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Rat Strain Specific Attenuation of Estrogen Action in the Anterior Pituitary Gland by Dietary Energy Restriction

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The purpose of this study was to compare the effects of a 40% restriction of dietary energy consumption, relative to that consumed by rats allowed to feed *ad libitum*, on the ability of 17β-estradiol (E2) to induce pituitary tumorigenesis in two inbred rat strains, ACI and Copenhagen (COP), which are very closely related genetically. Ovary-intact ACI and COP rats were fed either a control or an energy-restricted diet beginning at 8 wk of age. Continuous treatment with E2, released from subcutaneous Silastic tubing implants, was initiated at 9 wk of age and the animals were killed 12 wk later. Estrogen-induced pituitary tumorigenesis is associated with rapid induction of lactotroph hyperplasia, increased pituitary mass, and hyperprolactinemia. E2 significantly increased pituitary mass and circulating prolactin (PRL) in both ACI and COP rats, and this response was significantly greater in ACI rats relative to COP. Dietary energy restriction did not inhibit E2-induced pituitary growth in the ACI rat. By contrast, E2-induced pituitary growth in COP rats was attenuated by dietary energy restriction, as evidenced by quantification of pituitary mass, pituitary weight to body weight ratio, circulating PRL, and pituitary cell proliferation. This study indicates that sensitivity to the inhibitory actions of dietary energy restriction on E2-induced pituitary tumorigenesis is genetically determined.

Key Words: ACI rat; COP rat; prolactin; lactotroph; estrogen; tumorigenesis.

Introduction

Estrogens exert several well-defined actions on the prolactin (PRL)-producing lactotroph of the mammalian anterior pituitary gland (reviewed in *1,2*). Specifically, estrogens stimulate lactotroph proliferation *(3–5)*, promote lactotroph survival *(6,7)*, and activate transcription of the PRL gene *(8–11)*. In certain inbred rat strains, including Fischer 344 (F344) *(12,13)*, ACI *(14–16)*, and Copenhagen (COP) *(16,17)*, several weeks of continuous treatment with estrogens results in up to a 20-fold increase in pituitary mass and associated hyperprolactinemia. Histologically, the grossly enlarged pituitary glands of the estrogen-treated rats exhibit lactotroph hyperplasia and hypertrophy, significantly increased proliferation within the lactotroph population, and, in some instances, exhibit lactotroph adenoma *(1,2,4,5,18,19)*. Pituitary mass in the estrogen-treated rats correlates with both the DNA content of the pituitary gland *(12,20,21)* and the level of PRL in the circulation *(16)*, indicating that the increase in pituitary mass results in large part from an expansion of the lactotroph population. Although prolonged treatment of rats with estrogens can on occasion lead to development of pituitary carcinoma, morbidity and/or mortality generally result from the mass effect of the markedly enlarged pituitary gland on the brain and/or hyperprolactinemia before these malignant lesions develop *(2)*. Consequently, these rat models of estrogen-induced pituitary tumorigenesis are best suited for studying the early events associated with estrogen-induced carcinogenesis.

The lactotroph of the anterior pituitary gland also provides a valuable model for the study of the genetic factors that impact cellular responsiveness to estrogens. Whereas the F344, ACI, and COP rat strains each exhibit a significant and highly reproducible increase in pituitary mass in response to continuous estrogen treatment, these three rat strains differ quantitatively in the extent to which pituitary mass and circulating PRL increase in response to a defined duration of estrogen treatment *(2,12,16,22,23)*. Moreover, other rat strains, including Brown Norway (BN) *(16,23)* and Holtzman *(12,20)*, exhibit very little increase in pituitary mass in response to continuous estrogen treatment. Several studies indicate that the extent to which estrogens increase pituitary mass and hyperprolactinemia in different rat strains is genetically determined *(2,12,13,16,21,24)*. Wendell et al., in genetic crosses between the F344 and BN rat strains, have mapped to rat chromosomes 2, 3, 5, and 9 six distinct loci that determine the extent to which estrogens increase pituitary mass *(21,24)*. Our laboratory has mapped to rat chromosomes 1, 3, 6, and 10 five genetic loci that determine

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estrogen-induced pituitary mass in crosses between the ACI and COP rat strains (Strecker et al., manuscript submitted). Interestingly, the genetic loci mapped in the crosses between the F344 and BN strains are for the most part distinct from those mapped in the crosses between the ACI and COP rat strains, indicating that multiple genes determine the manner and/or the extent to which the pituitary gland responds to estrogens. Although the identities of the genes that reside within each of these loci and determine the actions of estrogens on the lactotroph population are not currently known, the recent initial release of the rat genome sequence and the continuing development of rat genetic databases is expected to facilitate greatly the identification of these genes.

