The effect of GH on estrogen receptor expression in the rat mammary gland

M Feldman, W Ruan, I Tappin, R Wieczorek and D L Kleinberg

Department of Veterans Affairs Medical Center, and Department of Medicine, New York University Medical Center, New York, New York 10010, USA

(Requests for offprints should be addressed to D L Kleinberg, DVA Medical Center, Room 16043W, 423 E 23 Street, New York, New York 10010, USA; Email: kleind02@popmail.med.nyu.edu)

Abstract

Both GH and insulin-like growth factor I (IGF-I) synergize with estrogen to induce normal mammary gland development. However, the nature of this synergy has not been explored. To gain insight into the mechanism of these interactions we have examined the effects of these substances on the estrogen receptor (ER). ER levels in the mammary gland cytosols from hypophysectomized and oophorectomized rats, were measured using two assay systems: a dextran-coated charcoal procedure to measure binding to radiolabeled steroid, and an immunologic assay employing a specific antibody to the receptor. In both assays, levels of ER were at or near baseline detection (1–2 ng/mg protein). Treating animals with either bovine or human GH significantly increased ER activity (P<0·001), whereas prolactin (PRL) and/or estradiol treatment had no effect. That this increase was at the level of transcription was demonstrated by reverse transcriptase/polymerase chain reaction. Following a single injection of GH (50 µg), a substantial increase in ER mRNA was observed by 10 h, with levels returning to baseline within 24 h; a concomitant increase in ER itself was also observed at the 10 h time point. The effect of GH appeared to occur mainly in the mammary stroma, because there were no differences in GH stimulation of ER between gland-free and gland containing mammary fat pads. Furthermore, analysis of mammary gland ER by immunocytochemistry demonstrated that while ER was present in the epithelial cells of non-treated animals, only GH treated animals had ER clearly visible in both glandular and fat cells of the tissue. In contrast, treating animals with des(1–3)-IGF-I did not result in reproducible increases in ER, nor in the staining of fat cell nuclei for ER. These data demonstrate a specific GH effect on the ER in the mammary fat cell.


Introduction

The development of the rodent mammary gland is dependent on both steroid and pituitary hormones (Lyons et al. 1958, Nandi 1958, Kleinberg et al. 1990, Lyons 1993). We have shown that growth hormone (GH) is the pituitary factor essential for mammary development (Kleinberg et al. 1990, Feldman et al. 1993), and that the effect of GH is likely mediated by local production of insulin-like growth factor (IGF)-I (Ruan et al. 1992, 1995). Recent evidence indicates that GH exerts its effect within the stromal compartment of the mammary gland, suggesting that a paracrine event causes induction of terminal end buds (TEBs), and subsequent pubertal mammary development (Walden et al. 1998).

Estrogen action depends on the presence of the estrogen receptor (ER). Although ER is clearly present in many mammary gland tumors (Osborne et al. 1980, McClelland et al. 1986, Moncharmont et al. 1991), the levels in normal mammary tissue are often quite low, with only a small percentage of cells expressing ER (Petersen et al. 1987, Ricketts et al. 1991). The regulation of ER activity in the rodent mammary gland is still not well understood, although variations due to hormonal influences (Yu & Leung 1982, Kelly et al. 1983) and diet (Hilakivi-Clarke et al. 1998) have been reported. In this study we set out to determine if the synergy between GH and/or IGF-I and estradiol (E2) might have to do with changes in ER.

Materials and Methods

Animals

Female Sprague–Dawley rats, which were hypophysectomized and oophorectomized at 21 days of age, were obtained from Charles River (Wilmington, MA, USA). After surgeries, animals were kept between 2 and 7 weeks before being used in experiments. In any given experiment, the animals used were all from the same grouping (i.e. same age and date of surgery). The animals were treated with either recombinant human GH (rhGH), des(1–3)-insulin-like growth factor I (des(1–3)-IGF-I ) (both gifts from Genentech, South San Francisco, CA, USA), recombinant bovine GH (rbGH) (a gift from
Monsanto, St Louis, MO, USA), or human prolactin (hPRL) (obtained from the National Hormone and Pituitary Program, NIDDK, Baltimore, MD, USA), by implantation in Alzet minipumps (200 µg/pump). The minipumps (model #2001) release 1 µl/h for periods of up to 7 days.

