Energy Restriction Reduces the Number of Advanced Aberrant Crypt Foci and Attenuates the Expression of Colonic Transforming Growth Factor β and Cyclooxygenase Isoforms in Zucker Obese (fa/fa) Rats

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ABSTRACT

Several epidemiological studies have supported the concept that high energy intake, obesity, and/or hyperinsulinemia are risk factors for colon cancer. Previously, it was shown that Zucker obese rats are more sensitive to chemically induced colon cancer than their lean counterparts. The present study investigated whether moderate (20–25%) dietary energy restriction (ER) would attenuate colon carcinogenesis in the Zucker obese rat model. Six-week-old Zucker obese (fa/fa) rats and lean (Fa/Fa) rats received s.c. injections of azoxymethane at a dose of 10 mg/kg body weight once weekly for 2 weeks. A week later, obese rats (n = 16) were assigned to an ER diet (Ob-ER group), based on a low-fat AIN-93G semisynthetic diet. The remaining obese and lean rats (n = 16 rats/group) were fed the low-fat diet ad libitum (Ob group and Ln group, respectively). All rats were euthanized after 8 weeks, and their colons were assessed for aberrant crypt foci (ACF; n = 8/group) or for the expression of transforming growth factor (TGF)-β and cyclooxygenase (COX) isoforms at the protein and mRNA transcript levels (n = 8/group). Ob rats had a higher number of advanced ACF (crypt multiplicity ≥ 7) than Ln rats. Dietary ER significantly reduced the appearance of advanced ACF in Ob-ER rats without significantly affecting the blood insulin level or body weights. TGF-β1 and COX isoforms were differentially expressed in the colonic mucosa of Ob and Ln rats. Dietary ER significantly reduced TGF-β1/β2 and COX-1/2 protein expression in obese rats. This study is the first to demonstrate that moderate ER attenuated TGF-β and COX protein expression and the carcinogenic process in Zucker obese rats. These findings provide insights leading to the proposal that the mechanism(s) underlying the early events of colon carcinogenesis in Zucker obese rats may extend beyond the role of excessive body weight and hyperinsulinemia per se.

INTRODUCTION

Colon cancer is a major cause of morbidity and mortality in the Western world (1, 2). Specific aspects of lifestyle, including overnutrition, physical inactivity, and resulting obesity may play an important role in the etiology and pathogenesis of colon cancer (3–5). A substantial amount of epidemiological evidence has accumulated suggesting that obesity and/or hyperinsulinemia is an augmenting factor of colon cancer (reviewed in Refs. 6 and 7). The multistep process of colon carcinogenesis involves the sequential transformation of “normal” colonic epithelial cells into preneoplastic lesions identified as ACF, which then progress into adenomas, carcinomas, and, finally, metastatic tumors (8–10). Bird (11) first identified and defined ACF in the colons of carcinogen-treated rodents by a simple microscopic methodological approach, and subsequently, ACF were identified in other animal models and humans (12–14). Systemic levels of various mitogens and cytokines act as positive or negative modulators of colonic epithelial cells and tumors (15–18). There has been considerable debate in the past decade addressing the hypothesis that elevated blood insulin promotes colon cancer. Whereas in vivo and in vitro studies support the hypothesis that insulin, when exogenously administered, acts as a proliferative agonist/stimulus to the colonic epithelial cells (19), there is still no conclusive evidence that obesity-associated hyperinsulinemia promotes colon cancer.

Zucker obese rats inherit obesity, as an autosomal Mendelian recessive trait (fa/fa, homozygous for nonfunctional leptin receptors) as compared with their lean (Fa/Fa or Fa/Fa) counterparts (20). At 6 weeks of age, all Zucker (fa/fa) rats are hyperinsulinemic and obese compared with their lean (Fa/Fa or Fa/Fa) counterparts (21). Their response to exposure to any external stimuli (i.e., diet and exogenous treatments of drugs) is more like that of normal rats than rats with hypothalamic obesity (21). Hence Zucker obese and lean rats represent an excellent animal model to test and refine hypotheses linking genetic and environmental factors in the etiology and prevention of chronic diseases such as cancer.

Weber et al. (22) reported that obesity, not high-fat diet exposure, was associated with augmented colon carcinogenesis in the Zucker (fa/fa) rats. However, it remained unclear whether hyperinsulinemia, ubiquitously associated with obesity in the Zucker obese rats, acts as a critical factor in the pathogenesis of colon cancer in obese rats. Lee et al. (23) reported that female Zucker obese rats exhibiting hyperinsulinemia were not susceptible to N-methyl-N-nitrosourea-induced mammary tumorigenesis but were susceptible to colon tumorigenesis. Because Zucker obese rats exhibit hyperphagia, Lee et al. (23) concluded that any differences observed between obese and lean rats in response to glucose, insulin, and metabolic markers of hyperinsulinemia associated obesity in the Zucker obese rats remained to be evaluated.

