Food Restriction Differentially Affects mRNAs Encoding the Major Anterior Pituitary Tropic Hormones

Eun-Soo Han, Deedee H. Lu, and James F. Nelson

Department of Physiology, The University of Texas Health Science Center, San Antonio.

Chronic food restriction (FR) markedly extends life span, apparently by retarding the aging process and the development of age-related disease (1). Although the greatest extension of life span is achieved when FR is initiated early and sustained throughout life, an increase in longevity has even been observed when rats are food-restricted for only a few months during early adulthood (2). These data indicate that anti-aging effects begin shortly after the onset of FR and are cumulative. This concept is important theoretically as well as practically, since it indicates that insight into the anti-aging mechanisms induced by FR may be obtained by studying young FR animals. Although short-term FR lowers metabolic rate (3), after 6 weeks of FR, metabolic rate returns to that of ad libitum (AL)–fed rats. The absence of a difference in metabolic rate (per unit body mass) indicates that the characteristic rather than the magnitude of fuel utilization are altered in ways that retard aging. Evidence that fuel is utilized differently by FR animals includes the fact that their reproductive processes are often attenuated (4), protein synthesis and turnover are enhanced (5), and metabolic pathways are qualitatively altered (6). Many of these changes have occurred by 6 weeks after the onset of FR. Although the mechanisms whereby FR prolongs life are not established, growing evidence suggests that even after a few months of FR, animals exhibit enhanced resistance to carcinogenic insult (7), greater detoxification capacity (8), and more rapid recovery from inflammatory processes (9). Thus, in addition to changes in metabolic processes, candidate mechanisms for the anti-aging actions of FR are also activated shortly after the onset of FR.

A key question is what induces and coordinates the potentially protective anti-aging mechanisms of food restriction. One possibility is that neuroendocrine processes mediate the perception of reduced caloric availability into a series of hormonal changes that alter cellular metabolism. Chronic FR is associated with several endocrine changes that could play a role (10–12). FR may coordinate these hormonal changes by altering the biosynthesis of the tropic hormones of the anterior pituitary (AP) gland.

A longstanding hypothesis is that food restriction acts by functionally hypotheysecomyzizing animals and thereby reducing presumptive deleterious hormonal exposure (1,13,14). However, we had previously shown that FR does not reduce propiomelanocortin (POMC) mRNA levels in the pituitary (15). This result, along with the fact that FR animals exhibit elevated plasma corticosterone levels in the afternoon, indicated that at least one endocrine system regulated through the pituitary was not suppressed by FR. This study was designed to complete the assessment of the effect of FR on pituitary tropic hormone mRNAs in young rats as an initial step in determining the extent of modulation of AP hormone biosynthesis by FR. The focus on young rats that had been under FR for only 6 weeks was designed to gain insight into the initial neuroendocrine effects of FR that might play a role in the induction of the anti-aging effect of FR.

Materials and Methods

Animals and dietary procedures.—Male Fischer 344 rats were obtained at 4 weeks of age from Charles River Laboratories (Kingston, NY) and housed singly in plastic cages (10 X 9.5 X 8) with wire mesh floors suspended on a Hazleton-Enviro Rack System (Hazleton Systems, Aberdeen, MD) in a barrier facility (2). Animals were kept on a cycle of 12 h darkness and 12 h light (lights on at 0530 h).
The presence of murine virus antibodies (CAR Bacillus, H-1 Virus, Kilham Rat Virus, Lymphocytic Choriomeningitis Virus, Mycoplasma pulmonis, Parovirus, Pneumovirus Virus of Mice, Rat Coronavirus/Sialodacryoadenitis Virus, Reovirus, and Sendai Virus) and mycoplasma antibodies was monitored quarterly with serum samples from sentinel animals by Microbiological Associates (Rockville, MD). All tests for pathogenic organisms were negative. The procedures and experiments involving use of rats were approved by the Institutional Animal Care and Use Committee and are consistent with the NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Education, The Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (National Academy Press, Washington, DC).

