

Effects of Every-Other-Day Feeding on Prolactin Regulatory Mechanism in Transgenic Human Growth Hormone Mice

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Transgenic mice overexpressing human growth hormone (hGH) exhibit accelerated aging with functional hyperprolactinemia and greatly depressed endogenous prolactin. Calorie restriction (CR) is widely recognized as the most effective experimental intervention to delay aging. The aim of the present work was to analyze the effects of lifelong overexpression of hGH on prolactin-gene expression as well as the dopamine production at the pituitary level and discern whether this mechanism changes as a function of feeding patterns. Ten-month-old mice fed every other day (EOD) were killed after one day of fasting. The results confirmed typical phenotypic features of these transgenic mice: an increase in body weight, very high hGH plasma concentrations, and hyperinsulinemia. There was a marked inhibition of the expression of the prolactin gene, together with an increased tyrosine hydroxylase (TH) and the long isoform of dopamine receptor type 2 (D2LR) gene expression at the pituitary level. These parameters were not affected by the EOD feeding pattern. These data may suggest an autocrine or paracrine effect of dopamine at the hypophyseal level on prolactin secretion that is independent of the feeding pattern. *Exp Biol Med* 233:434–438, 2008

Key words: growth hormone; prolactin; every-other-day feeding; tyrosine hydroxylase; dopamine receptor type 2

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Introduction

Transgenic mice (Tg) bearing the human growth hormone (hGH) gene with the mouse metallothionein-I promoter (MT) (MT-hGH Tg mice) express the foreign growth hormone (GH) in multiple organs including the liver, kidney, heart, lung, spleen, intestine, skin, gonads, and brain, with expression starting during fetal development and continuing throughout the animal's entire life span (1). The foreign GH genes fused to an MT promoter are not subject to the control mechanisms that normally regulate the synthesis and release of GH from the pituitary gland, because the MT promoter is regulated by heavy metals (1) rather than the hypophysiotropic peptides and/or catecholamines.

In rodents, hGH binds to both GH and prolactin receptors, exerting both somatotrophic and lactotrophic effects (2–5). As a result, a state of functional hyperprolactinemia is observed in Tg mice (1, 6). The excess of prolactin stimulus from birth results in a larger population of prolactin-inhibiting tuberoinfundibular dopamine (TIDA) hypothalamic neurons, demonstrated by immunocytochemical detection (ICC) for the catecholamine synthetic enzyme tyrosine hydroxylase (TH) and in a qualitative enhancement of dopamine expression measured by induced fluorescence (7, 8). These results reflect an increase in dopamine turnover in hypothalamic neurons (8), but this parameter is not altered at the median eminence level (9), although a reduction in prolactin secretion has been detected (6, 10). These findings may suggest that the difference in plasma prolactin levels between normal and Tg mice may be mediated through changes in prolactin-regulating factors other than hypothalamic dopamine (9) or by changes in the regulatory mechanism at the pituitary level.

Tg mice are, like other mice overexpressing GH, short-lived (11–13). Calorie restriction (CR) has been proven to extend life span in mice (14–17). Moreover, CR or changes in feeding patterns have been related to modifications in the

Table 1. Sequence of Primers for Gene Expression Used in Real-Time PCR Reactions

Gene	Genbank accession #	Forward primers	Reverse primers
PRL ^a	NM_011164	(+291) 5'-CAAGGTCATCAATGACTGCC	CTGAGGATCAGGTTTCAGAAG-3'(-400)
TH ^b	NM_009377	(+822) 5'-CTGGAGGATGTGTCTCACTT	GTGCACTGAAACACACGGAA-3'(-940)
D2LR ^c	NM_010077	(+611) 5'-TCCTGTCCTTACCACATCTCT	ACGATGAAGGGCACGTAGAA-3'(-740)

^a PRL, prolactin.

^b TH, tyrosine hydroxylase.

