammary carcinomas from ER rats compared with the activity observed in carcinomas from control rats (1.6-fold increase for caspase 3 and 1.8-fold increase for caspase 9).

Table 1 Body weight, tumor age, and tumor size<sup>a</sup>

	Control	40% ER <sup>b</sup>	ER-REP
No. of rats	18	18	5
Body weight (g)	185 ± 5 <sup>c</sup>	126 ± 1 <sup>d</sup>	177 ± 6 <sup>c</sup>
Tumor age (days)	18 ± 3 <sup>c</sup>	17 ± 3 <sup>c</sup>	8 ± 1 <sup>d</sup>
Tumor volume (mm <sup>3</sup> )	299 ± 85 <sup>c</sup>	32 ± 11 <sup>d</sup>	88 ± 18 <sup>c</sup>
Estimated growth rate (mm <sup>3</sup> )/day	13 ± 4 <sup>c</sup>	2 ± 0.6 <sup>d</sup>	11 ± 0.9 <sup>c</sup>

<sup>a</sup> Values are means ± SE. All rats were sacrificed at 75 days of age, the planned termination date of the study. Only one tumor per rat was evaluated.

<sup>b</sup> ER: energy restriction, ER-REP: energy repletion.

<sup>c</sup> Values within a row with different superscripts were statistically different,  $P < 0.05$ .

<sup>d</sup> Values within a row with different superscripts were statistically different,  $P < 0.05$ .

<sup>e</sup> Values within a row with different superscripts were statistically different,  $P < 0.05$ .

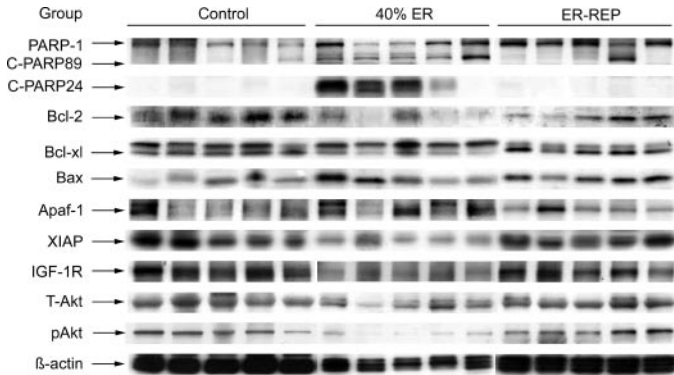


Fig. 1. Levels of proteins in mammary carcinomas of rats fed *ad libitum* (control), 40% energy-restricted (ER) continuously, or 40% energy restricted for 6 weeks but *ad libitum* until euthanized (ER-REP) as described in "Materials and Methods." Representative protein expression in tumor lysates determined by Western blotting as detailed in "Materials and Methods."

Table 2 Effect of energy restriction (ER) and energy repletion (ER-REP) on the cleavage of poly(ADP-ribose) polymerase 1 (PARP-1)<sup>a</sup>

	Control	40% ER	ER-REP
No. of rats	18	18	5
PARP-1	0.11 ± 0.03 <sup>b</sup>	0.18 ± 0.04 <sup>c</sup>	0.18 ± 0.02 <sup>c</sup>
89-kDa PARP-1	0.06 ± 0.01 <sup>b</sup>	0.15 ± 0.03 <sup>c</sup>	0.05 ± 0.02 <sup>b</sup>
24-kDa PARP-1	0.04 ± 0.01 <sup>b</sup>	0.42 ± 0.06 <sup>c</sup>	0.01 ± 0.01 <sup>d</sup>

<sup>a</sup> Only one tumor per rat was evaluated. Each value is expressed in relative density units and is a mean ± SE. Data were evaluated by multivariate analysis of variance as described in "Material and Methods." PARP-1, intact 113 kDa protein; 89-kDa PARP-1 and 24-kDa PARP-1, the two cleavage products into which the 113 kDa protein is cleaved during apoptosis.

<sup>b</sup> Values within a row with different alphabetical superscripts were statistically different, *P* < 0.025.

<sup>c</sup> Values within a row with different alphabetical superscripts were statistically different, *P* < 0.025.

<sup>d</sup> Values within a row with different alphabetical superscripts were statistically different, *P* < 0.025.

This effect was reversed by ER-REP. No significant differences in caspase 8 activity were observed among treatment groups.