For the first two weeks (i.e., until 6 weeks of age) all rats were fed ad libitum a standard semisynthetic diet: 21% Rat-Purina 101 soy protein isolate, 15% sucrose, 3% Solka Floc, 3.33% Rat-Purina vitamin mix, 5% Rat-Purina mineral mix with reduced sodium, .35% DL-methionine, .33% choline chloride, 6% corn oil, and 45.99% dextrose. At 6 weeks, approximately half of the rats (Group AL) were allowed to continue to eat this diet ad libitum until sacrifice (12 weeks of age). The other half (Group FR) were restricted to 60% of the mean food intake of Group AL until sacrifice (12 weeks of age). Fire intake by AL rats was measured twice a week and the amount ingested per day calculated. FR rats were given their food allotment one hour before the start of the dark phase of the light cycle.

The mean body weight of FR rats was 66% of that of AL rats (diet main effect, p = .0001). It is important to note that FR rats are healthy, more active than AL rats, and live longer and show fewer diseases than AL rats (16,17).

**Tissue collection.** — For measurement of proopiomelanocortin (POMC), follicite-stimulating hormone (FSHp), luteinizing hormone (LHp), thyroid-stimulating hormone (TSHp), growth hormone (GH), and prolactin (PRL) ribonucleic acid (RNA), tissue was collected at 0500 and 1500 h. Food intake by AL rats was measured twice a week and the amount ingested per day calculated. FR rats were given their food allotment one hour before the start of the dark phase of the light cycle.

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**RNA preparation.** — RNA was extracted separately from each pituitary, as previously described (18), with minor modifications. Frozen tissue was homogenized by polytron in 1 ml of phosphate buffered saline (PBS; sodium phosphate (0.1 M) and NaCl (9g/l), pH 7.5) plus diethiothreitol (DTT: .5 mM). An aliquot (.3 ml) of the homogenate was transferred to a fresh tube and stored at -20°C for other experiments. Two volumes (1.4 ml) of guanidine isothiocyanate (IBI, New Haven, CT) buffer (6 M guanidine isothiocyanate, 7.5 mM Nacitrate pH 7.0, .15 M β-mercaptoethanol, and .75% sarkosyl) were added to the remaining 0.7 ml of the homogenate and centrifuged over cesium chloride (IBI). The RNA yield of each sample was determined spectrophotometrically, assuming 1 OD$_{260}$ unit = 40 μg/ml. Samples were stored in diethylpyrocarbonate (DEPC)-treated water at -70°C. The quality of the RNA extracted from each sample was monitored by 1.0% agarose formaldehyde gel electrophoresis. All samples had 260:280 ratios of about 2 and exhibited discrete 28S and 18S bands.

**cRNA probe syntheses and Northern blot analyses.** — To synthesize riboprobe for POMC, cDNA probe complementary to rat POMC mRNA from pSP64 plasmid containing a 396 base pair AluI fragment from exon 3 of the rat POMC gene (19) was used. Other cDNA probes complementary to rat FSHp, TSHp, LHp, GH, and PRL were obtained as follows: FSHp (rat FSH pGEM 3) and TSHp (RP21) probes were obtained from Dr. Richard A. Maurer (20,21). For riboprobe synthesis, an EcoRV-PstI fragment of TSHp clone in pBR322 (RP21) was subcloned to pBluescript II KS in our laboratory. LHp (Sp645LH#1) probe was obtained from Dr. James L. Roberts (22). GH (pRGH-1) and PRL [pPrl (pSP65)] probes were obtained from Dr. Beth Schaechter (23,24). For riboprobe synthesis, a PstI-KpnI fragment of GH clone in pBR322 (pRGH-1) was subcloned to pBluescript II KS in our laboratory. The riboprobes were synthesized from these cDNA probes with T7 RNA polymerase for FSHp and TSHp, T3 RNA polymerase for GH and SP6 RNA polymerase for POMC, LHp and PRL following reaction conditions specified by the vendor (Promega, Madison, WI) (labeled to ~1.5 X 10^8 cpm/μg input DNA for FSHp and TSHp, T3 RNA polymerase for GH and SP6 RNA polymerase for POMC, LHp and PRL, -8 X 10^7 cpm/μg input DNA for GH and LHp with ^32P CTP). Analyses for POMC, FSHp, TSHp, GH, and PRL mRNAs were carried out by slot blot. Preliminary Northern blot analyses (25) using our riboprobes revealed single discrete positive bands for these mRNAs. Because Northern blot for LHp mRNA revealed multiple bands, LHp message was studied by solution hybridization and electrophoresis of the hybridized sample on nondenaturing polyacrylamide gel.