Whether dietary ER influences the growth and development of ACF and metabolic markers of hyperinsulinemia associated obesity in the Zucker obese rats remained to be evaluated.

The molecular mechanism by which obesity and/or hyperinsulinemia augments colon carcinogenesis has not been addressed, but there is substantial evidence that modulation of the multistep carcinogenic process in the colon is associated with altered expression of a number of signaling molecules including TGF-β and COX isoforms (32–34). We recently reported the elevated expression of TGF-β1 and β2 in colon tumors and a selected subset of microscopic ACF, thus con-
firming the critical role TGF-βs play in early and late stages of colon carcinogenesis (35). Similarly, the COX-2 isoform catalyzing the conversion of arachidonic acid to prostaglandin H₂ (36) plays a significant role in colon tumorigenesis (37, 38). Previous studies have demonstrated that colonic tumors of experimental animals and humans have increased expression of COX isoforms (34, 39). Whether these findings were demonstrated that colonic tumors of experimental animals and humans have increased expression of COX isoforms (34, 39). Whether these findings were demonstrated that colonic tumors of experimental animals and humans have increased expression of COX isoforms (34, 39).

The primary objective of the present study was to determine whether ER, an important modulator of colon carcinogenesis, is able to attenuate the appearance of precancerous ACF in the colons of AOM-injected Zucker obese (fa/fa) rats. The secondary objective of the study was to assess the expression of TGF-β and COX isoforms in the colonic mucosa of obese rats and the effects of ER on their expression. We report that moderate ER markedly affected expression of TGF-β and COX isoforms in the colonic mucosa of obese rats. These observations provide important insights into the role of obesity in modulating the early events of colon carcinogenesis in vivo and suggest that the mechanism(s) underlying the carcinogenic process may extend beyond the role of excessive body weight and hyperinsulinemia.

MATERIALS AND METHODS

Animals, Care, and Diets. Five-week-old female Zucker obese (fa/fa) rats (n = 32) and their lean (Fa/Fa) counterparts (n = 16) were procured from Charles River Laboratories (Wilmington, MA) and housed in suspended wire cages approximately 10 cm above sawdust bedding trays with a 12-h light/12-h dark cycle in the animal housing facility of the University of Manitoba. Temperature and relative humidity were controlled at 22°C and 55%, respectively. All animals were acclimatized to the above conditions for 1 week with free access to standard laboratory rodent chow and drinking water until initiation of the experiment and over the duration of carcinogen induction. All animals were cared for according to the guidelines of the Canadian Council on Animal Care. The experimental diets were ad libitum, and ER diets were based on a semisynthetic AIN-93G standard diet containing 5% corn oil by weight (Table 1; Ref. 40). The ER diet contained 20% more essential nutrients (at the expense of corn starch) than the ad libitum diet. In principle, animals fed the ER diet consumed 20% less energy derived from carbohydrate but the same amounts of fat, protein, vitamins, minerals, fiber, and other nutrients relative to that consumed by the animals fed ad libitum (28). The rats were allowed continuous access to tap water. As the rats gained weight, they changed their food intake to consume more; thus, ER was adjusted on a daily basis, depending on the food intake of each rat for the previous day.

Experimental Design, Carcinogen Injection, and Euthanasia. After 1 week of acclimatization, Zucker obese (fa/ fa) rats (n = 32) and lean (Fa/Fa) rats (n = 16) received s.c. injection with AOM, a colon-specific carcinogen, once a week for 2 weeks at a dose of 10 mg/kg body weight. A week after the second injection, all injected obese rats were divided into two subgroups receiving either ad libitum (Ob) or ER (Ob-ER) diets. The injected lean rats received only the ad libitum (Ln) diet. All groups were on the experimental diets for a period of 8 weeks. Body weights and food intake were monitored routinely on a daily basis. After 8 weeks of feeding, all animals were fasted for 12 h overnight and euthanized by CO2 asphyxiation. Colons were removed, flushed with ice-cold PBS, and slit open along the length from the anus to the cecum on a cold plate. Colons were assessed for any macroscopic changes. The colons (n = 8/group) were fixed flat between filter papers in 70% ethanol and coded for blind scoring. For histological observation, segments of colons (2 cm in length from the distal/rectal end) were fixed flat in 70% ethanol for a period of 48 h and processed for serial sectioning. Four-μm transverse sections were made and stained with H&E. After euthanasia, colons were excised, and the mucosal layer of the colons (n = 8/group) was quickly scraped and snap-frozen in liquid N₂ to be used for Western blot and RNA isolation.

