Mammary and Submandibular Gland Epidermal Growth Factor Expression Is Reduced by Calorie Restriction1

Robert W. Engelman,2 Una E. Owens, W. Guy Bradley, Noorbibi K. Day, and Robert A. Good
All Children’s Hospital Department of Pediatrics, University of South Florida, St. Petersburg, Florida 33701

ABSTRACT

Calorie restriction reduces mammary mitogenesis and tumorigenesis. To test whether epidermal growth factor (EGF) levels are influenced by calorie intake, 72 four-week-old C3H/HeOu mice were separated into two groups and either fed ad libitum (group AL) or calorie-restricted at a mean 19% (group CR). Three mice from each group were evaluated when 6, 8, 10, and 12 weeks old for submandibular gland transcription of EGF and β-actin RNA for levels of EGF protein in the submandibular gland, mammary gland, and serum and for immunohistological evidence of EGF protein within the submandibular and mammary glands. Submandibular levels of EGF RNA and protein and mammary and serum levels of EGF protein were similar between dietary groups when mice were 6 and 8 weeks old. Mean EGF:β-actin RNA transcription in submandibular glands of 12-week-old mice were ~10-fold greater in AL compared to CR mice (ratio means, 1.499 versus 0.157, respectively; P < 0.01). Mean submandibular levels of EGF protein were greater in 10-week-old AL compared to CR mice (7017±4 versus 4098.5 ±mg/mg protein, respectively; P < 0.05) and even greater in 12-week-old AL compared to CR mice (4342.6 ± mg/mg protein; P < 0.001). Mean mammary levels of EGF protein were greater among 12-week-old AL compared to CR mice (7.8 ± 0.5 ng/mg protein; P < 0.05). Serum levels of EGF did not differ between dietary cohorts. More anti-EGF immunoprecipitate was present in submandibular and mammary gland sections of 10- and 12-week-old AL compared to CR mice. Lowered EGF levels may contribute to the antiproliferative and antineoplastic effects of calorie restriction.

INTRODUCTION

Peripubertal mammary development begins in pubescent, ~4-week-old mice and involves extension and arborization of the primordial ductal tree (1). Stimulated by estrogen, and perhaps augmented by progesterone, EGF1, and transforming growth factor α (2–8), mammary ducts develop intensely mitotic end buds which invade the surrounding fatty stroma, form secondary and tertiary branches, and bud alveoli. Peripubertal mammary development concludes when mice are ~12 weeks old, by which time the growing ductal tree has extended to the outer margins of the fat pad, end bud epithelial proliferation subsides, and end buds involute, perhaps in response to growth-inhibiting transforming growth factor β (9, 10).

Mammary transcription of the type B retrovirus, MMTV, and the proviral insertional activation of proto-oncogenes, such as Wnt-1 and int-2, contribute to regular and early development of mammary adenocarcinoma in female C3H/HeOu mice (11). Efficient proviral expression requires frequent and active host cell division (12–14). When the dietary calories of C3H/HeOu mice are restricted, mammary epithelial proliferation is reduced (15, 16), peripubertal mammary development is delayed (16), MMTV transcription is suppressed (17–20), and mammary tumorigenesis is abrogated (19–22).

Mammary carcinogenesis is inhibited most effectively when calorie restriction is initiated during early peripubertal life (23–25). Even an 8-week interval of calorie restriction only during the peripubertal mammary development of 4–12-week-old C3H/HeOu mice significantly lowers cumulative mammary tumor incidence by 33% (16).

Calorie restriction during mammary development results in the formation of fewer, smaller, and less mitotic end buds, which migrate more gradually toward fat pad margins, and of fewer and less mitotic alveoli (Fig. 1; Ref. 16).

This relationship between calories, mammary development, and carcinogenesis, demonstrated experimentally (16), is comparable to an apparent relationship in women (26), where a degree of breast cancer risk is established during adolescence by influences of calories on breast development (26–32). Endocrine and molecular mechanisms of this relationship, and of the antiproliferative and antineoplastic effects of calorie restriction have not been delineated.