**Slot blot analyses for POMC, FSHp, TSHp, GH, and PRL mRNAs.** — Duplicates of each anterior pituitary RNA sample (250 ng for POMC, GH, and PRL, 2.5 μg for FSHp and TSHp) were brought to 80 μl with DEPC-treated H$_2$O, diluted with 100 μl deionized formamide (BRL, Gaithersburg, MD), heated for 5 minutes at 65°C, chilled on ice, and diluted with 20 μl 20X SSC. An 80 μl aliquot from each duplicate was applied to GeneScreen (NEN, Boston, MA) presoaked in 20X SSC, using a Schleicher and Schuell (Keene, NH) slot blot minifold. To normalize values between blots for same hormone, aliquots from pooled rat pituitary RNA (25, 50, 100, 300, and 500 ng for POMC, 100 ng for GH and PRL, 1 μg for FSHp and TSHp) were applied in triplicate to each membrane. Liver RNA, which contains no detectable POMC, FSHp, TSHp, GH, and PRL mRNA, was added to the membranes as a negative control (100 ng and 500 ng for POMC, 100 ng for GH and PRL, 1 μg for FSHp and TSHp in duplicate).
2P-labeled riboprobes for POMC, FSHβ, TSHβ, GH, and PRL were synthesized as described in the previous section. Hybridization was performed as previously described (25). Signal quantitation was performed with a storage phosphor-imaging system (Molecular Dynamics, Sunnyvale, CA).

Solution hybridization for LH mRNA.—The solution hybridization assay was performed as described previously (26) except using 1 µg of anterior pituitary RNA. To normalize values between gels, pooled rat pituitary RNA (1 µg) was hybridized with 32P-labeled probe and loaded on each gel in triplicate. Liver RNA (1 µg), which contains no detectable LHβ, was also hybridized with 32P-labeled probe and loaded on a gel in duplicate as a negative control. Signal quantitation was performed with a storage phosphor-imaging system.

Polyadenylated RNA.—The blots used for probing TSHβ were used for polyadenylated (polyA) RNA measurement after the TSHβ signals had decayed. Quantitation of polyA in anterior pituitary RNA provided a determination of whether there were overall changes in steady-state levels of mRNA. Radiolabeled oligo-dT was synthesized using oligo-dT12-18 (Collaborative Research, Bedford, MA), terminal deoxynucleotide transferase (BRL), and radioisotope 32PdTTP (New England Nuclear, Boston, MA). This probe was prepared to a specific activity of 3.3 × 1010 cpm/µg input of oligo-dT12-18. The filters were prehybridized at 42°C overnight in a solution of 50% formamide, 750 mM NaCl, 20 mM tris (pH 7.5), 500 µg/ml salmon sperm DNA, 5X Denhardt (1X Denhardt = 200µg/ml each of BSA, polyvinyl pyrrolidone, and Ficoll), 200 µg/ml yeast RNA, .1% sodium dodecyl sulfate (SDS), and .05% sodium pyrophosphate. The filters were hybridized with oligo-dT at 37°C overnight in a buffer of 6X SSC, 20 mM sodium phosphate (pH 6.8), .1 mg/ml salmon sperm DNA, 1X Denhardt, .1 mg/ml yeast RNA, .1% SDS, .2% sodium pyrophosphate, and 10% dextran sulfate. The filters were then washed at room temperature for 20 min in 2X SSC wash buffer (2X SSC, .1% SDS, .02 M sodium phosphate, and .05% sodium pyrophosphate) and for 1 hr at 42°C in 0.2X SSC wash buffer (0.2X SSC, .1% SDS, .05% sodium pyrophosphate, and .02 M sodium phosphate). Signal quantitation was carried out with a storage phosphor-imaging system.