**Effects of ER and ER-REP on Apoptosis Regulatory Molecules.**

The effects of ER and ER-REP on the levels of Bcl-xl, Bcl-2, Bax, Apaf-1, and XIAP in mammary carcinomas were determined by Western blotting. The results are shown in Fig. 1 and summarized in Table 5. The amount of Bcl-2 and Bcl-xl were decreased significantly in carcinomas from ER rats in comparison with those from control (*P* = 0.003) or ER-REP (*P* = 0.004) rats. No statistically significant difference was observed in the level of Bcl-2 between ER and ER-REP rats, or in the levels of both proteins between carcinomas from control and ER-REP rats. The protein level of Bax in mammary

carcinomas from ER or ER-REP rats was increased significantly in comparison with the level observed in carcinomas from control rats (*P* = 0.005 for both). No statistically significant difference was observed in the level of Bax in carcinomas from ER and ER-REP rats. The amount of Apaf-1 protein in mammary carcinomas from ER rats was increased significantly in comparison with the level observed in carcinomas from control (*P* = 0.014) or ER-REP (*P* = 0.002) rats. No statistically significant difference was observed in the level of Apaf-1 protein in carcinomas from control and ER-REP rats. The protein level of XIAP in mammary carcinomas from ER rats was decreased significantly in comparison with that observed in carcinomas from control (*P* = 0.009) or ER-REP (*P* = 0.009) rats. No statistically significant difference was observed in the level of XIAP between control and ER-REP rats.

**IGF-IR and Akt.** The effects of ER and ER-REP were studied by Western blotting on two elements of a signal transduction pathway that could down-regulate Bcl-2 family mediated initiation of apoptosis, namely levels of IGF-IR and activation (phosphorylation) of Akt. As reported in Table 6, the level of IGF-IR protein in mammary carcinomas from ER rats was decreased significantly in comparison with the level observed in carcinomas from control (*P* = 0.002) or ER-REP (*P* = 0.007) rats. Statistically significant differences (*P* < 0.02) were also observed among the three groups in the level of pAkt with an order of ER < control < ER-REP, whereas no statistically significant difference was observed in the level of total Akt.

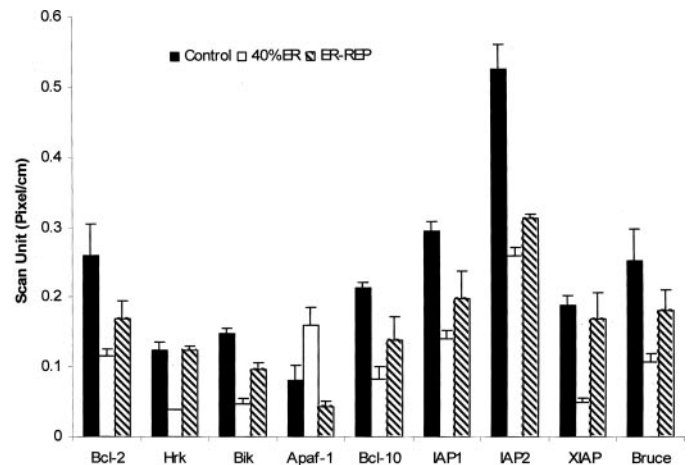


Fig. 2. Gene array analysis in mammary carcinomas of rat fed *ad libitum* (control), 40% energy-restricted (ER) continuously, or 40% energy restricted for 6 weeks but *ad libitum* until euthanized (ER-REP) as described in "Materials and Methods." These data are representative of four mammary carcinomas excised from four rats in each treatment group. Each data point is a mean of four carcinomas; bars, ± SE.

Table 3 Summary of functional gene grouping evaluated by cDNA microarray analysis