EGF peptide is derived from a large precursor protein (preproEGF) encoded by a 4.7-kb mRNA species transcribed within many tissues, including the submandibular salivary gland, kidney, and mammary gland (33–35). In neonatal mice, synthesis of EGF peptide becomes detectable just prior to puberty (36, 37) and predominantly within the convoluted tubular epithelium of the submandibular salivary gland (35). EGF is mitogenic for mammary epithelium both in vitro and in vivo (6, 8, 38–40) and mitogenic for cultured neoplastic mammary cells (41). It appears to contribute to both peripubertal mammary development (6, 8) and prelactational mammmogenesis (7, 39, 42, 43) and causes the reappearance of regressed mammary end buds and localized mammary growth when administered into the growth-static mammary glands of ovariectomized mice (6, 8). EGF enhances the transformation of cultured granulosa cells by the Kirsten murine sarcoma virus (44) and the carcinogenic potential of methylcholanthrene in skin (45). Submandibular sialoadenectomy reduces the incidence of mammary tumors in C3H/He mice (46) and the growth of transplanted mammary tumors in nude mice (47). Mammary tumorigenesis is potentiated in both of these models when EGF is administered to the sialoadenectomized mice (46, 47).

In the present report, to determine whether EGF expression is regulated by calorie intake and may mediate the antiproliferative and antineoplastic effects of calorie restriction, C3H/HeOu mice were fed ad libitum or were calorie restricted when 4–12-weeks-old and evaluated for levels of submandibular gland transcription of EGF and β-actin RNA; for radioimmunoassayable levels of EGF protein within the serum and submandibular and mammary glands; and for immunohistological expression of EGF protein within the submandibular and mammary glands.

MATERIALS AND METHODS

Animals. Seventy-two 4-week-old, female C3H/HeOu mice (The Jackson Laboratory, Bar Harbor, ME) were separated into two dietary groups. Thirty-six mice consumed a similar but calorie restricted diet and were designated group AL. Thirty-six mice consumed a similar but calorie restricted diet and were designated group CR to indicate calorie restriction. More anti-EGF immunoprecipitate was present in submandibular and mammary glands.

Experimental Plan. Nine mice in metestrus/diestrus (48), each from groups AL and CR, were euthanized when 6, 8, 10, and 12 weeks old. At each interval of assessment, three mice from each of the two dietary groups were separated into two dietary groups. Thirty-six mice consumed a similar but calorie restricted diet and were designated group CR to indicate calorie restriction.
evaluated for submandibular gland transcription of EGF and β-actin RNA by Northern blot hybridization; three other mice from each group were evaluated for submandibular gland, mammary gland, and abdominal vena cava serum levels of EGF protein by RIA, and three other mice from each group were evaluated for EGF immunolocalization in histosections of submandibular and mammary glands.

**Semipurified Diets.** Group AL mice were allowed free access to an excess of high-calorie, semipurified diet. Mammary restriction of CR mice was made at increments of 5%, less dietary calories offered each feeding, relative to the feeding of group AL mice. Group CR mice were offered 80% of the calories offered AL mice at 6 weeks, 70% at 7 weeks, and 60% of the calories offered group AL mice when 8–12 weeks of age. Food consumption was determined by weighing the food offered and reweighing the remnant food at the end of each feeding interval. Calorie intake was determined as a product of the weight of diet consumed (grams) multiplied by the caloric concentration of the diet offered (calories/gram). Mice were weighed weekly.

The composition of diets was formulated with each incremental increase in calorie restriction to ensure an equivalent intake of essential nutrients by all mice, while limiting only total dietary calories. Composition of the diets is presented in Table 1. All dietary constituents were obtained from ICN Biochemicals (Costa Mesa, CA). Diets were low in dietary fat. Calories were restricted principally by limiting calories from carbohydrate.