Dietary energy consumption appears to be an important determinant of cancer risk in human populations and is a potent modulator of carcinogenesis in several animal models *(25–30)*. It has often been suggested that dietary energy consumption might impact carcinogenesis by modulating the endocrine system. For the past few years our laboratory has been evaluating the effects of differing levels of dietary energy consumption on estrogen-induced pituitary tumorigenesis in inbred rat strains in which the genetic bases of sensitivity to the pituitary tumor-inducing actions of estrogens are relatively well defined. Data from these studies demonstrate rat strain-specific effects of dietary energy consumption on estrogen-induced tumorigenesis in the pituitary gland. A 40% restriction of dietary energy consumption virtually abolished the increase in pituitary mass in F344 rats treated continuously with either the synthetic estrogen diethylstilbestrol (DES) or the naturally occurring estrogen 17β-estradiol (E2), relative to that observed in F344 rats that were allowed to feed *ad libitum (5,20,31)*. In contrast, no inhibitory effect of dietary energy restriction was observed in E2-treated ACI rats *(31–33)*. In order to define further the interactions between dietary energy consumption and estrogens in the regulation of the lactotroph population of the anterior pituitary gland, we have in this study directly compared the effect of dietary energy restriction on estrogen-induced hyperplastic growth and hyperprolactinemia in the genetically related ACI and COP rat strains. The data presented herein indicate that the ACI and COP strains differ significantly in sensitivity to the antitumorigenic actions of dietary energy restriction and set the stage for future studies in which diet-estrogen-gene interactions can be assessed.

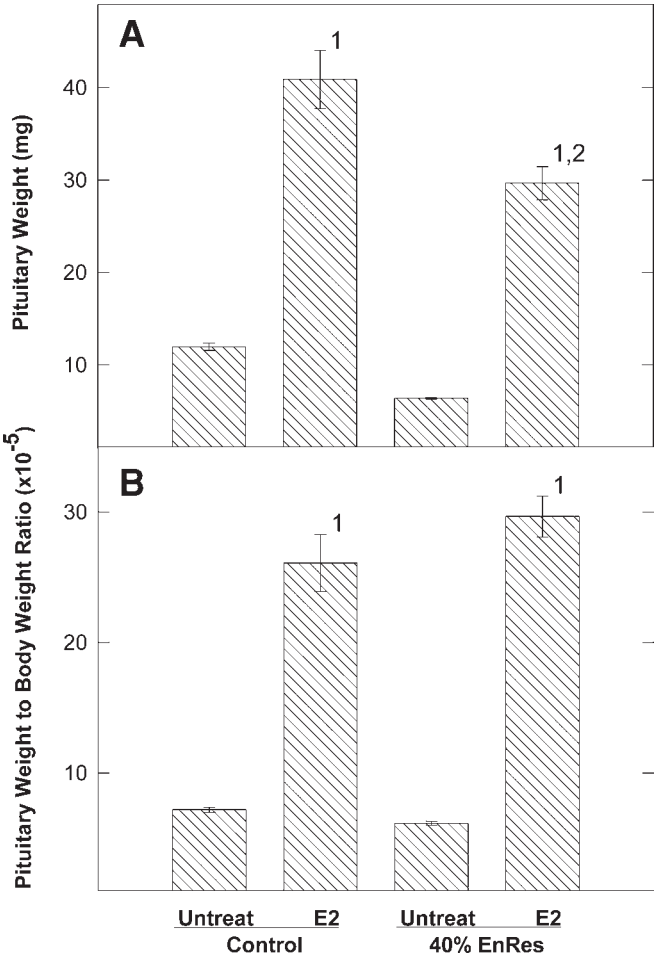

Fig. 1. Dietary energy restriction does not inhibit 17β-estradiol-induced pituitary growth in ovary-intact ACI rats. Female ACI rats were fed the control or energy restricted diet and treated with E2 for 12 wk as described in Materials and Methods. The anterior pituitary gland was removed and weighed immediately following death. (**A**) Each bar represents the mean (± SEM; $n = 7–8$) anterior pituitary wet weight. (**B**) Each bar represents the mean (± SEM; $n = 7–8$) ratio of anterior pituitary wet weight to final body weight. Numerals: **1**, indicates a statistically significant difference ($p \leq 0.05$) between untreated and E2-treated animals fed the same diet; **2**, indicates a statistically significant difference ($p \leq 0.05$) between similarly treated animals fed the different diets.

Results

Rat-Strain-Specific Inhibitory Action of Dietary Energy Restriction on Estrogen–Induced Pituitary Growth and Hyperprolactinemia

Twelve weeks of E2 treatment significantly increased pituitary mass in female ACI rats fed the control diet, from a mean of 12.0 mg in untreated rats to 41.0 mg in E2-treated rats ($p < 0.01$) (Fig. 1A). Treatment with E2 also significantly increased pituitary mass in ACI rats fed the energy restricted diet, from 6.4 mg in untreated rats to 29.7 mg ($p < 0.01$). Because energy restriction significantly inhibits overall growth, the mass of the anterior pituitary gland was normalized to body mass at the termination of the experiment. The ratio of pituitary mass to body mass was increased similarly in response to E2 in ACI rats fed either the control or the energy-restricted diet (Fig. 1B). These data indicate that a 40% restriction of dietary energy consumption did not inhibit induction of pituitary growth in female ACI rats treated continuously with E2 for 12 weeks, and confirm and extend previous studies from our laboratory in which the effect of dietary energy restriction on E2-induced pitu-