Some animals also received simultaneous administration of E2 (Sigma Chemical Co., St Louis, MO, USA), in silastic capsules implanted subcutaneously as described previously (Newman et al. 1987). These studies were approved by the institutional committee on animal care, and animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Preparation of mammary gland cytosol

After 1 or 5 days of hormonal treatment, rats were killed, and the lumbar mammary glands removed. One gland from each animal was homogenized in 1:5 ml TED buffer (0.01 M Tris base, 0.0015 M EDTA, 0.0005 M dithiothreitol, 0.01 M sodium molybdate and 10% glycerol, adjusted to pH 7.4 at 4 °C). The homogenate was spun at 100,000 × g to obtain a particulate-free cytosol fraction. The protein concentration of the cytosol was determined using the Biorad dye reagent as per the directions of the manufacturer.

Isolation of mammary gland RNA

Total RNA was isolated essentially as described by Chomczynski & Sacchi (1987). Individual mammary glands were homogenized in 1 ml 4 M guanidine thiocyanate containing 25 mM citrate (pH 7), 0.1 M 2-mercaptoethanol and 0.5% sarcosyl (GIT buffer). The preparations were acidified by adding 0.1 ml 2 M sodium acetate (pH 4), and the homogenates centrifuged at 1000 × g to remove a large layer of fat. The soluble material was mixed with 1 ml water-saturated phenol and 0.3 ml chloroform–isoamylalcohol (49:1), and separated into two phases by centrifugation. The aqueous phase was collected and mixed with an equal volume of isopropanol. After incubation overnight at −20 °C, RNA was pelleted by centrifugation at full speed in a microfuge, and resolubilized in GIT buffer. An equal volume of isopropanol was added, and after overnight incubation at −20 °C, the RNA was again centrifuged. The pelleted RNA was solubilized in water, and kept at −40 °C until needed.

Reverse transcriptase-polymerase chain reaction (RT-PCR) of ER mRNA

For the RT phase of the reaction, 2 µg total RNA from individual mammary gland samples was incubated with oligo (dT)12-18 and Superscript II (GibcoBRL, Grand Island, NY, USA) using the directions specified by the manufacturer for synthesis of cDNA. ER primers used for PCR were: 5' GCT CCT AAC TTG TCT TTG GAC A3' and 5' ATC TCC AGC AGC AGG TCA TAG A 3'. These primers encompass a 415 bp fragment in the E2 binding domain of ER (Steinmetz et al. 1997). Additional reactions were run using primers for ribosomal protein L19, to serve as a control: 5' AGT ATG CTT AGG CTA CAG AAG 3' and 5' TTC CTT GGT CTT AGA CCT GCG 3'. These primers encompass a 500 bp fragment of L19.

The PCR was for 40 cycles (30 s at 94 °C, 30 s at 57 °C, 30 s at 72 °C) using TAQ DNA polymerase (Gibco-BRL) according to directions supplied by the manufacturer. Following PCR, aliquots of the products were run on 1% agarose gels containing ethidium bromide, and the gels photographed.

Immunocytochemistry

Mammary glands were removed from animals and immediately fixed in phosphate-buffered formalin for approximately 24 h. Blocks of tissue were embedded in paraffin wax using a standard laboratory processor, and 4 micron thick sections cut and placed on positively charged slides. After dewaxing in xylene, and passing through 100 and 70% ethanol, endogenous peroxidase was blocked by treating slides for 15 min with H2O2 (final concentration

Analysis of ER

ER was measured by either direct binding or immunoassay. For direct binding, the cytosol preparations were incubated with a fixed saturating concentration (0.5 nM) of 3H-estradiol, in the presence or absence of 10−6 M diethylstilbestrol. Incubation was at 4 °C for 22 h. All preparations were analyzed in triplicate. After incubation, the cytosols were treated with five volumes suspension of 0.25% activated charcoal, 0.025% dextran in TED buffer (DCC), and 15 min later the preparations centrifuged at 1000 × g for 10 min. Aliquots of the supernatants were counted in a scintillation counter, and the difference in radioactivity between the tubes without and with the diethylstilbestrol competitor used as a measure of specific binding of estradiol. Immunoassay of ER was done using the Abbot ER–EIA kit, which employs a double monoclonal antibody sandwich procedure; detailed methodology is provided in a brochure accompanying the kit.