**Preparation of Probes for EGF and β-actin RNA.** Probes for mouse EGF and β-actin were purified from PCR products of mouse submandibular gland cDNA. Submandibular gland total RNA was isolated using RNAzol B (Biotex Laboratories, Houston, TX) in a single-step RNA extraction method (49). Purified submandibular RNA was used as template to synthesize cDNA using recombinant MMTV reverse transcriptase, and an oligo (dT)15 primer (Clonetech Laboratories, Palo Alto, CA). cDNA was amplified by PCR using Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), EGF, or β-actin primer pairs. For EGF, cDNA was amplified for 45 cycles (1 cycle at 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min); for β-actin, cDNA was amplified for 40 cycles (1 cycle at 95°C for 45 s, 60°C for 45 s, and 72°C for 2 min). EGF primers corresponded to the mouse sequence (33, 34), nucleotide position 1424 (5'-GAAGTGACTGCGAAGATG-3') and nucleotide position 1882 (5'-AAAGTCCTCGACCGG-3'), β-actin primer corresponded to the mouse sequence (50), nucleotide position 1417 (5'-GTGATGTGGCGCATG-3') and nucleotide position 2369 (5'-TAAATGTCACCGCAGTATTCC-3'). PCR products were separated by electrophoresis through a 1.2% TAE-agarose gel, and the EGF and β-actin bands visualized by ethidium bromide staining. Bands were excised from gels, and EGF or β-actin PCR products were eluted and purified using a Qiaex DNA gel extraction kit (Qiagen, Chatsworth, CA). Purified PCR products were used to prepare 32P-labeled EGF or β-actin probes using random oligonucleotides as primers and Klenow enzyme (Boehringer-Mannheim, Indianapolis, IN).

**Northern Blot Hybridization of Submandibular Gland RNA for EGF and β-actin Transcription.** Submandibular gland total RNA from individual 6–12-week-old AL or CR mice was isolated using a single step RNA extraction method (49) and RNAzol B (Biotex). Purity and quantity of isolated submandibular RNAs were ascertained by spectrophotometric analysis.

Ten μg of submandibular gland RNA from each mouse was denatured in a loading premixture containing 8.0% formaldehyde, 60% deionized formamide, 0.12% ethidium bromide, and 2.5X MOPS buffer (diluted from 20X Mops buffer stock comprised of 0.4 M MOPS, 0.1 M sodium acetate, and 20 mM EDTA). Samples were heated for 15 min at 55°C, chilled on ice, and then submitted to electrophoresis in formaldehyde denaturing gels (2.2 M formaldehyde, 1% agarose, and 1X Mops buffer). RNA was transferred from gels to Zeta-probe GT nylon blotting membranes (Bio-Rad, Hercules, CA) with a vacuum blotting apparatus (Pharmacia LKB, Piscataway, NJ) and bound to the membranes by exposure to UV light (Stratagene, La Jolla, CA).

Prehybridization (duration, 1–4 h) and hybridization (16–24 h) were at 65°C in buffer containing 7% SDS, 1 mM EDTA, and 500 mM NaH₂PO₄. Each 11 x 14-cm membrane was incubated with 1.5 x 10⁶ cpm of radiolabeled probe in 15 ml of hybridization buffer. After hybridization, membranes were washed at 65°C two times for 15 min each in 5% SDS, 1 mM EDTA, 40 mM NaH₂PO₄, and then twice for 15 min in 1% SDS, 1 mM EDTA, and 40 mM NaH₂PO₄. Membranes were exposed to Kodak X-Omat LS film at ~70°C (Sigma Chemical Co., St. Louis, MO). Hybridization signals specific for EGF or β-actin RNA were semiquantified by densitometric scanning using a Sparc-1 computer (Sun Microsystems, Fremont, CA), a Truval scanner (Chatsworth, CA), and whole band analysis software (BioImage, Ann Arbor, MI) and reported as ODs. Mean ± SD ODs of bands were determined for each dietary group at each interval of assessment. EGF OD/β-actin OD ratios were determined for each mouse, and the mean ± SD of these ratios was determined for each dietary group at each interval of assessment.

**RIA for Mammary, Submandibular Gland, and Serum EGF Protein.** Mice were euthanized by CO₂ inhalation and immediately exsanguinated via the abdominal vena cava at the level of the renal veins. Blood was allowed to clot at room temperature for 30 min, cooled on ice, and clarified by centrifugation; then the serum was stored at ~20°C. Mammary and submandibular glands were each weighed, homogenized in 20 volumes (wt/vol) of PBS at 37°C, and centrifuged at 15,000 × g for 20 min at 4°C; then the supernatants were stored at ~20°C. All samples were processed within 2 weeks of collection.