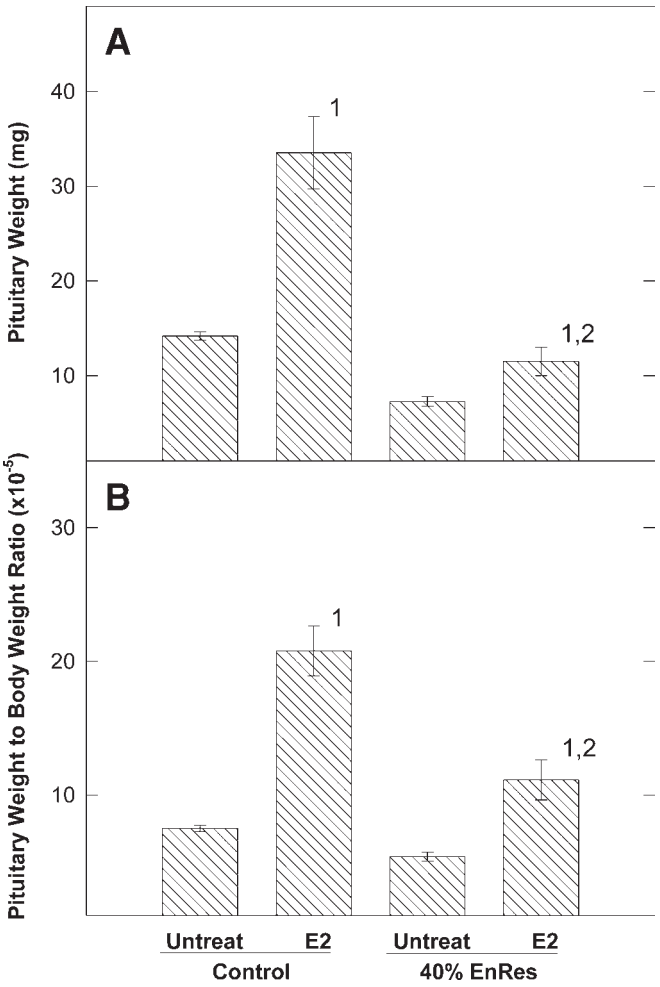

Fig. 2. Dietary energy restriction inhibits 17β-estradiol-induced pituitary growth in ovary-intact COP rats. Female COP rats were fed the control or energy-restricted diet and treated with E2 for 12 wk as described in Materials and Methods. The anterior pituitary gland was removed and weighed immediately following death. (**A**) Each bar represents the mean (± SEM; n = 7–8) anterior pituitary wet weight. (**B**) Each bar represents the mean (± SEM; n = 7–8) ratio of anterior pituitary wet weight to final body weight. Numerals: **1**, indicates a statistically significant difference ($p \leq 0.05$) between untreated and E2-treated animals fed the same diet; **2**, indicates a statistically significant difference ($p \leq 0.05$) between similarly treated animals fed the different diets.

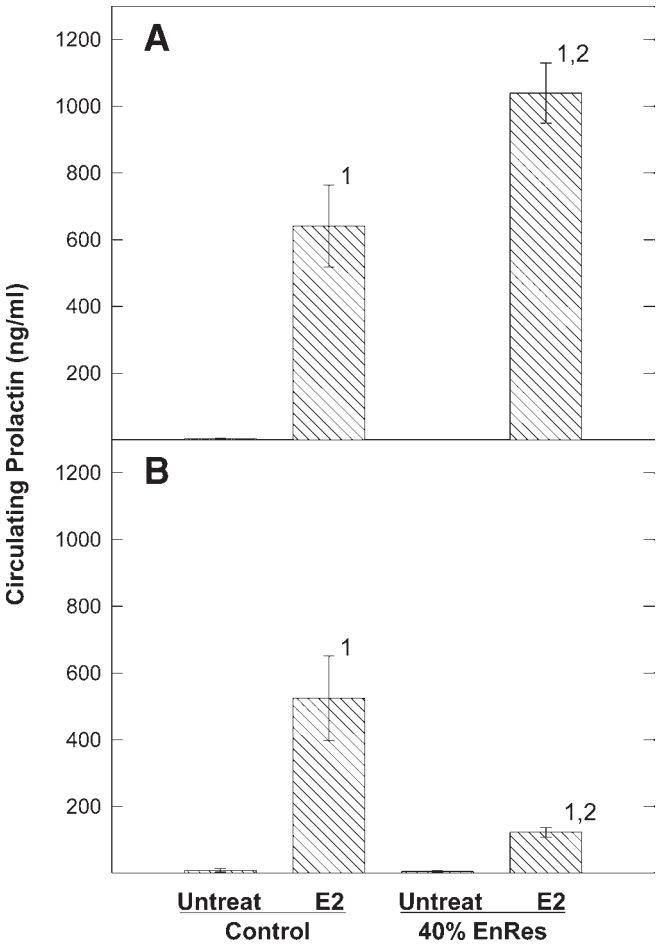

Fig. 3. Rat-strain-specific effects of dietary energy restriction on 17β-estradiol-induced hyperprolactinemia. PRL in serum from trunk blood collected from (**A**) ACI and (**B**) COP rats was quantified by radioimmunoassay as described in Materials and Methods. Each data point represents mean (± SEM; n = 7–8) level of circulating PRL at the time of sacrifice. Numerals: **1**, indicates a statistically significant difference ($p \leq 0.05$) between untreated and E2-treated animals fed the same diet; **2**, indicates a statistically significant difference ($p \leq 0.05$) between similarly treated animals fed the different diets.

itary growth was examined in ovariectomized ACI rats *(32)* or ovary-intact ACI rats examined in the context of a mammary carcinogenesis study *(33)*.