Statistical analysis and normalization of data.—Data are expressed as means and SEM, and were analyzed by two-way (dietary treatment and time of sampling) analysis of variance (ANOVA) (27). The Box-Cox transformation (28) was used to meet the assumption of normality of ANOVA. Differences among feeding conditions and sampling times were evaluated by the t test (30). Differences with p < .05 were considered significant. Power analysis was provided for each factorial design with a significance level of α = .05 and 80% power (31). Power analysis revealed that our experimental design enabled detection of 25–49% differences in most dependent variables for main effects of diet, time, and their interaction. The only exceptions were for total TSHβ, GH, and PRL mRNAs per anterior pituitary, in which we were able to detect differences less than 10%.

How to normalize data in comparisons between food-restricted and ad libitum–fed animals depends on the question being asked. In this article, mRNA levels are expressed in two ways: (a) total amount in the pituitary and (b) normalized to µg of total RNA. The former measure provides an index of the effect of FR on the total pool of substrate available to the animal for translation. The latter provides a measure of specificity of the effect of FR on a given mRNA species beyond any general effect of FR, because polyA RNA levels normalized to total RNA were unaffected by FR (see Figure 1). Thus, any differences between FR and AL animals in levels of mRNA/µg total RNA reflect effects not generally observed across mRNA species.

Results

Polyadenylated RNA.—Anterior pituitary (AP) polyadenylated (polyA) RNA was measured to determine the influence of food restriction on overall mRNA levels. When expressed per µg of RNA, the polyA RNA of AL and FR rats did not differ in the early morning (p = .30, t test) or afternoon (p = .37, t test) (Figure 1A). However, the total amount of RNA in the AP of FR rats was about half that of AL rats (diet main effect, p = .0001, Figure 1B). Thus, the AP content of polyA RNA was significantly reduced in FR rats (diet main effect, p = .0001, Figure 1C).

POMC RNA.—Figure 2 shows the effect of FR on the levels of POMC mRNA in the AP. The data in Figure 2 were published previously, but are presented because they enable a direct comparison to the mRNAs encoding the other pituitary hormones, which were measured from the same animals. POMC mRNA levels in a separate cohort of FR and AL rats confirmed the results shown in Figure 2 (data not shown). When expressed per µg of RNA, POMC mRNA was significantly increased in FR rats (diet main effect, p = .0015, Figure 2A; diet main effect, p = .014, with the separate cohort). The increases were about 80% in the morning and 40% in the afternoon (Figure 2A). However, because the total amount of RNA in the AP of FR rats was about half (Figure 1B) that of AL rats, the AP content of POMC mRNA of AL and FR rats did not differ (Figure 2B).

LHβ and FSHβ RNA.—Figure 3 shows the effect of FR on the levels of the gonadotropic hormone β subunit mRNAs. Expressed per µg of RNA, LHβ mRNA was significantly increased (~15–20%) in FR rats (diet main effect, p = .0078, Figure 3A). Time of day had no effect on LHβ mRNA expression (time main effect, p = .46). However, in contrast to POMC mRNA, the AP content of LHβ mRNA was significantly reduced in FR rats (diet main effect, p = .0016, Figure 3B). Figure 3C shows the level of FSHβ mRNA per µg of AP RNA. Although there were no significant differences between the two dietary groups (diet main effect, p = .33) and between the morning and afternoon values (time main effect, p = .15), there was a significant interaction effect (p = .036). In the morning, but not in the afternoon, FSHβ mRNA/µg RNA in the FR group was lower.
Figure 1. Effect of food restriction on polyadenylated (polyA) RNA in the anterior pituitary. Within each panel, bars with differing alphabetical letters are significantly different (p < 0.05 by t test) (n = 9-11 per group). (A) polyA RNA per μg anterior pituitary RNA; (B) total anterior pituitary RNA; (C) total polyA content in the anterior pituitary.