^c D2LR, dopamine D2 receptor, long isoform.

activity of the hypothalamic pituitary axis (18, 19) and can cause the induction of several different neurotrophic-factor gene expression in the brain (19, 20), as well as changes in the circadian patterns of prolactin, GH, or their hypothalamic neuromodulators (21). The neuroendocrine mechanisms associated with feeding patterns may explain the effects of fasting on the aging process (14–17).

The references cited above led us to study the effects of lifelong overexpression of hGH on prolactin gene expression as well as dopamine production at the pituitary level and investigate whether this mechanism changes as a function of an every-other-day (EOD) feeding pattern.

Materials and Methods

Animals and Tissue Preparation. Tg male mice were originally produced by fusing the hGH structural gene with MT promoter and injecting the solution of hybrid gene into male pronuclei in the laboratory of Dr. T. E. Wagner (22). The animals were produced in our breeding colony derived from mice he kindly provided. The male mice evaluated in this study were produced by mating hemizygous Tg males with normal C57BL/6 × C3H F₁ hybrid females and were housed under conditions of controlled illumination (12:12-hr light:dark cycle) and temperature (22 ± 2°C). At the age of 4–6 months, the wild-type (WT) and Tg mice were divided into two groups: a group fed *ad libitum* (Lab Diet Formula 5008, Purina Mills Inc., St. Louis, MO) (WT-AL and Tg-AL) and a group fed every other day (WT-EOD and Tg-EOD), which had *ad libitum* access to food for 24 hours and no food on alternate days. All groups had constant access to water.

At the age of 10 months, Tg and WT animals were fasted for 24 hrs, anesthetized with isoflurane and bled by cardiac puncture between 0800 hrs and 1000 hrs. To minimize the effects of stress, the time between removing the animal from the cage and bleeding was 1 min. After collection of the blood sample, the animals were killed by decapitation; the brains were rapidly removed to access the pituitary gland, which was quickly frozen on dry ice and stored at –80°C until processed. The Laboratory Animal Care and Use Committee at SIU School of Medicine approved the protocols for maintenance and killing of the animals.

Plasma Chemical Analyses. Insulin levels were determined using Ultra Sensitive Rat Insulin ELISA Kits (Crystal Chem Inc., Downers Grove, IL) and plasma hGH

levels were measured by the Immunoassay hGH-ELISA kits (BioSource International, Inc. Camarillo, CA).

Total RNA Extraction and Complementary DNA (cDNA) Synthesis. Total RNA was extracted from the pituitary by the guanidinium thiocyanate-phenol chloroform method (23) (BioRad, Hercules, CA). RNA concentrations were measured spectrophotometrically at 260 nm. Three micrograms of total RNA were electrophoresed on a 1.5% agarose gel to confirm RNA integrity. Potentially contaminating residual genomic DNA was eliminated using DNase I (Promega, Madison, WI). The cDNA was made using iScript cDNA Synthesis Kit (BioRad, Hercules, CA) as instructed by the manufacturer.

Real-Time PCR. The real-time polymerase chain reaction (RT-PCR) amplification was carried out with the iQTM SYBR Green PCR Supermix (BioRad, Hercules, CA) using the SmartCycler (Cepheid, Sunnyvale, CA). The primers used are listed in Table 1. The primer used in this work, for the dopamine receptor type 2, was for the long isoform (D2LR) of this receptor.

The RT-PCR reaction program included a 94°C denaturation step for 2 mins followed by 45 cycles of 95°C denaturation for 15 secs, 62°C annealing for 30 secs, and 72°C extension for 30 secs. Detection of fluorescent product was carried out at the end of the 72°C extension period. Melting curve and agarose gel electrophoresis were used to confirm PCR products. Data were analyzed and quantified using Cepheid SmartCycler Software 7.