In contrast to the observed lack of inhibition of E2-induced pituitary growth in female ACI rats, energy restriction significantly inhibited E2-induced pituitary growth in female COP rats. Pituitary mass in COP rats fed the control diet was increased in response to 12 wk of E2 treatment from 14.2 to 33.5 mg ($p < 0.01$), whereas in COP rats fed the energy restricted diet pituitary mass was increased only from 7.3 to 11.5 mg ($p < 0.05$) (Fig. 2A). The inhibitory effect of dietary energy restriction on E2-induced pituitary growth in the COP rat remained apparent when pituitary weight was normalized to body weight (Fig. 2B). Whereas the ratio of pituitary mass to body mass was significantly increased ($p < 0.05$) in response to E2 in COP rats fed either the control or the energy-restricted diet, this ratio was significantly greater in E2-treated rats fed the control diet relative to treated rats fed the energy restricted diet ($p < 0.05$) (Fig. 2B).

Treatment with E2 increased circulating PRL 160-fold ($p < 0.01$), from 4 to 640 ng/mL, in ACI rats fed the control diet (Fig. 3A). Induction of hyperprolactinemia was even greater in ACI rats fed the energy restricted diet. In the energy restricted rats, circulating PRL was increased 520-fold ($p < 0.01$) in response to E2, from 2 to 1040 ng/mL. Dietary energy restriction significantly inhibited induction

of hyperprolactinemia in COP rats. Whereas E2 increased circulating PRL 58.2-fold, from 9 to 524 ng/mL, in COP rats fed the control diet, circulating PRL was increased only 20.5-fold, from 6 to 123 ng/mL, in COP rats fed the energy-restricted diet (Fig. 3B). Although circulating PRL in E2-treated ACI and COP rats fed the control diet was proportional to pituitary mass, this relationship was not evident in rats fed the energy restricted diet.

The level of circulating E2 in the treated rats was not affected by either rat strain or diet. In E2-treated ACI rats fed either the control or energy-restricted diet, serum E2 levels averaged 165.1 ± 24 and 157 ± 18 pg/mL ($p = 0.81$), respectively, at the time of death. In treated COP rats fed these diets, serum E2 levels averaged 306.3 ± 119 and 255 ± 77 pg/mL ($p = 0.75$), respectively. Although mean E2 levels were lower in treated ACI rats than in COP rats, this difference was not statistically significant ($p = 0.32$). The levels of circulating E2 in the treated animals were within the range observed in rats during pregnancy.

Effects of Dietary Energy Restriction and Estrogen on Pituitary Histology and Cell Proliferation

The pituitary glands of untreated, ovary-intact, ACI and COP rats were indistinguishable when examined by light microscopy (Figs. 4A and 4B), and the PRL-producing lactotroph was the most common cell type in these glands (Figs. 5A and 5B). Twelve weeks of treatment with E2 induced a diffuse hyperplasia in the pituitary glands of the ACI and COP rats. The nuclei were enlarged and the nucleoli were more prominent in the pituitary glands of the treated rats (Figs. 4C and 4D), relative to those of untreated females (Figs. 4A and 4B). Moreover, the pituitary cells in the E2-treated rats often contained juxtanuclear inclusions (Figs. 4C and 4D). Lactotrophs comprised the most common cell type in the glands of the E2-treated ACI and COP rats (Figs. 5C and 5D). Lactotroph hypertrophy was evident in the pituitary glands of the E2-treated ACI and COP rats fed either experimental diet upon estimation of average cell volume (data not shown). No reproducible discernable differences in pituitary gland histology were noted between the ACI and COP rat strains or in E2-treated ACI or COP rats fed the two experimental diets (Figs. 4 and 5).

The number of pituitary cells incorporating BrdU (cells exhibiting black nuclear staining in Fig. 5) was similar in untreated ACI and COP rats and was not significantly impacted by dietary energy restriction (Fig. 6). Administered E2 significantly induced pituitary cell proliferation in both ACI and COP rats. E2-induced cell proliferation was similar in ACI rats fed either the control or energy-restricted diets (Fig. 6A). By contrast, dietary energy restriction significantly ($p < 0.05$) attenuated the ability of E2 to stimulate pituitary cell proliferation in the COP rat. Whereas the number of pituitary cells staining positive for BrdU was increased 8.9-fold in E2-treated COP rats fed the control diet, the number of BrdU positive cells was increased only 3.6-fold in E2-treated COP rats fed the energy restricted diet (Fig. 6B).

Discussion

Although it is clear from numerous epidemiologic and laboratory studies that diet is a strong determinant of cancer risk, the mechanisms underlying these diet–cancer associations are not currently understood. Our studies in this regard address the premise that the amount of energy consumed in the diet alters the responsiveness of specific target cell populations to estrogens and thereby impacts tumorigenesis in estrogen-responsive tissues such as the pituitary and mammary glands. In this study, we have compared the actions of a 40% restriction of dietary energy consumption on the ability of administered E2 to induce hyperplastic growth in the pituitary lactotroph population of two genetically related inbred rat strains. The data presented herein indicate that the ACI and COP rat strains differ dramatically in sensitivity to the inhibitory actions of dietary energy restriction on E2-induced pituitary growth and associated hyperprolactinemia. These data indicate that presently unidentified genetic factors determine whether or not dietary energy restriction attenuates the responsiveness of the pituitary lactotroph to estrogens.