Quantification of ACF. Topographical analysis of the colonic mucosa was performed after a minimum of 24 h in histologic CCH method by methods previously (11, 28). Colons were bathed in a 0.2% methylene blue solution for 5–10 min, placed mucosal side up on a microscopic slide, and viewed using a light microscope. The total number of ACF in the entire colon was determined in every 2-cm section of the colon starting from the rectal or distal (taken as 0 cm) to cervical or proximal end of the colons. The sections of the colons were categorized into distal region (0–6 cm), middle region (6–12 cm), and proximal region (>12 cm). Average number of ACF/section was determined as the mean number of ACF/region/colon. Defined criteria used in the quantification of ACF have been described previously (8). Crypt multiplicity refers to the number of crypt(s)/focus. Average crypt multiplicity was determined as the mean number of crypt/focus/colon. Growth features of ACF in each group were further categorized into primed (1–3 crypts/focus), intermediate (4–6 crypts/focus), and advanced (>7 crypts/focus). After evaluation of the colon, H&E stained sections were prepared for histology. All histological specimens were viewed in a Nikon ECLIPSE E400 light microscope equipped with a Nikon COOLPIX 990 digital camera. Transverse sections of ACF were further evaluated for the presence of dysplasia as described previously (41, 42). Atypia of ACF is an eventuated by the nucleus epithelial cells. Dysplastic ACF are characterized by nuclear elongations. They are further stratified into mild, moderate, or severe dysplasia, depending on the number of dysplastic epithelial cells within the crypt (41, 42).

Western Blot Analyses. Whole homogenates of colon mucosa were prepared with protein inhibitors, and the total proteins were quantified using a Bradford assay (43). The proteins were separated by SDS-PAGE using a Mini-PROTEIN Bio-Rad-II system (Bio-Rad Laboratories Ltd.). The separated proteins were transferred to Hybond-enhanced chemiluminescence nitrocellulose membranes (Amer sham). Membranes were blocked for 1 h at room temperature with 5% skim milk powder and then probed with primary antibodies at 4°C overnight on a shaker. Rabbit anti-TGF-β1 and rabbit anti-TGF-β2 antibodies (sc-146 and sc-90, respectively; 1:100 dilution; Santa Cruz Biotechnologies) were the primary antibodies. Blots were washed and incubated with secondary antibody for TGF-β1/β2 and COX-2) or antitrust antibody for COX-2) conjugated with horseradish peroxidase (Santa Cruz Biotechnologies) at a 1:1,000 dilution for 1 h at room temperature. After washing, the blots were incubated with ChemiGlow Chemiluminescent Substrate (Alpha Innotech Corp.) for 5 min and exposed in a FluorChem Imaging Systems (Alpha Innotech Corp.). Densitometric analysis was conducted on visible protein bands using the FluorChem Imaging Systems (Alpha Innotech Corp.).

RNA Extraction. All reagents and enzymes were obtained from Life Technologies, Inc., unless otherwise specified. Extraction of total RNA was based on the method developed by Chomczynski and Sacchi (44).
Reverse Transcription. CDNA was synthesized by reverse transcription from 1 μg of total RNA in a 20-μl reaction volume according to the method detailed by Life Technologies, Inc. Initially, 1 μg of RNA, 8 μl of water, and 1 μl of oligodeoxynucleotidyl acid (500 μg/ml) were combined and heated at 65°C for 10 min and then placed immediately on ice for 5 min. After cooling, 1 μl (39 units) of RNA Guard (Pharmacia), 2 μl of 100 mM DTT, 2 μl of 5 μM deoxynucleotide triphosphate, and 5× first-strand buffer were added and vortexed briefly. Reverse transcription was performed by the addition of 1 μl (200 units/μl) of Moloney murine leukemia virus at 42°C for 2 h.