Figure 2. Effect of food restriction on POMC mRNA in the anterior pituitary. Within each panel, bars with differing alphabetical letters are significantly different (p < 0.05 by t test) (n = 9-11 per group). (A) POMC mRNA per μg anterior pituitary RNA; (B) total POMC mRNA content in the anterior pituitary.

than that of the AL group (p = .0058, t test). Also, in contrast to AL rats, morning FSHβ mRNA levels in FR rats were less than afternoon values (p = .017, t test). FSHβ mRNA content was markedly reduced in FR rats compared to AL rats (diet main effect, p = .0001, Figure 3D). Thus, FR had different effects on the two gonadotropic hormone β subunit mRNAs. A major difference in the effect of FR on the gonadotropic subunit mRNAs from its effect on POMC mRNA was the marked reductions in total AP content of both LHβ and FSHβ mRNAs in FR animals.

TSHβ, GH, and PRL RNA.—Figure 4 shows the effect of FR on the levels of TSHβ mRNA, GH mRNA, and PRL mRNA in the AP. Figure 4A shows the level of TSHβ mRNA per μg of AP RNA, which differed significantly between the two dietary groups (diet main effect, p = .0032). In addition, there was a significant interaction effect of diet group and time of day on this variable (p = .040) although there was no time main effect (p = .17). This interaction effect was reflected in a lack of dietary effect on TSHβ mRNA/μg RNA in the morning but a significant reduction of this variable in FR rats in the afternoon (p = .0045, t test). The AP content of TSHβ mRNA was markedly reduced in FR rats (diet main effect, p = .0001).
DISCUSSION

This study demonstrates that 6 weeks of food restriction in young rats has marked and differential effects on steady state levels of the mRNAs encoding the major tropic hormones in the anterior pituitary gland. Whether all of these changes persist throughout the life span of FR rats remains to be determined. However, these changes are of potential importance to the initiation as well as maintenance of the altered state of the FR animal and to the anti-aging mechanisms of FR, because even short periods of FR can reduce reproductive activity (4), enhance cytoprotective actions (7-9), and prolong life span (2). Rats that have been restricted for 6 weeks exhibit altered metabolic and hormonal characteristics that persist in many cases into old age. For example, blood glucose and insulin levels are lowered by 3 months and remain lowered throughout the life span (3). Plasma concentrations of triiodothyronine are similarly lowered throughout the life span of FR rats (11). Diurnal levels of plasma total corticosterone levels are elevated by FR in young and middle-aged rats, but not in older rats (12), although plasma free corticosterone levels appear to be maintained at an elevated level by FR throughout the life span (12). Moreover, enhanced cytoprotective mechanisms are seen in young FR animals (7-9), suggesting that the altered physiological status of FR that may contribute to the anti-aging effects of FR is present in young FR animals. Thus, the changes that have been observed in this study may be important to the altered metabolic and cytoprotective state that contributes to the extended life span of FR animals. Changes that are sustained throughout the life span of FR rats
are most likely to play important roles in the anti-aging actions of FR, however, and it will ultimately be important to assess which of the changes observed in the present study are sustained into old age by FR.

The effect of FR on POMC mRNA was unique. POMC mRNA levels are regulated by FR in a markedly different way in comparison to overall polyA or total RNA levels. POMC mRNA content was not downregulated by FR,
whereas mRNA contents of all the other hormones were reduced 40–80% by FR. POMC mRNA levels, whether expressed as total pituitary content or normalized to RNA, were maintained by FR at higher levels relative to those of the other tropic hormone mRNAs. It is debatable whether of the two ways of normalizing the data—as content or per µg of RNA—is biologically more meaningful. Regardless of the normalization mode, POMC mRNA levels were uniquely affected by FR. Although LHβ mRNA, when expressed per µg RNA, was also elevated by FR, its elevation was modest. POMC mRNA was elevated two- to fourfold more than was LHβ mRNA.