A standard curve was prepared by duplicate measurements of six dilutions of purified standard mouse submandibular gland EGF at 0.2–50.0 ng/ml (Biomedical Technologies, Stoughton, MA). Two dilutions of each serum, or submandibular or mammary supernatant sample, were assayed in duplicate using a competitive binding technique, rabbit anti-mouse submandibular EGF, and [125I]-EGF (Biomedical Technologies). Radioactivity of the bound fraction was determined in a gamma counter with <5% variability between duplicates. For EGF, [125I]EGF (Biomedica Technologies) was added to each supernatant sample, and [125I]EGF was added to each serum sample for determination of total EGF levels. Standard curves were prepared by duplicate measurements of six dilutions of 3.8 x 10⁸ to 3.8 x 10⁵ cpm [125I]EGF. A standard curve was prepared by duplicate measurements of six dilutions of purified standard mouse submandibular gland EGF at 0.2–50.0 ng/ml (Biomedical Technologies, Stoughton, MA). Two dilutions of each serum, or submandibular or mammary supernatant sample, were assayed in duplicate using a competitive binding technique, rabbit anti-mouse submandibular EGF, and [125I]-EGF (Biomedical Technologies). Radioactivity of the bound fraction was determined in a gamma counter with <5% variability between duplicates.
RESULTS

Calories and Body Weight. Group CR mice consumed 14% fewer calories when 5 weeks old (10.2 versus 11.8 kcal/day), 26% fewer calories when 12 weeks old (9.5 versus 12.9 kcal/day), and an overall mean 19% fewer calories during the 8-week study (9.9 versus 12.2 kcal/day). Mean body weights of AL and CR mice were comparable when mice were 4–9 weeks of age. When 10–12 weeks old, CR mice (20.8 ± 0.7 g) weighed less than AL mice (24.2 ± 0.9 g; P < 0.01).

Influence of Calories on Submandibular Gland Transcription of EGF RNA. Submandibular gland transcription of EGF RNA and EGF-β-actin RNA ratios were comparable when AL and CR mice were 6 and 8 weeks old. The mean band density of submandibular gland EGF RNA was greater in 10-week-old AL mice compared to CR mice (6.869 versus 2.505; mean lODs, respectively; P < 0.05) and even greater among 12-week-old AL mice (12.016 versus 4.483; mean IODs, respectively; P < 0.01). Consequently, the means of individual ratios of EGF:β-actin RNA transcription was 10-fold greater for AL mice (1.499 ± 0.310) compared to CR mice (0.157 ± 0.036; P < 0.01), indicating selective regulation of EGF transcription by calorie intake.

Influence of Calories on Mammary Gland, Submandibular Gland, and Serum Levels of EGF Protein. Levels of EGF protein in submandibular glands of AL and CR mice were similar when mice were 6 and 8 weeks old, but significantly greater in AL compared to CR mice when mice were 10 and 12 weeks old (Fig. 4). Calculated relative to total submandibular protein, levels of EGF protein in submandibular glands of 10-week-old AL mice (7017.4 ± 892.3 ng/mg protein) were greater than those of CR mice (4098.5 ± 1163.7 ng/mg protein; P < 0.05) and even greater among 12-week-old AL mice (12.049 ± 3.100 compared to CR mice (0.157 ± 0.036; P < 0.01).
CALORIES AND EPIDERMAL GROWTH FACTOR

Fig. 5. Immunohistological preparations of submandibular glands from 12-week-old group AL (A) or group CR (B) mice. Appreciably more granular brown immunoprecipitate, indicating the presence of EGF protein, overlaid the submandibular tubular epithelium of group AL mice (hematoxylin stain; × 630).

Fig. 6. Immunohistological preparations of mammary glands from the 12-week-old group AL (A) or group CR (B) mice. Appreciably more granular brown immunoprecipitate, indicating the presence of EGF protein, overlaid mammary ductal epithelium of group AL mice (hematoxylin stain; × 1000).

mice (4342.6 ± 675.6 ng/mg protein) compared to CR mice (137.9 ± 59.9 ng/mg protein; P < 0.001).