The reference gene used for normalization was β-2-microglobulin (B2m) (24). Relative mRNA expression was calculated using the threshold cycle numbers (Ct), represented by the equation:

$$2^{A-B}/2^{C-D}$$

A: ¼ Cycle Threshold (Ct) number

B: ¼ Ct number for the gene of interest in the analyzed sample

C: ¼ Ct number for the housekeeping gene in the first control sample

D: ¼ Ct number for housekeeping gene in the analyzed sample

Statistical Analysis. Multiple comparisons were performed with the program SPSS (SPSS Inc., Chicago, IL) using two-way analysis of variance (ANOVA) followed

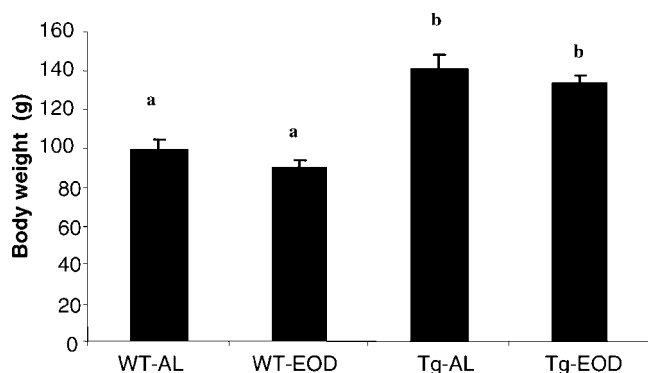


Figure 1. Body weight of control (WT) and transgenic mice (Tg) fed *ad libitum* (AL) or subjected to every-other-day (EOD) feeding. Different superscripts denote significant difference at $P < 0.05$. Seven to ten animals per group were analyzed.

by a post hoc Tukey's test. Comparisons between two study groups were performed with Student's t test for independent samples. Confidence levels for statistical significance were set at $P < 0.05$. The results are presented as the mean $\pm$ standard error of the mean (SEM).

Results

Effects of hGH Overexpression and EOD Feeding Pattern on Body Weight and Blood Parameters.

Consistent with hGH overexpression, the body weights of Tg mice were significantly greater than in their WT littermates ($F = 71.47$, $P < 1 \times 10^{-9}$; Fig. 1). Body weight was not significantly affected by EOD, although there was a tendency for reduced weight in both phenotypes under this feeding regimen.

As expected, measurements of plasma hGH show significantly higher values in Tg as compared to normal mice ($F = 17.45$, $P < 4 \times 10^{-4}$). EOD fasting did not affect plasma hGH levels in either normal or GH-Tg mice (Fig. 2A).

As expected, there was also a significant increase in plasma insulin concentration in Tg as compared to WT mice ($F = 8.96$, $P = 0.015$; Fig. 2B). However, WT-EOD mice showed lower plasma insulin concentration than WT-AL mice ($F = 11.018$, $P = 0.007$). In contrast, in Tg mice the result was opposite, i.e., EOD feeding further increased plasma levels of insulin ($F = 8.5$, $P = 0.019$; Fig. 2B), as compared with the Tg mice fed *ad libitum* ($F = 107.5$, $P = 1 \times 10^{-6}$).

Effects of hGH Overexpression and EOD Feeding on Pituitary Prolactin and TH Gene Expression.

The pituitary prolactin gene expression was markedly decreased in Tg-AL mice as compared to the values measured in their WT littermates ($F = 33.24$, $P = 4 \times 10^{-6}$; Fig. 3A). EOD feeding did not influence the prolactin gene expression in either WT or Tg mice.

TH gene expression in the pituitary was barely detectable in WT male mice and was greatly increased in