Why is POMC mRNA uniquely affected by FR? One possibility is that there is a relationship between POMC and the hyperadrenocortical status of the FR animal (15). The diurnal peak of plasma corticosterone is elevated by FR in rats (12) and mice (9), whereas all of the other major hormones appear to be reduced by FR (10,11,32). One might postulate, therefore, that adrenocorticotropic hormone (ACTH), which stimulates corticosterone release, would be elevated by FR and, accordingly, so would its precursor mRNA, POMC. However, our earlier observation that diurnal plasma ACTH concentrations are not increased by FR (15) indicates that the relationship between POMC mRNA and ACTH secretion in FR rats is not straightforward. There appears to be drive to stimulate POMC mRNA levels in the FR animal which is blocked distally (i.e., at the level of translation or secretion) such that circulating levels of ACTH are not concomitantly elevated. It is tempting to speculate that the unique elevation of POMC mRNA provides an enhanced substrate reserve to the FR animal that enables greater production of glucocorticoid-stimulating ACTH, in the event that an unusual stressor may present itself to the FR animal.

Although there are well-established circadian rhythms in circulating ACTH concentrations and evidence for a circadian rhythm in corticotropin releasing hormone (CRH), precursor of ACTH, mRNA in paraventricular nucleus (33,34), we did not observe any circadian rhythm in anterior pituitary POMC mRNA in either AL or FR rats in the present measurement and in another repeated measurement with six timepoints across the 24-hour period (unpublished data). There is a possibility that the strain difference may account for the discrepant results. Kwak et al. (33) and Cai and Wise (34) used male and female Sprague-Dawley rats, respectively, whereas we used male Fischer 344 rats.

Total contents of the mRNAs of all the other tropic hormones were reduced 50% or more by FR, except for LHβ, whose content was only reduced 25–30% by FR. With the exception of LHβ, the mRNA levels of these hormones normalized to total RNA were also reduced at one or both of the sampling times. These results indicate that FR suppresses steady-state levels of these mRNAs at one or both sampling times beyond the overall suppression of total RNA content. Whether this repression involves transcription, RNA processing or degradation remains to be determined. These findings, however, are consistent with previous reports of FR effects on circulating levels of the cognate hormones. For example, blood levels of TSH and GH have been reported to be repressed by chronic FR (10). Sonntag et al. (35) suggested that acute and chronic restriction differ in their effects on GH; FR decreases GH pulse amplitude early in life but chronic FR increases GH pulse amplitude. In a study involving short-term FR, TSH levels were unaffected (32). Although the linkages between mRNA level, intercellular synthesis, and secretion of peptide in the circulation do not necessarily correlate and can be affected by multiple intervening processes, these results provide a plausible explanation for the reduction in circulating levels of TSH in the chronically food-restricted rat. Bronson and Hiedeman (32) reported that the blood levels of luteinizing hormone, GH, and prolactin were suppressed by short-term FR. In their study, circulating levels of follicle-stimulating hormone and TSH were unaffected by short-term FR. Other studies (12,36,37), however, reported reduced thyroid hormone levels in chronically FR rats.

In summary, POMC mRNA content in the anterior pituitary was uniquely maintained from the general FR-induced reduction of anterior pituitary RNA. All of the other pituitary tropic hormones showed evidence of selective regulation by FR beyond the general suppressive effect of FR on RNA levels in the pituitary. These results provide new evidence that the neuroendocrine status of the FR rat is markedly different from the ad libitum state, and provide new probes for ascertaining how FR exerts its anti-aging and other actions.

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Address correspondence to Dr. Eun-Soo Han, Department of Physiology, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284-7756. E-mail: han@uthscsa.edu

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