Estrogens induce hyperplastic growth in the pituitary gland by stimulating lactotroph proliferation and enhancing lactotroph survival *(2)*. Administered E2 stimulated lactotroph proliferation in both ACI and COP rats, as evidenced by BrdU labeling indices. Dietary energy restriction attenuated E2-stimulated cell proliferation in the COP rat, but not in the ACI rat. It is probable that this attenuation of the proliferative response of the COP lactotroph population to E2 contributed to the observed inhibitory effect of dietary energy restriction on induction of increased pituitary mass and hyperprolactinemia observed in this strain. Although these experimental endpoints were affected by dietary energy restriction in a rat strain-specific manner, neither rat strain nor dietary energy restriction had any discernable effect on pituitary gland histology. The effect of dietary energy restriction on lactotroph survival was not examined in this study, because apoptotic cells in the pituitary gland are rapidly phagocytosed making their quantification very difficult *(5–7,34)*. We have previously demonstrated that dietary energy restriction markedly inhibits induction of E2-induced and DES-induced pituitary growth and associated hyperprolactinemia in the F344 rat strain *(5,20,31)*. However, in contrast to the current finding in the female COP rat, inhibition of pituitary tumorigenesis in the F344 rat did not appear to be associated with an attenuation of estrogen-stimulated lactotroph proliferation *(5,20)*. That observation led us to suggest that dietary energy restriction inhibits estrogen-induced pituitary tumorigenesis in the F344 rat by inhibiting the ability of administered hormone to enhance lactotroph survival. Together, these data suggest that dietary energy restriction

might act through multiple mechanisms to inhibit estrogen-induced hyperplastic growth in the pituitary lactotroph population and that these mechanisms may be rat strain specific.

The mechanisms through which estrogens regulate lactotroph proliferation and survival are under active investigation in several laboratories (reviewed in *1,2*). Published studies indicate that autocrine and/or paracrine pathways involving galanin *(35–39)* and members of the transforming growth factor beta family *(40–44)* contribute to this regulation. Additional pathways involving the tuberoinfundibular dopaminergic neurons of the hypothalamus are also likely to play an important role in this regulation *(45,46)*. Whether or not dietary energy consumption modulates regulation of any of these pathways by estrogens remains to be determined.

Genetic studies indicate that induction of lactotroph hyperplasia by estrogens is a highly complex process that is regulated through the actions of multiple genes (*2,16,21,23,24*). Our laboratory has mapped five distinct genetic loci that determine estrogen-induced pituitary growth in male F2 progeny generated in reciprocal crosses between the ACI and COP strains (Strecker et al., manuscript submitted). A model based on these genetic data suggests that three of these loci contribute to estrogen-induced pituitary growth in the ACI strain, whereas the remaining two loci contribute to estrogen-induced pituitary growth in the COP strain. Future studies will determine the impact of dietary energy restriction on the actions of each of these five genetic loci.

In summary, the data presented herein extend our previous studies of modulation of estrogen action in the rat anterior pituitary gland by dietary energy restriction. It is clear from these studies that dietary energy restriction attenuates induction of lactotroph hyperplasia in a rat-strain-specific manner. These findings are significant in that they indicate that dietary energy consumption can modulate at least two estrogen-regulated processes, i.e., cell proliferation and cell survival, and that this modulation is strongly determined by genetic background. It is also apparent that the inhibitory effects of dietary energy restriction on estrogen-induced tumorigenesis are cell type specific. In this study, no inhibitory effect of dietary energy restriction was observed in the pituitary gland of the ACI rat. In contrast, dietary energy restriction markedly inhibits development of estrogen-induced mammary cancer in the ACI rat strain *(33,47)*. Because estrogens are implicated in the etiology of several cancer types, these findings suggest potential mechanisms through which dietary energy consumption might modify cancer risk.