Analysis of ER

ER was measured by either direct binding or immunoassay. For direct binding, the cytosol preparations were incubated with a fixed saturating concentration (0.5 nM) of 3H-estradiol, in the presence or absence of 10−6 M diethylstilbestrol. Incubation was at 4 °C for 22 h. All preparations were analyzed in triplicate. After incubation, the cytosols were treated with five volumes suspension of 0.25% activated charcoal, 0.025% dextran in TED buffer (DCC), and 15 min later the preparations centrifuged at 1000 × g for 10 min. Aliquots of the supernatants were counted in a scintillation counter, and the difference in radioactivity between the tubes without and with the diethylstilbestrol competitor used as a measure of specific binding of estradiol. Immunoassay of ER was done using the Abbot ER–EIA kit, which employs a double monoclonal antibody sandwich procedure; detailed methodology is provided in a brochure accompanying the kit.

Analysis of ER

ER was measured by either direct binding or immunoassay. For direct binding, the cytosol preparations were incubated with a fixed saturating concentration (0.5 nM) of 3H-estradiol, in the presence or absence of 10−6 M diethylstilbestrol. Incubation was at 4 °C for 22 h. All preparations were analyzed in triplicate. After incubation, the cytosols were treated with five volumes suspension of 0.25% activated charcoal, 0.025% dextran in TED buffer (DCC), and 15 min later the preparations centrifuged at 1000 × g for 10 min. Aliquots of the supernatants were counted in a scintillation counter, and the difference in radioactivity between the tubes without and with the diethylstilbestrol competitor used as a measure of specific binding of estradiol. Immunoassay of ER was done using the Abbot ER–EIA kit, which employs a double monoclonal antibody sandwich procedure; detailed methodology is provided in a brochure accompanying the kit.
0.75% in ethanol). After washing the slides in water, heat-induced epitope retrieval, to aid in the visualization of ER, was performed using 0.01 M citrate buffer (pH 6.0) in a benchtop Napco autoclave at 125 °C for 4 min. After cooling, the slides were sequentially washed in phosphate-buffered saline (PBS), treated with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA), blocked with 100% donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) for 20 min and then incubated overnight (16–20 h) at 4 °C with a 1:20 dilution of a primary mouse monoclonal antibody against ER (clone #1D5; Dako, Carpinteria, CA, USA). The next day slides were washed twice in PBS and then incubated for 30 min with a 1:150 dilution of a biotinylated second antibody (donkey anti-mouse IgG; Jackson ImmunoResearch). Following two washes in PBS, slides were incubated with avidin-biotin-peroxidase conjugate (ABC vectastain reagent; Vector Laboratories) for 30 min according to the instructions of the manufacturer. The slides were then washed twice in PBS and stained with peroxidase substrate solution (0.5 mg/ml diaminobenzidine tetrahydrochloride in citrate–phosphate buffer containing 0.05% H2O2) for 4 min. Slides were counterstained with hematoxylin.

Results

Hormonal effects on ER levels in rat mammary glands

ER levels in mammary glands from hypophysectomized and oophorectomized rats treated for 5 days with hGH were significantly higher than in their non-treated counterparts (Fig. 1). ER levels in untreated animals were at or near the level of detection using two different assays; a functionality assay (DCC) measuring binding of 3H-estradiol, and an assay kit measuring immunologic activity (ER–EIA). GH treated animals had mammary gland ER levels varying between 5 and 15 fmol/mg protein (P<0.001 vs controls). Treating animals with PRL or E2 had no effect on the level of ER. Treating with des(1–3)-IGF-I did not stimulate ER activity in most cases (Fig. 1). However, in one experiment, des(1–3)-IGF-I increased ER levels over controls at the 5–day time point but not at 24 h (Fig. 2); after 5 days treatment with des(1–3)-IGF-I, ER levels increased from a mean value of 5.5 fmol/mg protein to 9.5 fmol/mg protein (P<0.01). These values were still significantly lower than those following GH treatment (mean ER = 25 fmol/mg protein; P<0.005). Interestingly, the levels of ER in control animals in this experiment were also higher than those we normally obtain.