TNF ligand family	<i>Tnf</i> (TNF- <i>a</i> ), <i>Lta</i> (TNF- <i>b</i> ), <i>Ltb</i> (LT- <i>b</i> ), <i>Tnfsf4</i> (OX40 ligand), <i>Tnfsf5</i> (CD40 ligand), <i>Tnfsf6</i> (Fas L), <i>Tnfsf7</i> (CD27 ligand), <i>Tnfsf8</i> (CD30 ligand), <i>Tnfsf9</i> (4-1BB ligand), <i>Tnfsf10</i> (TRAIL), <i>Tnfsf11</i> , <i>Tnfsf12</i> (Apo3L), <i>Tnfsf14</i> (HVEM-L)
TNF receptor family	<i>Libr</i> (LT- <i>bR</i> ), <i>Tnfrsf1a</i> (TNFR1), <i>Tnfrsf1b</i> (TNFR2), <i>Tnfrsf4</i> (OX40), <i>Tnfrsf5</i> (CD40), <i>Tnfrsf6</i> (Fas), <i>Tnfrsf7</i> (CD27), <i>Tnfrsf8</i> (CD30), <i>Tnfrsf9</i> (4-1BB), <i>Tnfrsf10b</i> (TRAILR), <i>Tnfrsf11a</i> , <i>Tnfrsf11b</i> (osteoprotegerin), <i>Tnfrsf12</i> (DR3), <i>April</i> , <i>Dr6</i>
Bcl-2 family <sup>a</sup>	<i>Bcl2</i> , <i>Bcl2l</i> ( <i>bcl-x</i> ), <i>Bcl2a1d</i> ( <i>bfl-1</i> ), <i>Bcl2l2</i> ( <i>bcl-w</i> ), <i>Bcl2l10</i> , <i>Bax</i> , <i>Bak</i> , <i>Bokl</i> , <i>Bad</i> , <i>Bid</i> , <i>Bid3</i> , <i>Biklk</i> ( <i>bik</i> ), <i>Bim</i> , <i>Blk</i> , <i>Bnip3</i> ( <i>Nip3</i> ), <i>Mcl1</i>
Caspase family	<i>Casp1</i> , <i>Casp2</i> , <i>Casp3</i> , <i>Casp6</i> , <i>Casp7</i> , <i>Casp8</i> , <i>Casp9</i> , <i>Casp11</i> , <i>Casp12</i> , <i>Casp14</i>
IAP family <sup>a</sup>	<i>Birc1a</i> (NAIP1), <i>Birc1b</i> (NAIP2), <i>Birc1e</i> (NAIP5), <i>Birc2</i> (IAP1), <i>Birc3</i> (IAP2), <i>Birc4</i> (XIAP), <i>Birc5</i> (survivin), <i>Birc6</i> (bruce)
TRAF family	<i>Traf1</i> , <i>Traf2</i> , <i>Traf3</i> (CRAF1), <i>Traf4</i> , <i>Traf5</i> , <i>Traf6</i> , <i>Tank</i> (I-TRAF), <i>Traip</i>
CARD family <sup>a</sup>	<i>Apaf1</i> , <i>Asc</i> , <i>Arc</i> , <i>BCL10</i> (HuE10), <i>Nop30</i> -like
Death domain family	<i>Fadd</i> , <i>Myd88</i> , <i>Ripk1</i> , <i>Cradd</i> , <i>Dapk2</i>
Death effector domain family	<i>Cash</i> (Casper), <i>Fadd</i> , <i>Bar-like protein</i> , <i>Casp8ap2</i> (Flash)
CIDE domain family	<i>Dffa</i> , <i>Dffb</i> , <i>Cidea</i> , <i>Cideb</i>
p53 and ATM pathway	<i>Trp53</i> (p53), <i>Cdkn1a</i> (p21Waf1), <i>Mdm2</i> , <i>Gadd45a</i> , <i>Atm</i> , <i>Rpa-like protein</i> , <i>chek1</i> , <i>Rad53</i> , <i>Hus1</i>

<sup>a</sup> Multivariate analysis of various provided evidence of differential expression of genes in this functional grouping, *P* < 0.05.

Table 4 Effects of 40% energy restriction (ER) and of energy repletion (ER-REP) on activities of caspases in mammary carcinomas<sup>a</sup>

Caspase activity (U/mg protein) <sup>b</sup>	Control n = 18	ER n = 18	ER-REP n = 5
Caspase 3	58.4 ± 8.2 <sup>c</sup>	95.2 ± 9.6 <sup>d</sup>	42.1 ± 5.1 <sup>c</sup>
Caspase 8	15.1 ± 1.4 <sup>c</sup>	11.4 ± 1.5 <sup>c</sup>	17.8 ± 0.9 <sup>c</sup>
Caspase 9	15.1 ± 1.5 <sup>c</sup>	27.5 ± 2.2 <sup>d</sup>	13.7 ± 1.3 <sup>c</sup>

<sup>a</sup> Each value is mean ± SE. Data were analyzed by analysis of variance. *Post hoc* comparisons were made using the Bonferroni multiple-range test.

<sup>b</sup> U, unit; one unit of caspase enzymatic activity was defined as the release of 1 nmol of substrate (pNA) per h at 37°C. The extinction coefficient for pNA at 405 nm is 9750 M<sup>-1</sup> · cm<sup>-1</sup>.

<sup>c</sup> Values in a row with different alphabetical superscript were significantly different (*P* < 0.05).

<sup>d</sup> Values in a row with different alphabetical superscript were significantly different (*P* < 0.05).