Materials and Methods

Care and Treatment of Animals

The Institutional Animal Care and Use Committee of the University of Nebraska Medical Center approved all procedures involving live animals. Ovary-intact ACI (Harlan, Indianapolis, IN) and COP rats (National Cancer Institute Breeding Program, Frederick, MD) were obtained at approx 7 wk of age and housed one animal per cage within a barrier animal facility under controlled temperature, humidity, and lighting (12-h light/12-h dark cycle) conditions. Upon arrival at our facility, the rats were initially fed a semipurified control diet that was formulated in accordance with guidelines established by the American Society of Nutritional Science *(48)*. Approximately 1 wk later, the rats were randomly assigned to groups fed either this control diet or an energy-restricted diet. The compositions of these diets and the methods used in their preparation have been described previously *(33)*. Animals fed the control diet were allowed to eat *ad libitum* and their food consumption was monitored twice weekly. Animals maintained on the energy-restricted diet were fed each day at the beginning of the dark phase of the lighting cycle. Each rat fed the energy-restricted diet received 0.64 g of food per g of food consumed per day by rats fed the control diet. Because of the manner in which the diets were formulated, animals fed the energy-restricted diet consumed 40% less energy, derived from carbohydrate and fat, but equivalent amounts of protein, vitamins, minerals, fiber, and other nutrients, relative to that consumed by animals fed the control diet. The rats were allowed continuous access to water. Half of the rats on each of the two experimental diets were treated with E2, beginning at approx 9 wk of age. The remaining rats received empty implants. Silastic tubing implants, empty or containing 27.5 mg of E2, were prepared and surgically inserted subcutaneously in the interscapular region while the rats were under ether anesthesia *(15,17)*. Published studies from our laboratory indicate that animals treated with E2 in this manner for various lengths of time exhibit circulating E2 levels at sacrifice that are within the physiologic range *(5,15,33,49)*. Body weights were monitored weekly. Four hours prior to killing, each rat received an intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) solubilized in sterile phosphate-buffered saline and administered at a dose of 50 mg/kg body weight, to allow pituitary cells in the S phase of the cell cycle to be identified using immunohistochemical techniques. Each experimental group consisted of seven or eight rats.

Collection of Pituitary Tissues and Analysis of Circulating Hormones

The rats were killed by decapitation following 12 wk of E2 treatment. Trunk blood was collected, allowed to clot at 4ºC and centrifuged at 1300x*g* for 15 min. Serum was retained and stored at −80ºC. Circulating E2 and PRL in serum from trunk blood were measured by radioimmunoassay as previously described *(5,15,33,49)*. Pituitary glands were removed immediately following death, weighed, fixed in 10% neutral buffered formalin and processed for histology. Because pituitary mass correlates directly with pituitary DNA content and circulating PRL in rats treated con-