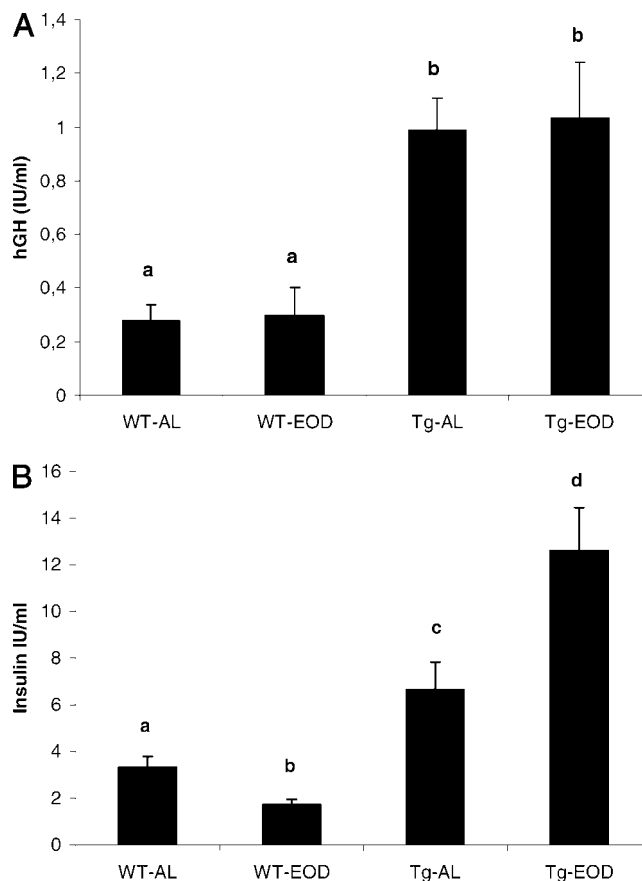


Figure 2. Plasma concentration of hGH (A) and insulin (B) from WT and Tg male mice, as assayed by ELISA ($n = 7-10$ per group). Group values are expressed as the mean $\pm$ SEM, values marked with a different superscripts (a and b) denote significant differences at $P < 0.05$.

Tg mice ($F = 98.27$, $P = 1 \times 10^{-8}$; Fig. 3B). EOD feeding did not influence TH gene expression in either genotype.

Effects of EOD Feeding on Dopamine Receptor Type 2 (D2LR) Gene Expression in Tg Male Mice.

Pituitary expression of D2LR gene was significantly higher in Tg than in WT mice ($F = 8.877$, $P = 0.006$) and was not affected by the feeding regimen (EOD vs. AL) in either genotype (Fig. 3C).

The main novel finding of the present study is that unregulated hGH expression in adult male Tg mice enhanced pituitary expression of the TH and the D2LR genes while reducing prolactin gene expression. These data suggest that paracrine effects of locally produced dopamine are likely to contribute to the inhibition of prolactin biosynthesis and secretion in these animals.

Although direct hGH effects cannot be ruled out in mice, hGH binds not only to GH receptors but also to the prolactin receptors, thus inducing a functional hyperprolactinemic state (3, 4, 6, 25, 27), although endogenous (mouse) prolactin levels are suppressed. The low expression of the prolactin gene detected in Tg animals in the present study may also be related to the lower number of lactotrophs