Table 5 Levels of apoptosis-related proteins in mammary tumors of rats fed control, 40% energy-restricted (ER), or ER-repletion (ER-REP) diet<sup>a</sup>

Proteins investigated	Control n = 18	ER n = 18	ER-REP n = 5
Bcl-xl	0.12 ± 0.02 <sup>b</sup>	0.06 ± 0.01 <sup>c</sup>	0.12 ± 0.02 <sup>b</sup>
Bcl-2	0.22 ± 0.03 <sup>b</sup>	0.12 ± 0.01 <sup>c</sup>	0.14 ± 0.04 <sup>b,c</sup>
Bax	0.14 ± 0.02 <sup>b</sup>	0.31 ± 0.05 <sup>c</sup>	0.27 ± 0.03 <sup>c</sup>
Apaf-1	0.32 ± 0.04 <sup>b</sup>	0.55 ± 0.08 <sup>c</sup>	0.20 ± 0.04 <sup>b</sup>
XIAP	0.60 ± 0.08 <sup>b</sup>	0.14 ± 0.02 <sup>c</sup>	0.50 ± 0.06 <sup>b</sup>

<sup>a</sup> Each value is expressed in relative density units and is a mean ± SE. Data were analyzed by multivariate analysis of variance. *Post hoc* comparisons were made using the Bonferroni multiple-range test.

<sup>b</sup> Values in a row with different alphabetical superscript were significantly different (*P* < 0.05).

<sup>c</sup> Values in a row with different alphabetical superscript were significantly different (*P* < 0.05).

The ratio of phosphorylated Akt to total Akt between ER and control (*P* = 0.024) or ER-REP (*P* = 0.002) rats also was affected.

**DISCUSSION**

A number of laboratories have reported that acute or chronic ER is associated with an elevated rate of apoptosis in premalignant or malignant pathologies (1, 14–24); however, studies of the mechanism(s) by which ER induces this cell death pathway are limited and fail to provide a cohesive view of apoptosis induction. Moreover, the reports of apoptosis induction by ER in various cancer models are in marked contrast to reports in experimental models of aging that ER inhibits apoptosis (25–27). This situation underscores the importance of identifying the mechanisms that account for changes in rates of apoptosis induced by ER under both physiological and patho-physiological conditions.

We have reported previously that ER not only inhibited the occurrence of mammary carcinomas in a rat model for breast cancer, but that it also reduced the rate of tumor growth and the size of the carcinomas that did occur (6). As shown in Table 1, reduced tumor growth rate and size were observed in the carcinomas investigated in this study, and these effects (85% reduction in tumor size and 90% reduction in growth rate) were markedly greater than the reduced rate of body weight gain observed in response to ER (2.4 versus 1.4 g body weight gain per day, *i.e.*, a 40% reduction in growth rate); these effects were reversed, although not completely, by 7 days of ER-REP.

Using morphological criteria, we have observed that apoptosis is induced in the mammary carcinomas that arise in ER rats and that the rate of cell proliferation is suppressed (1). We hypothesize that these effects work in concert to inhibit carcinogenesis. On the basis of our previous observation that apoptosis is induced in carcinomas of ER rats, we set out to determine the elements of the cell death pathway affected by ER.

Consistent with our previous work (1), the data presented in Table

2 provide biochemical evidence that levels of the *M<sub>r</sub>* 24,000 and 89,000 cleavage products of PARP-1, a recognized early marker of apoptosis that is detected before morphological evidence of apoptotic cell death (8), are markedly elevated by ER. However, the data in Table 2 extend the initial observation and show that this effect can be rapidly reversed when ER is discontinued. This observation also is consistent with the rapid loss of protection against carcinogenesis that occurs when ER is discontinued (7).

Whereas evidence continues to accumulate demonstrating that levels of gene transcript expression do not necessarily parallel changes in the amount and/or activity of the translated proteins (28), transcript expression analyses remain a useful tool for screening for signaling pathways involved in explaining a particular biological event, in this case apoptosis induction. We had speculated at the outset of this work that apoptosis induction would be via an intrinsic pathway, *i.e.*, one in which mitochondrial function is known to be involved (29). This speculation was based on evidence that ER alters energy metabolism and signaling pathways that have been implicated in apoptosis induction via the mitochondrial release of cytochrome C (30). As summarized in Table 3 and Fig. 2, the results of the cDNA microarray analyses were consistent with apoptosome formation mediated by Apaf-1, and with cytoplasmic and mitochondrial conditions that are proapoptotic (31). On the other hand, no evidence was found to implicate the involvement of the extrinsic pathway of cell death induction.