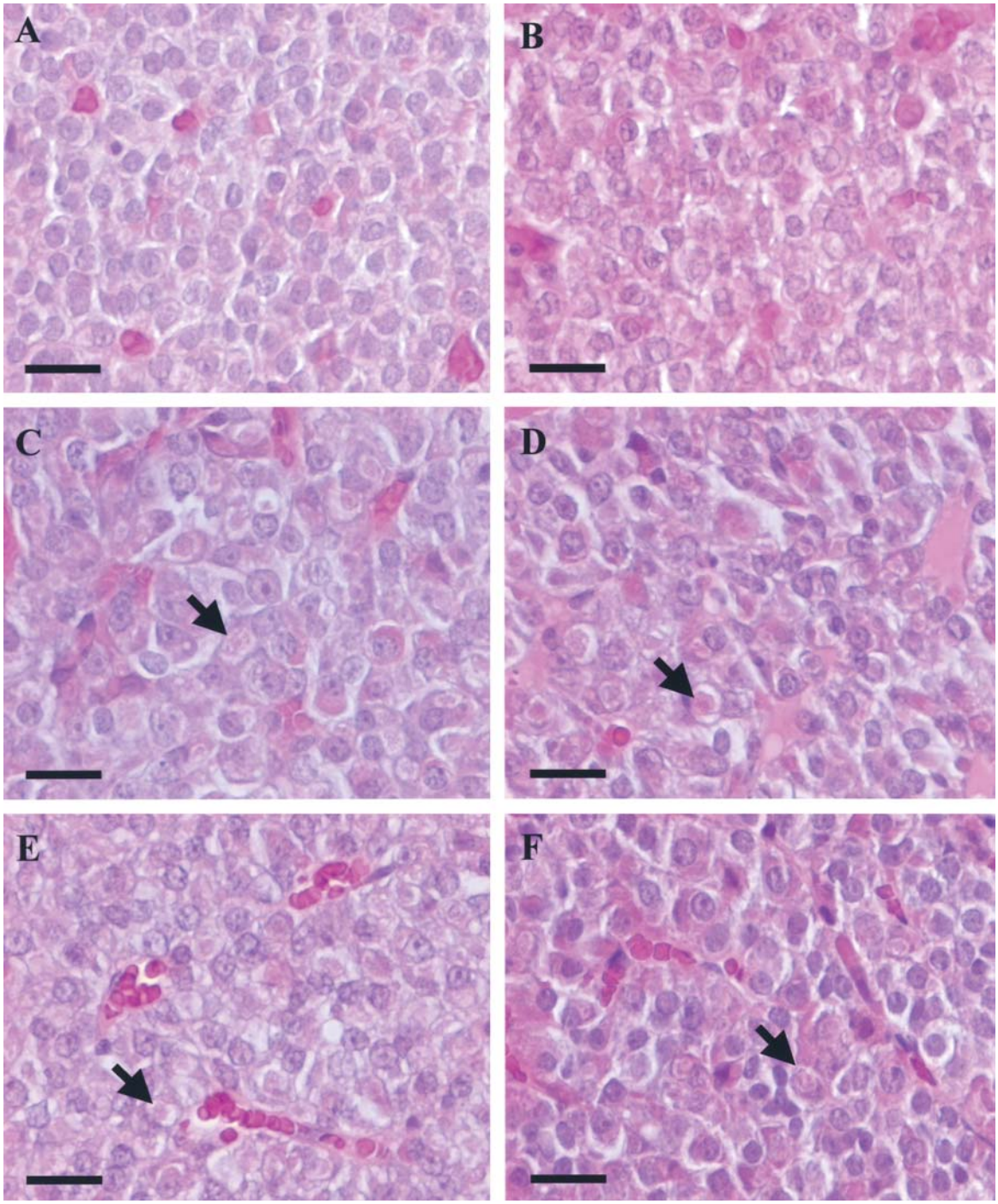

Fig. 4. Effects of 17β-estradiol and dietary energy restriction on pituitary histology. Pituitary glands were collected, fixed, sectioned, and stained with hematoxylin/eosin. Anterior pituitary glands from untreated ovary intact ACI (**A**) and COP (**B**) rats fed the control diet were indistinguishable from one another. Hyperplastic and hypertrophic changes were observed in the pituitary glands of ACI (**C**) and COP (**D**) rats fed the control diet and treated with E2 for 12 wk, as evidenced by increased cellular volume, increased nuclear size, and prominent nucleoli. Juxtanuclear inclusions, indicated by arrows, were prominent in the pituitary glands of E2-treated ACI and COP rats relative to untreated rats. The anterior pituitary glands of E2-treated ACI (**E**) and COP (**F**) rats fed the energy-restricted diet were similar in histologic appearance to those of treated rats fed the control diet. The bar in each of the panels corresponds to 20 μm.

tinuously with estrogens *(12,16,20,21)*, pituitary mass is a useful and valid surrogate indicator of absolute lactotroph number.

Immunohistochemical Analysis of Pituitary Tissue

Pituitary lactotrophs were identified immunohistochemically using an antibody to rat PRL (National Hormone and