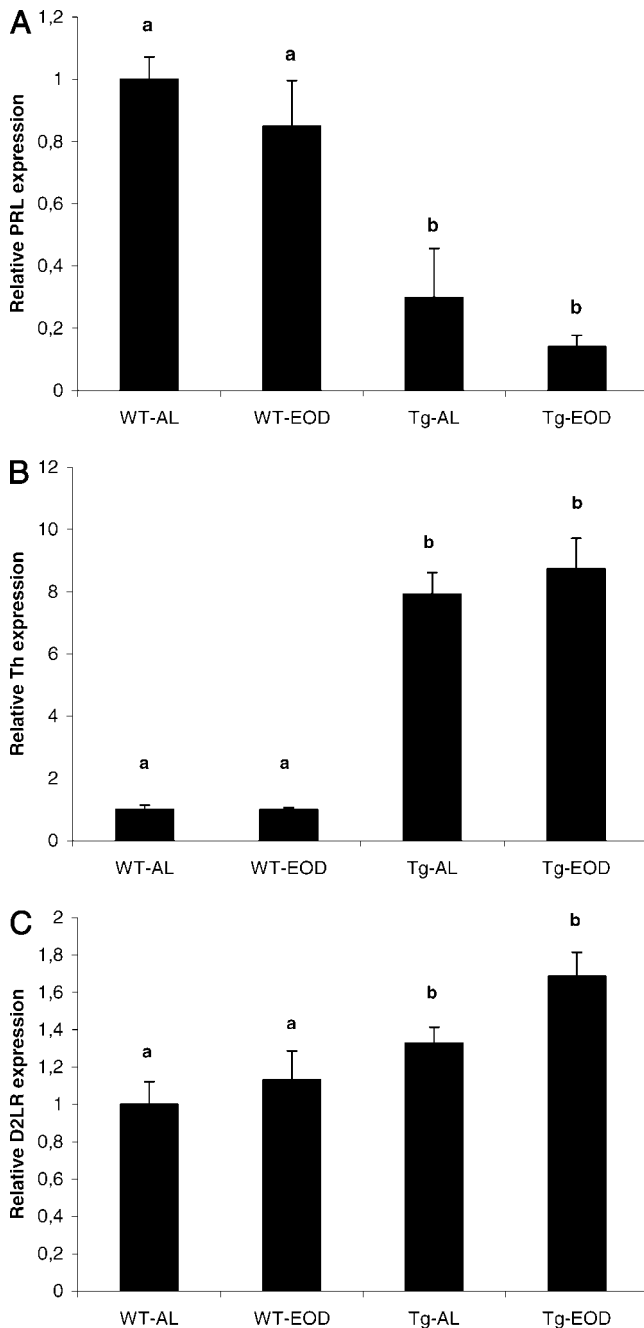


Figure 3. Expression of prolactin (PRL) (A), TH (B), and D2LR (C) genes in the pituitary gland of control (WT) and transgenic (Tg) mice subjected to an EOD feeding pattern or *ad libitum* (AL) feeding. The data from real-time PCR were normalized by the housekeeping gene β -2-microglobulin (B2M) and expressed as mean $\pm$ SEM. Different superscripts denote significant difference at $P < 0.05$. Seven to ten animals per group were analyzed.

previously reported (10) and likely accounts for the low plasma prolactin levels observed in Tg mice from the same line (3, 9, 26, 27).

Dopamine, synthesized primarily at the arcuate and periventricular nuclei, provides the main regulatory input for prolactin gene expression at the pituitary level, thus regulating the synthesis (28) and release of prolactin from

the pituitary gland (29, 30). Dopamine acts on the lactotrophs through its association to its D2-type receptors (28). Whether dopamine is synthesized in the anterior pituitary is a matter of controversy. The results of the present study indicate that TH gene expression is almost undetectable in the pituitary gland of normal mice and thus, almost all of the dopamine present in the pituitary must be derived from the tuberoinfundibular system via median eminence as was documented previously (28). However, significant expression of this enzyme was detected in Tg mice, indicating that dopamine was also synthesized in the pituitary gland, likely as a response of the hyperprolactinemic state induced by the high plasma levels of hGH in these animals.

In the present study, pituitary expression of the D2LR receptor gene was greater in Tg as compared to the WT mice. These results support the findings obtained using the *in situ* hybridization technique (31) for the TG mice. It is interesting to note that there was an increased expression of both TH and D2LR genes in the pituitary of Tg mice, thus indicating a modification of the mechanisms that regulate prolactin secretion at the pituitary level in the presence of high levels of circulating hGH.

The EOD feeding pattern did not alter the expression of any of the studied genes in WT animals. This may have been due to the relatively mild reduction of the average daily calorie intake in EOD animals in the present study. However, the Tg animals were apparently more sensitive to this feeding regimen, as both TH and D2LR gene expression in Tg, but not in WT mice showed a non-significant tendency to be higher in the EOD than in the control group. Previous studies in rats have shown that changes in the feeding pattern can alter plasma prolactin levels (32, 33). Interestingly, plasma ACTH levels increase with variations in the feeding pattern so that the metabolic stress is activating the adrenal gland (33–35). The chronically increased corticosterone levels in GH Tg mice (36, 37) may be responsible, at least in part, for the increases in TH gene expression, in agreement with earlier reports (28, 38).

In summary, we report that in the presence of chronic excess of a lactogenic GH, adenohypophyseal expression of the TH and the D2R gene is enhanced, suggesting a local paracrine mechanism of suppression of endogenous prolactin release.

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