Comparable results were obtained when determinations of EGF protein in submandibular glands were made on the basis of submandibular tissue weight. EGF protein levels were greater in submandibular glands of 10-week-old AL mice (279.4 ± 33.9 ng/mg tissue) compared to CR mice (120.9 ± 24.4 ng/mg tissue; P < 0.01) and 10-fold greater in 12-week-old AL mice (148.7 ± 6.6 ng/mg tissue) compared to CR mice (14.5 ± 12.1 ng/mg tissue; P < 0.0001).

Mammary levels of EGF protein, relative to mammary total protein, were similar for 6-, 8-, and 10-week-old AL and CR mice but greater among 12-week-old AL mice (7.8 ± 0.5 ng/mg protein) compared to CR mice (5.0 ± 0.9 ng/mg protein; P < 0.05; Fig. 4). Calculated relative to mammary fat pad weight, mammary EGF protein levels of 6–12-week-old AL and CR mice were similar at each age of assessment. For example, mammary EGF protein/mammary weight for 12-week-old CR mice (0.13 ± 0.07 ng/mg mammary fat pad) was similar to that of AL mice (0.14 ± 0.09 ng/mg mammary fat pad). These mammary EGF determinations on the basis of tissue weight were influenced by significant differences in the mammary fat pad
weights of AL (0.741 ± 0.156 g) compared to CR (0.322 ± 0.099 g) mice (P < 0.01).

Serum levels of EGF protein ranged between 97–604 pg/ml. Mean serum EGF protein levels of 6-, 8-, 10-, and 12-week-old AL and CR mice were comparable at each age of assessment. For example, when mice were 12 weeks old, mean serum EGF levels of AL and CR mice were 234 ± 86 and 225 ± 43 pg/ml, respectively. No correlation between mammary, submandibular gland, and serum EGF protein levels was noted.

**Immunolocalization of Mammary and Submandibular Gland**

EGF. Granular, brown immunoprecipitate, indicative of EGF protein, was present over the convoluted tubular epithelium of the submandibular gland and over the mammary ductal and alveolar epithelium, the inner aspect of mammary end buds, and extended into the mammary ductal lumen. Patterns and amounts of immunoprecipitate in submandibular and mammary glands of 6- and 8-week-old AL and CR mice were comparable. Appreciably more precipitate was present in the submandibular glands of 10- and 12-week-old AL compared to CR mice, with only faint brown precipitate overlaying the submandibular tubular epithelium of 12-week-old CR mice (Fig. 5). Appreciably more precipitate overlaid the mammary epithelium of 10- and 12-week-old AL compared to CR mice. Ducts, alveoli, and end buds in glands of 12-week-old CR mice often lacked appreciable immunoprecipitate (Fig. 6).

**DISCUSSION**

Autoradiographs of the mammary gland of 4–12-week-old ad libitum-fed mice during peripubertal mammary development indicate that more than 20% of ductal end bud cells, and more than 14% of alveolar cells within [3H]thymidine-labeled alveoli, are actively mitotic (16). Calorie restriction reduces the proportion of actively mitotic end bud and alveolar cells to ≤13 and ≤7%, respectively (16). Even these reduced mitotic rates are relative bursts of epithelial proliferation compared to the vegetative rates of epithelial division in the mature mammary gland (≤3%; Refs. 15, 16, and 51–53). The intensely mitotic, undifferentiated epithelium of the developing mammary gland of the ad libitum-fed rodent is particularly vulnerable to chemical carcinogenic challenge (54, 55), sensitive to oncogenic mutation (56–58), and has a limited capacity for enzyme-mediated DNA repair (54).

Concurrently, 10-fold lower submandibular gland expressions of EGF RNA and protein, and lower mammary levels of EGF protein in calorie-restricted mice described herein, coincide temporally with distinctly lower rates of mammary end bud and alveolar epithelial mitosis, delayed mammary growth, and lower rates of intestinal epithelial mitosis and precede a lower cumulative mammary tumor incidence described previously in mice similarly calorie restricted from 4–12 weeks of age (16).