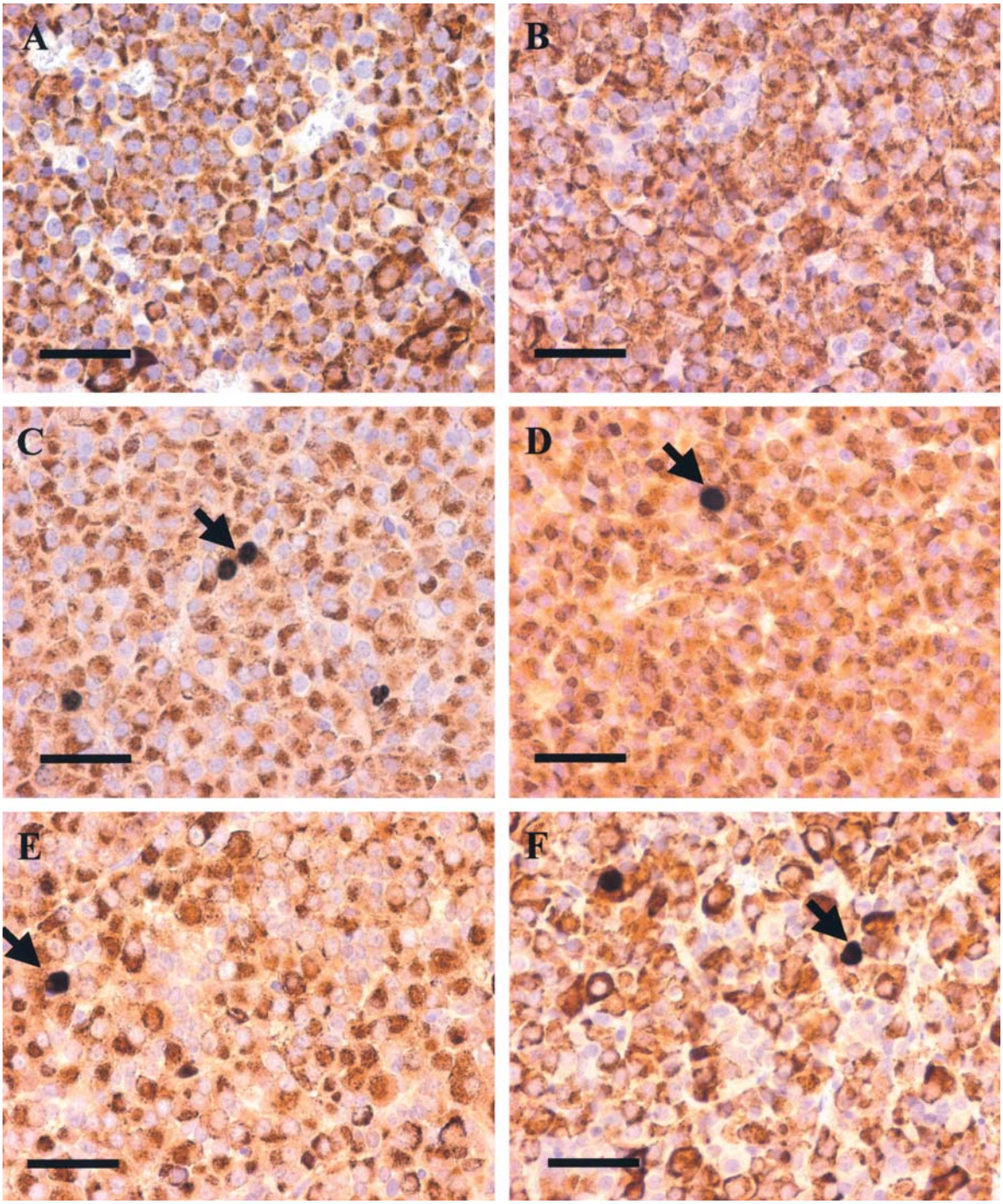

Fig. 5. Effects of 17β-estradiol and dietary energy restriction on lactotroph proliferation. Lactotrophs were identified immunohistochemically using an antibody to rat PRL. Cells exhibiting brown staining in the cytoplasm were defined as lactotrophs. Cells in S phase were identified using an antibody to BrdU. Cells exhibiting black staining over the nucleus, indicated by arrows, were defined as being in the S phase of the cell cycle during the 4 h period preceding death. Lactotrophs were the most prevalent cell type in the pituitary glands of untreated ovary-intact ACI (**A**) and COP (**B**) rats fed the control diet. Twelve weeks of E2-treatment stimulated lactotroph proliferation in ACI (**C**) and COP (**D**) rats fed the control diet. Note that the BrdU positive nuclei are usually observed in cells exhibiting PRL-positive cytoplasm and/or juxtanuclear inclusions. Pituitary glands from E2-treated ACI (**E**) and COP (**F**) rats fed the energy-restricted diet are similar in appearance to the pituitary glands of rats fed the control diet. The bar in each of the panels corresponds to 40 μm. Quantitative data on the proportion of the pituitary cell population staining positive for BrdU are presented in Fig. 6.

Pituitary Program, NIDDK, NIH; lot number AFP425_10_91) and proliferating pituitary cells in the S phase of the cell cycle were identified using a mouse monoclonal antibody to BrdU (Amersham, Arlington Heights, IL) as described previously *(5,17)*. At least 1000 cells from each pituitary section were defined as positive or negative for PRL and BrdU

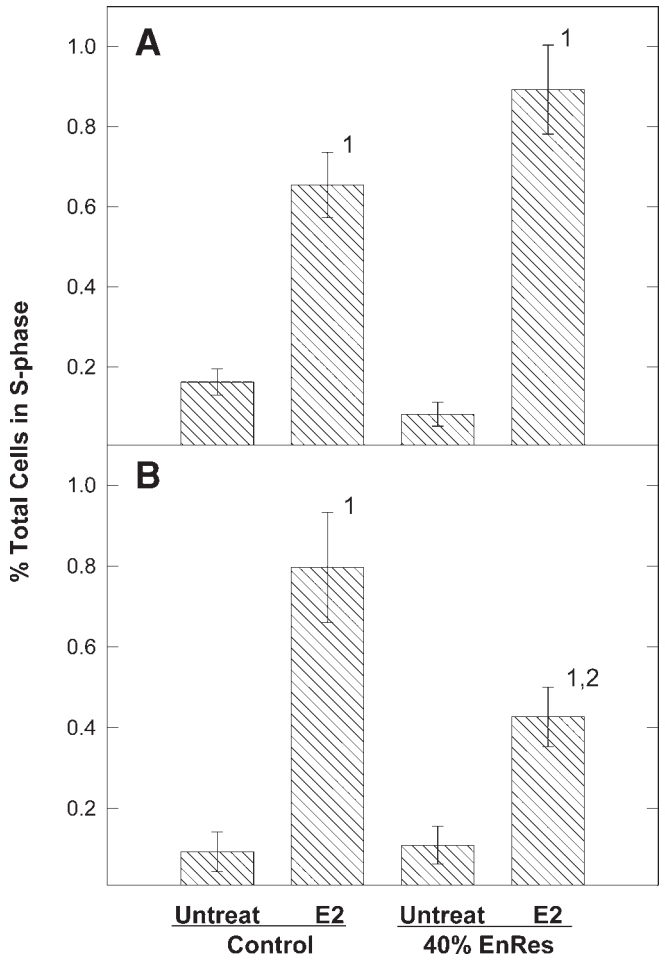

Fig. 6. Effects of 17β-estradiol, genetic background, and dietary energy restriction on pituitary cell proliferation. Female ACI (**A**) and COP (**B**) COP rats were treated as described in Fig. 1 and Materials and Methods. Each animal received an intraperitoneal injection of BrdU, administered at a dose of 50 mg/kg, 4 h prior to death. Anterior pituitary cells in S phase of the cell cycle were identified by immunohistochemical detection of BrdU-positive cells. A minimum of 1000 cells was counted for each pituitary gland. Each bar represents the average (± SEM) number of BrdU positive pituitary cells expressed as a percentage of total anterior pituitary cells. Numerals: **1**, indicates a statistically significant difference ($p \leq 0.05$) between untreated and E2-treated animals fed the same diet; **2**, indicates a statistically significant difference ($p \leq 0.05$) between similarly treated animals fed the different diets.

using a computer-assisted image analysis system (Optimas, Seattle, WA).

Statistical Analysis of Data

Data are presented as the mean ± standard deviation (SD) or standard error of the mean (SEM), where $n = 6–8$ rats. Differences between the means were evaluated by two-way analysis of variance (ANOVA) with Scheffe's test for multiple comparisons among group means or Student's *t*-test. *p* values ≤ 0.05 were considered statistically significant.

Acknowledgments

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