PCR. PCR reactions were carried out using a PTC-100 Thermocycler (MJ Research Inc.). After CDNA synthesis, 0.5–5 μl of 10× diluted cDNA was amplified in a 20-μl PCR reaction mixture. The PCR reaction mixture contained 1.87 units of Taq DNA polymerase, 0.2 mM deoxynucleotide triphosphate, 1.5 mM MgCl2, 20 mM Tris (pH 8.4), 50 mM KCl, and 0.5 μM 5′ and 3′ primers for either TGF-β1/2 or β-actin (Life Technologies, Inc.). Primer sequences were as follows: (a) β-actin, 5′-GGT-GGC-CCC-AGG-CAC-CA-3′ (sense) and 5′-CTC-ATT-AAT-GTC-AGC-GAC-GAT-TTC-3′ (antisense); (b) TGF-β1, 5′-GCT-GAA-CCC-AAA-3′ (sense) and 5′-AAA-TGG-ATC-CAC-GAG-CCC-AA-3′ (antisense); (c) TGF-β2, 5′-GCT-GCA-ATT-GCA-GGA-GGC-CAC-3′ (sense) and 5′-AAA-TGG-ATA-CAC-GGA-CCC-AA-3′ (antisense); (d) COX-1, 5′-TGC-ATG-TGG-GTG-ATT-TCA-TCA-A-3′ (sense) and 5′-CAC-AAA-AGA-AAG-CAC-3′ (antisense). The reaction was mixed gently and overlaid with mineral oil (Sigma) and preheated for 2 min at 94°C, and then 25, 30 cycles for β-actin, 33 cycles for TGF-β1, 35 cycles for TGF-β2, 29 cycles for COX-1, and 35 cycles for COX-2 were performed at 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min. All samples were subjected to a final elongation period of 10 min at 72°C for 3 min. At the end of the PCR reaction, 5 μl of 10× PCR loading buffer were added, and all samples were stored at -20°C until further analysis.

Visualization and Quantification of PCR Products. PCR products were separated on a 2% agarose gel in 1× Tris-borate EDTA and 0.5 μg/ml ethidum bromide in Tris-borate EDTA buffer using the Life Technologies, Inc. Horizon 11-14 gel electrophoresis apparatus at 150 V for 25 min. Equal volumes of each sample were loaded (15 μl), and all primers for one sample were loaded on a single gel to reduce variation among samples. The resulting gels were exposed to UV illumination using the FluorChem Imaging Systems (Alpha Innotech Corp.). Densitometric analysis was conducted on visible bands using the FluorChem Imaging Systems (Alpha Innotech Corp.). The area of the band corresponding to the particular primer was expressed as a ratio relative to the area of the band corresponding to β-actin. β-Actin is considered to be a housekeeping gene, which is present in equal amounts in all cells and has been used previously (45).

Statistical Analysis. Data were analyzed by ANOVA and Duncan’s multiple range test using the SPSS SigmaStat software for microcomputers. In all statistical tests, P < 0.05 was considered significant.

RESULTS

Dietary ER and Body Weight in Zucker Obese Rats. Obese and lean rats were monitored for food intake from day 1 of their arrival in the animal housing facility (Table 2). The food intake of Ob rats was significantly higher than that of the Ob-ER and Ln rats (P < 0.001). Ob-ER rats ate 20–25% less food than Ob rats, and the food intake of the former was similar to that of the Ln rats (Table 2). At the time of intervention with ER diets, mean body weight ± SE was 287.53 ± 4.86 g for obese rats and 171.75 ± 5.08 g for lean rats. This difference in body weight between obese and lean rats, irrespective of their dietary groups, was maintained throughout the study duration (Table 2). ER did not affect body weights of obese rats (Table 2).

Dietary ER and Plasma Parameters in Zucker Obese Rats. Obesity is a plurimetabolic syndrome with characteristic disarray in metabolic as well as hormonal parameters. Hence we assessed the levels of fasting plasma insulin, glucose, lactate, and total triglyceride and cholesterol levels in Ob, Ob-ER, and Ln rats to make any functional interrelationship between the metabolic states encountered in obesity to colon carcinogenesis. Compared with Ln rats, Ob rats had significantly higher fasting plasma insulin, lactate, triglyceride, and cholesterol levels (P < 0.001; Table 3). Fasting glucose levels were the same in all three groups (Table 3). With the exception of plasma triglyceride level, ER did not alter the levels of any of the other plasma parameters studied (Table 3).

Dietary ER Reduces the Number of Advanced ACF in Zucker Obese Rats. ACF were present throughout the length of the colon in all rats (n = 8/group), irrespective of their genotype. There was no significant difference in the total number of ACF among the Ob, Ob-ER, or Ln rats (Fig. 1), with most ACF falling in the primal (1–3 crypts) category. The number of primal ACF was significantly higher than the average number of intermediate (4–6 crypts) and advanced (7+ crypts) ACF (Fig. 2) in all of the groups (P < 0.001). There were no apparent differences in the average number of primal ACF between Ob and Ln rats fed ad libitum. The Ln rats had significantly fewer intermediate and advanced ACF than the Ob rats (P < 0.001). The average number of intermediate ACF in obese rats remained constant despite dietary ER. Ob-ER rats had a significantly lower number of advanced ACF than the Ob rats (P < 0.001; Fig. 2).