EGF may stimulate epithelial synthesis of type IV collagen (59), a component of the basal lamina required for epithelial attachment and proliferation (60) and required for growth of cultured mammary gland (61). EGF causes the reappearance and growth of invioluted mammary end buds in ovariectomized mice (6, 8) and reverses the intestinal hypoplasia which results from prolonged i.v. feeding (62). Administering EGF to calorie-restricted mice may reelevate lowered mammary and intestinal rates of epithelial mitosis, expedite mammary development, and reevaluate mammary tumor risk.

Submandibular levels of EGF protein typically ranged from ≥2000 ng/mg protein, while those of 12-week-old CR mice (≤200 ng/mg protein) approximated levels expressed in submandibular glands of 3–4-week-old, prepubescent mice (data not shown; Ref. 37). Sustained, stringent calorie restriction of adult rodents has been shown to lower and delay peak serum levels of estrogen and progesterone, which accompany each ovulatory cycle (63), and to reduce serum prolactin (21). Even a modest interval of peripubertal calorie restriction might lower peak mammogenic hormone levels. Peripubertal calorie restriction may lower EGF levels by modulating levels of estrogen or other hormones considered inductants of EGF synthesis (64–68). Lowered hormone levels could influence mammary development directly or by decreased induced synthesis of EGF. The delayed mammary development and reduced mitogenesis due to calorie restriction might be largely mediated directly by lowered EGF levels, since EGF alone induces epithelial mitogenesis within ovariectomized, adrenalectomized, and hypophysectomized mice in the absence of pituitary, reproductive, and adrenal hormones (69).

EGF physiology and homeostasis are not well understood. Inducers and regulators of preproEGF synthesis and processing to form EGF peptide are not well defined. Origin(s) of EGF peptide measured in the serum and in tissues from various organs, such as the mammary gland, are not known with certainty. For example, in spite of substantial renal transcription of preproEGF RNA, little EGF peptide is synthesized by the kidney (35). Although abundant EGF peptide is synthesized by the submandibular gland (35), levels of EGF within the submandibular gland may (67) or may not correlate with those of serum EGF (37), and submandibular sialoadenectomy may (67) or may not lower serum EGF levels (65). Whether EGF peptide measured in the mammary gland is synthesized entirely in situ or represents multiple sources of EGF protein, including the submandibular gland, is not known.

Proposed mechanisms of the antineoplastic effect of calorie restriction include influences on levels and rhythms of hormones, cytokines, and growth factors (21, 63, 70–74), rates of cellular proliferation (15, 16, 51–53), free radical production and detoxification (77–79), efficiency of enzyme-mediated DNA repair (80), and immunological responsiveness (25, 81–83). Herein, specific down-regulation of EGF expression by calorie restriction is demonstrated. This lowered EGF expression correlates temporally with reduced rates of mammary and intestinal epithelial mitosis described previously (16). The abrogation by calorie restriction of both mammary and intestinal carcinoma (84) may be partly attributable to reduced levels of EGF expression, lowered rates of epithelial mitosis, and diminished opportunity for fixing genetic lesions or activating genetic expressions, which contribute to neoplastic transformation (i.e., MMTV expression, oncogene activation, or tumor suppressor gene inactivation).

Understanding normal breast development and how external risk factors alter breast development and contribute to carcinogenesis are important antecedents to controlling breast cancer incidence. Controlling dietary calories may not only reduce the risk of breast cancer but may also limit development of neoplasia at multiple sites, impair the upswing in autoimmune phenomena associated with aging, and delay the development of cardiovascular disease (22, 25, 85, 86).

**ACKNOWLEDGMENTS**

We thank C. Thompson for excellent care, feeding, and monitoring of the animal collection; S. Sexton for excellent technical assistance; Dr. R. Haire (Department of Molecular Genetics) for synthesis of EGF and β-actin primers; and C. S. Hu (Department of Pediatrics) and Dr. C. H. Brown (Department of Biostatistics and Epidemiology) for assistance with statistical analyses.

**REFERENCES**

2. Daniel, C. W., Silberstein, G. B., and Strickland, P. Direct action of β-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autora-


Mammary and Submandibular Gland Epidermal Growth Factor Expression Is Reduced by Calorie Restriction


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/55/6/1289

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.