On the basis of the microarray analyses and the finding that PARP-1 cleavage was elevated in mammary carcinomas, we proceeded to evaluate the pattern of caspase activity observed in mammary carcinomas. It was found (Table 4), that the activity of caspase 3, the primary executioner caspase that is responsible for PARP-1 cleavage, was significantly increased in ER carcinomas in comparison with the activity observed in carcinomas from control or ER-REP rats. Interestingly, the increased caspase 3 activity was accompanied by an increase in caspase 9 activity, whereas the activity caspase 8 was unaffected. Consistent with the PARP-1 cleavage data, the ER-associated increases in the activity of caspases 3 and 9 were reversed by refeeding (ER-REP). These caspase activity data provide strong support for the hypothesis that the increased level of apoptosis by ER in mammary carcinomas is induced by the mitochondrial pathway (32).

To additionally explore this hypothesis, we conducted a series of Western blot analyses of *Bcl-2*, *CARD*, and *IAP* gene family members. As shown in Fig. 1 and Table 5, it was found that levels of the proapoptotic proteins Bax and Apaf-1 were increased, whereas levels of the antiapoptotic proteins Bcl-2 and Bcl-xl were decreased in carcinomas from ER rats. These findings are consistent with the observed increase in activity of caspase 9. Similarly, the finding that the amount of the XIAP was significantly lower in carcinomas from ER rats is consistent with increased activities of caspase 3 and 9,

Table 6 Levels of IGF-1R initiated signal transduction pathway proteins in mammary tumors of rats fed control, 40% energy-restricted (ER), or ER-repletion (ER-REP) diet<sup>a</sup>

Name of proteins	Control n = 18	ER n = 18	ER-REP n = 5
IGF-1R	0.85 ± 0.08 <sup>b</sup>	0.44 ± 0.03 <sup>c</sup>	0.82 ± 0.10 <sup>b</sup>
Total Akt	0.48 ± 0.05 <sup>b</sup>	0.40 ± 0.04 <sup>b</sup>	0.47 ± 0.03 <sup>b</sup>
Phospho-Akt	0.19 ± 0.01 <sup>b</sup>	0.06 ± 0.005 <sup>c</sup>	0.26 ± 0.02 <sup>d</sup>

<sup>a</sup> Each value is expressed in relative density units and is a mean ± SE. Data were analyzed by multivariate analysis of variance. *Post hoc* comparisons were made using the Bonferroni multiple-range test.

<sup>b</sup> Values in a row with different alphabetical superscripts were significantly different (*P* < 0.05).

<sup>c</sup> Values in a row with different alphabetical superscripts were significantly different (*P* < 0.05).

<sup>d</sup> Values in a row with different alphabetical superscripts were significantly different (*P* < 0.05).

because this protein and others in the IAP family function to block the activity of activated caspases (33). The finding of lower levels of XIAP also is consistent with the down-regulation of transcript expression of the IAP family in carcinomas from ER rats. Collectively, these findings imply that ER coordinately regulates the cellular machinery involved in apoptosis induction to maintain a proapoptotic state within carcinomas.

If regulation of apoptosis is indeed coordinated by ER, it prompts the question of what factor(s) could account for such an effect. One hint at an explanation comes from an article published recently by our laboratory indicating the ER blocks cell cycle progression possibly via modulation of the signaling pathway of which IGF-I is a component (2). Therefore, using the same strategy as reported in that article, we evaluated levels of IGF-I receptor and of total and phosphorylated Akt in the carcinomas that were evaluated in this study. As shown in Table 6, and consistent with the work reported previously (2), levels of IGF-IR and of phosphorylated Akt were lower in carcinomas obtained from ER rats, and this effect was reversed in the carcinomas obtained from ER-REP rats. Akt is a member of the signal transduction pathway regulated by IGF-IR and is known to be a critical cell survival factor. As reported previously (34), reduction in cellular levels of phospho-Akt have been reported to be associated with apoptotic cell death induction via the mitochondrial pathway.

In conclusion, levels of apoptosis are increased in mammary carcinomas that arise despite ER, and this effect appears to be achieved via induction of the intrinsic pathway of cell death induction that is mediated in part by the mitochondrion. Modulation of the IGF-I signaling pathway may account for the proapoptotic environment induced by ER due to a reduction in cell survival factor concentrations of molecules such as phospho-Akt. However, because ER has been reported to exert antiapoptotic effects in other contexts and model systems, it remains important to determine whether changes in cell signaling that lead to cell death induction are mediated by systemic *versus* local production and activity of growth factors such as IGF-I.

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