The total number of ACF in the distal 0–6 cm of the colons was similar in obese and lean rats fed ad libitum, and there was no significant difference due to ER among the obese rats (Fig. 3). The total number of ACF in the mid 6–12 cm of the colons was significantly lower (P < 0.001) in Ln rats than in Ob rats, and there was no significant difference due to ER in obese rats (Fig. 3). In the proximal

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Body weight and food intakea</th>
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</thead>
<tbody>
<tr>
<td>Ob</td>
<td>Ob-ER</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>479.3 ± 18.1b</td>
</tr>
<tr>
<td>Food intake (g/animal/day)</td>
<td>32.6 ± 0.4a</td>
</tr>
</tbody>
</table>

a All values are means ± SE, n = 16/dietary group.
b Values with different superscripts are significantly different (P < 0.001) from each other.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Blood plasma levels of various metabolites in obese and lean rats fed different dietsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ob</td>
<td>Ob-ER</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>289.2 ± 61.6b</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>206.7 ± 33.6b</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>154.7 ± 3.2b</td>
</tr>
<tr>
<td>Lactate (mg/dl)</td>
<td>91.8 ± 4.8b</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>39.5 ± 7.3b</td>
</tr>
</tbody>
</table>

b All values are means ± SE, n = 16/group.
c Values with different superscripts are significantly different (P < 0.001) from each other.
degree of dysplasia. As depicted in Fig. 4a, one ACF consisting of eight crypts showed negligible morphological changes (dysplasia); whereas in Fig. 4b, ACF with two crypts exhibit moderate dysplasia as evident by crowding of elongated nuclei, as described previously (41, 42).

**Dietary ER Attenuates the Protein Expression of Colonic TGF-β1/2 and COX-1/2.** The levels of TGF-β1/2 and COX-1/2 protein and mRNA transcript levels were determined in the colonic mucosae of Ob, Ob-ER, and Ln rats (n = 8/group) by Western blotting and RT-PCR techniques, respectively. Figs. 5 and 6 represent Western blots and RT-PCR products of TGF-β and COX isoforms in colonic mucosae of Ob, Ob-ER, and Ln rats, respectively.

The 12.5-kDa TGF-β1 and the 13-kDa TGF-β2 protein levels were detectable in the colonic mucosae of all of the experimental rats (Table 4). In comparison with the protein levels in the colonic mucosae of Ln rats, TGF-β1 and -β2 were significantly higher in the mucosae of Ob rats (P < 0.001; Table 4). Ob-ER rats had significantly lower TGF-β1 and -β2 than Ob rats (P < 0.001), and the values were similar to that of the Ln rats (Table 4). COX-1 protein level was significantly higher in the colonic mucosae of Ob rats than Ln rats (Table 4). In contrast, Ob-ER rats had significantly lower levels of COX-1 than Ob rats and had similar COX-1 protein levels as the Ln rats. COX-2 level was significantly lower in the colonic mucosae of Ob rats than Ln rats (P < 0.001; Table 4). ER lowered COX-2 further in the obese rats than in the other two groups (P < 0.001).

The TGF-β1 and -β2 mRNA transcripts were significantly lower in the Ob rats than Ln rats (P < 0.001; Table 5). Ob-ER rats had significantly lower levels of TGF-β2 mRNA transcripts than Ob and Ln rats, whereas the level of TGF-β1 mRNA transcripts was not affected by ER (Table 5). Semiquantitative RT-PCR analyses of COX-1 mRNA revealed no significant difference in the level of COX-1 mRNA between Ob and Ln rats. However, ER significantly upregulated COX-1 mRNA transcripts in Ob rats (P = 0.001; Table 5). ER significantly upregulated COX-2 mRNA transcripts in Ob rats (P = 0.001; Table 5).

![Fig. 4](image_url)

**Fig. 4.** Histological depiction of ACF from Zucker obese (fa/fa) rats (H&E-stained sections, ×400 magnification) as (a) no dysplasia to (b) mild to moderate dysplasia (arrow). The ACF in a consist of more than six crypts, whereas the ACF in b consist of two prominent crypts. The epithelial cells in ACF in b have distinctive elongated nuclei (arrow), whereas those in a show nuclear morphology similar to the epithelial cells in surrounding normal crypts.

![Fig. 5](image_url)

**Fig. 5.** ER attenuates the protein expression of TGF-β1/2 and COX-1/2 in Zucker obese rats. Representative Western blot analyses of TGF-β1/2 and COX-1/2 in colonic mucosae of Ob, Ob-ER, and Ln rats are shown, as described in “Materials and Methods”.
Rats are more sensitive to colon carcinogenesis than their lean counterparts. Advanced ACF compared with Zucker lean rats confirms and supports lean counterparts.

Methods

Colonic mucosae of Ob, Ob-ER, and Ln rats. Representative blots of mRNA transcripts separated on a 2% agarose gel after RT-PCR are shown, as described in “Materials and Methods.”

**DISCUSSION**

The most salient findings of the present study were as follows: (a) Zucker obese rats had a higher number of advanced colonic precancerous lesions than their lean counterparts; and obese rats exhibited a number of physiological differences from their lean counterparts by having higher plasma levels of insulin, cholesterol, triglycerides, and lactate. (b) Dietary ER retarded the appearance of advanced ACF in Zucker obese rats without affecting their body weight or measures of metabolic aberrations at the plasma level with the exception of triglycerides. (c) Zucker obese rats had higher protein levels of colonic TGF-β1, TGF-β2, and COX-1 but not COX-2 compared with their lean counterparts.

The observation that Zucker obese rats had a higher number of advanced ACF compared with Zucker lean rats confirms and supports the reports of Weber et al. (22) and Lee et al. (23) that Zucker obese rats are more sensitive to colon carcinogenesis than their lean counterparts. It is proposed that ACF are preneoplastic lesions and that their growth features may predict tumor outcome with some accuracy (8). Taking into consideration that by 6 weeks of age, Zucker obese rats achieve hyperinsulinemia, in the present study 6-week-old obese rats received injection with AOM and were simultaneously grouped into either the Ob or Ob-ER group. Thus, the carcinogenic process was evaluated not only by enumerating ACF but also by noting the growth features in conjunction with the hyperinsulinemic state in the obese rats. The assessment of blood levels of insulin, cholesterol, triglyceride, and lactate confirmed the possible interrelationships between the disarrayed hormonal or metabolic parameters encountered in obesity as reported previously (21). Moderate ER did not change the levels of insulin, cholesterol, and lactate. However, ER significantly reduced the level of plasma triglycerides, suggesting that ER is capable of modulating either the level of lipolysis, the secretion of hepatic lipoprotein, or the clearance of triglycerides in Zucker obese rats. Whether ER of longer experiment duration would affect any of the plasma parameters in Zucker obese rats as evaluated in this study remains to be investigated.

We have shown previously that moderate ER consistently reduces the number of advanced preneoplastic lesions and the incidence of colonic tumors in AOM-injected F344 rats (26, 28). Our findings that ER exerted an effect on colonic mucosal physiology independent from the body weight or blood parameters is worth noting and in part support the findings of Steinbach et al. (46), who demonstrated that individuals with a high body mass index who followed a dietary regime to restrict their energy intake by 35% had lower colonic proliferation status than when they had free access to food. The effect of ER on proliferative status of colon was unrelated to body mass index, resting metabolic rate, or body composition (46). By using an in vivo model of obesity and an approach to restrict the energy intake for 8 weeks after AOM injections, we were able to assess changes occurring in the colon, as reflected by metabolic adaptations to sustain a certain physiological state during colon carcinogenesis. The fact that ER retarded the appearance of advanced ACF in obese rats without affecting the total number of ACF alludes to the possibility that ER inhibited the growth of ACF rather than their survival, and the effect was more on the promotion phase than the initiation phase. Our preliminary observation that moderate ER inhibited severe dysplasia compared with the ACF in Ln rats warrants further investigation. Dysplasia is a well-known marker of preneoplastic state, and we have previously shown that a fraction of the ACF population exhibit dysplasia with time, regardless of the crypt multiplicity (41, 42). ER significantly reduced the number of ACF in the proximal colon rather than the distal colon, supporting the concept that the molecular mechanism(s) in the proximal colon as opposed to that in the distal colon may differ. Whether this observation translates into different tumor outcome in colonic regions remains to be explored.

Our finding that moderate ER did not change the body weight or blood insulin level in Zucker obese rats is consistent with previous reports (47) and argues against a direct relationship between excessive blood insulin level in Zucker obese rats is consistent with previous studies (47–49) and argues against a direct relationship between excessive

### Table 4

**Steady-state protein levels of TGF-β1/-2 and COX-1/-2 by Western blot analyses in colonic mucosa of obese and lean rats fed different diets**

<table>
<thead>
<tr>
<th></th>
<th>Ob</th>
<th>Ob-ER</th>
<th>Ln</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>1.78 ± 0.19a</td>
<td>1.23 ± 0.16b</td>
<td>1.39 ± 0.15c</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>1.58 ± 0.26a</td>
<td>0.78 ± 0.13b</td>
<td>1.05 ± 0.05c</td>
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<tr>
<td>COX-1</td>
<td>1.82 ± 0.24a</td>
<td>1.35 ± 0.11b</td>
<td>1.34 ± 0.11c</td>
</tr>
<tr>
<td>COX-2</td>
<td>1.09 ± 0.06a</td>
<td>0.77 ± 0.11b</td>
<td>1.75 ± 0.21b</td>
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</tbody>
</table>

*All values are pixels or arbitrary units and are expressed as means ± SE, n ≥ 6/group. b,c,d Values with different superscripts are significantly different (P < 0.001) from each other. For detailed description of the procedure see “Materials and Methods.”

### Table 5

**Steady-state mRNA transcript levels of TGF-β1/-2 and COX-1/-2 by RT-PCR analyses in colonic mucosa of Zucker obese and lean rats fed different diets**

<table>
<thead>
<tr>
<th></th>
<th>Ob</th>
<th>Ob-ER</th>
<th>Ln</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>0.76 ± 0.08a</td>
<td>1.10 ± 0.29b</td>
<td>1.21 ± 0.08c</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>0.57 ± 0.08a</td>
<td>0.37 ± 0.02c</td>
<td>1.59 ± 0.21d</td>
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<td>COX-1</td>
<td>0.37 ± 0.06a</td>
<td>0.25 ± 0.11b</td>
<td>0.46 ± 0.07b</td>
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<tr>
<td>COX-2</td>
<td>1.98 ± 0.40a</td>
<td>2.38 ± 0.14b</td>
<td>3.71 ± 0.46c</td>
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</tbody>
</table>

*All values are pixels or arbitrary units and are expressed as means ± SE, n ≥ 6/group. b,c,d Values with different superscripts are significantly different (P < 0.001) from each other. For detailed description of the procedure see “Materials and Methods.”
Zucker obese rats. Barzilai and Gabriely (48) reported that moderate ER, allowing the consumption of 75–80% of ad libitum intake, improved peripheral insulin sensitivity, which is often reduced in obesity. From our results, it seems that ACF in Zucker obese rats responded to ER in the presence of the hyperinsulinemic state. The reduction in the level of insulin in the Ob-ER rats as compared to the Ob rats was not significant. Whether this slight reduction after 8 weeks of ER has any implications in retarding the growth of ACF remains to be explored. The other possibility is that ER may retard insulin-mediated responses in the Zucker obese rats without significantly affecting the level of plasma insulin.

In light of these findings, it is reasonable to suggest that one or more factors that are the underlying cause of the obese/hyperinsulinemic state also could be involved in the early stages of colon carcinogenesis in the Zucker model. The role of ER in retarding the growth of preneoplastic lesions or appearance of tumors has been studied extensively in animal models (26–29, 49). Moderate ER could influence a plethora of responses at the cellular and molecular levels (50, 51). In the present study, we demonstrate that the colonic mucosae of Zucker obese rats differ from the mucosae of lean rats with respect to the status of TGF-β and COX isotypes. Obese rats had considerably higher protein levels of TGF-β1, TGF-β2, and COX-1 but not COX-2 compared with their lean counterparts. The level of mRNA transcripts for TGF-β and COX isosforms did not always corroborate the findings at the protein levels, demonstrating that under steady-state experimental conditions in vivo, the differences in the mRNA and protein expression of these target molecules could be a result of the mRNA stability, transcription and translation, and/or protein turnover rate directly influenced by the complex biological state of the colon.

TGF-β1 and -β2 are pleiotrophic cytokines with positive and negative growth-modulating abilities on the growth of preneoplastic and cancer cells, depending on their biological make up (32, 34, 52, 53). In addition, TGF-β1 and -β2 affect cellular processes that influence growth and development of preneoplastic lesions such as immunosuppression and angiogenesis (54). The distinction between the mechanism(s) by which TGF-β synthesized by normal colonic epithelial cells could affect the growth of preneoplastic lesions as opposed to tumor-derived TGF-β and its role in tumor growth remains to be explored. Our observations of the differences in TGF-β1 and -β2 expression in the colonic mucosae of Zucker obese and lean rats raise the question of what role they play in modulating preneoplastic changes. We reported previously that colonic mucosae of AOM-injected F344 rats express lower levels of TGF-β1 mRNA than the tumors, and the levels were influenced by dietary lipids, leading us to conclude that tumor-derived TGF-βs are important to tumor growth (35). Our present findings that mucosal TGF-β levels differ with the genetic makeup and physiological status of animals and may exert growth-modulating effects on ACF further strengthen this hypothesis.

The observation that increased colonic TGF-β1 and -β2 protein levels in the Ob rats compared with Ob-ER or Ln rats corroborates to the accelerated growth of ACF (presence of more advanced ACF) in Ob rats and emphasizes the role of mucosal-derived TGF-β as opposed to tumor-derived TGF-β. Our results suggest that the expression of colonic TGF-β1 in the Ob-ER rats was higher at the mRNA transcript level and lower at the protein level compared with the levels in the Ob rats. Although a similar pattern in the expression of colonic TGF-β2 in the Ob-ER rats was observed at the protein level, the TGF-β2 mRNA transcript levels were lower in the Ob-ER rats than in the Ob rats. It appears that ER was augmenting the expression of colonic TGF-β1 at the gene level in the Zucker obese rats, followed by decreased translational activity, whereas ER was suppressing the expression of colonic TGF-β2 at both the gene and protein levels. Attempts are being made to understand the relationship between TGF-βs and their receptors as well as the downstream signaling events in the colon during carcinogenesis to provide a better signaling perspective.

A number of studies have presented evidence in support of the contention that COX-2 plays an enhancing role during colon carcinogenesis (reviewed in Refs. 38 and 55). COX-2 has received much more attention than COX-1 because COX-2 is an inducible form, present in a number of pathological states including inflammation, tissue repair, and neoplasia (reviewed in Ref. 37). The COX-1 isoform is constitutively expressed and is believed to maintain the normal physiology of a tissue. However, the fact that some tumors may depend preferentially on COX-1 for survival and growth is being proposed (56–58). In our own laboratory, we have found that COX-1 and -2 levels in tumors vary a great deal, depending on the environment under which the tumors are appearing; some tumors express elevated levels of both COX-1 and -2, but others express an elevated level of COX-2 exclusively compared with the surrounding normal mucosa. One can argue that COX-1 may be more important in augmenting the growth of ACF during early stages than COX-2 because COX-2 level was lower in obese rats than lean rats, and its level was further lowered after ER. Elevated levels of COX-2 in tumor mass may be more crucial to tumor growth than mucosal COX-2 to ACF growth. It has been shown that elevated COX-2 expression may protect animals from skin carcinogenesis (59) and that COX-2 metabolites attenuate gastrointestinal tumorigenesis (60). Although the colonic epithelial cells may have their own molecular mechanism during tumorigenesis that differs from that of skin epithelial cells, the possibility that elevated COX-2 in mucosae of lean rats plays a protective role against early events during tumorigenesis cannot be ruled out. An opposite view would be that ER-mediated effect on the protein levels as observed in the present study are nonspecific and do not affect the growth of ACF.

In summary, the present study investigated the carcinogenic process taking place in the colon mucosae in an obese/hyperinsulinemic state. Zucker obese and lean rats exhibited remarkable differences in their sensitivity to AOM-induced colon carcinogenesis. ER affected the biochemical state of the colonic mucosae in Zucker obese rats, as exemplified by the reduced levels of both TGF-β and COX isosforms and attenuation in the number of advanced preneoplastic lesions, without affecting the plasma insulin level. Obesity appears to be associated with physiological events, which translate to complex cellular and molecular disarray in various tissues including the colon. An impressive literature exists outlining the complexity surrounding the biological modes of action of TGF-β and COX isosforms at the cellular levels (reviewed in Refs. 37 and 54). Our results call for further investigation to delineate the physiological importance of these and other growth modulators in normal and pathological states in vivo in relation to obesity-associated hyperinsulinemia. Moderate ER of a short duration could possibly attenuate colon carcinogenesis in obese rats by regulating the expression of important cytokines and enzymes in the colonic epithelia without significantly affecting excess body weight and hyperinsulinemia, the key pathological characteristics of Zucker obese rats. The augmented risk for colon cancer in Zucker obese rats may thus extend beyond metabolic abnormalities due to excessive body weight and plasma insulin level per se as pathological factors.

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4 R. P. Bird, unpublished data.
REFERENCES


Energy Restriction Reduces the Number of Advanced Aberrant Crypt Foci and Attenuates the Expression of Colonic Transforming Growth Factor β and Cyclooxygenase Isoforms in Zucker Obese (fa/fa) Rats

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