Analysis of ER mRNA

ER mRNA was analyzed by RT-PCR as described in the Methods section. After 40 cycles of nucleic acid synthesis, only a faint ER signal was picked up relative to the L19 reporter gene (Fig. 3). This faint signal was present in both GH- and des(1–3)-IGF-I-treated animals. However, in one experiment, des(1–3)-IGF-I increased ER levels over controls at the 5-day time point but not at 24 h (Fig. 2); after 5 days treatment with des(1–3)-IGF-I, ER levels increased from a mean value of 5.5 fmol/mg protein to 9.5 fmol/mg protein (P<0.01). These values were still significantly lower than those following GH treatment (mean ER = 25 fmol/mg protein; P<0.005). Interestingly, the levels of ER in control animals in this experiment were also higher than those we normally obtain.

Figure 1 ER levels in mammary gland cytosols. Hypophysectomized and oophorectomized rats were treated with hormones for 5 days, and ER measured in the lumbar mammary glands as described in Materials and Methods. (■), DCC assay; (□), EIA assay. Each point represents a separate animal.

Figure 2 Effect of hGH and des(1–3)-IGF-I on ER levels in mammary gland cytosols. Hypophysectomized and oophorectomized rats were treated for either 1 or 5 days with hGH or des(1–3)-IGF-I as described in Materials and Methods. ER was measured in the lumbar mammary glands by DCC assay. Values are mean ± S.E.M. (n=6). *P<0.01, compared with control; **P<0.005, compared with control. Statistical analysis was by Student’s t-test.
marker in the hypophysectomized/oophorectomized controls, or in control animals given E₂ (Fig. 3). In contrast, GH-treated animals, regardless of their estrogen status, demonstrated a stronger signal for ER mRNA in the mammary gland.

To determine the time course of ER mRNA synthesis, a single injection of 50 µg hGH was given to a group of hypophysectomized and oophorectomized rats, and the glands removed at varying times after injection for analysis. As shown in Fig. 4, a signal for ER mRNA was clearly visible within 10 h of a single injection of hGH, with levels returning to baseline within 24 h. Quantitation of the levels of ER protein demonstrated a corresponding increase at the 10 h time point (data not shown).

Analysis of ER in mammary fat pads

To determine whether the effect of GH on ER levels was predominantly in the glandular tissue, or whether the stromal tissue was affected, we treated hypophysectomized/oophorectomized rats with rbGH or des(1–3)-IGF-I for 1 day, and then divided whole mammary glands into areas of gland-free fat pads, and gland-containing fat pads. ER levels in the two groups were then compared. As shown in Fig. 5, the levels of ER in gland-free fat pads were significantly increased (P<0.001) by rbGH relative to control values (mean ± s.e.m. = 10.5 ± 1.9 vs 3.1 ± 2.8). This was similar to results obtained in gland-containing fat pads (mean ± s.e.m. = 13.3 ± 5.0 vs 1.8 ± 1.3; P<0.001), suggesting that GH is working primarily through an increase in stromal ER. Again, des(1–3)-IGF-I treatment did not
significantly affect ER activity, and there was no significant difference between values obtained in fat pads with or without glands within treatment groups. Treating animals for 5 days with GH gave similar results (data not shown).

**Immunocytochemistry of ER**

To determine which cell types were positive for ER, we analyzed tissue sections of mammary glands from immature hypophysectomized and oophorectomized rats by immunocytochemistry as described in the Material and Methods section. Animals were treated with either bGH or des(1–3)-IGF-I for 1 day. Treatment with bGH caused an increase in ER staining in fat cells of the mammary gland as evidenced by a deep brown color in the nuclei (Fig. 6C). This fat cell staining was not noted in control animals (Fig. 6A) or animals treated with des(1–3)-IGF-I (Fig. 6B). All groups had approximately 50% of the epithelial cell nuclei stained for ER. Although we did not observe an increase or decrease in epithelial ER, we cannot rule out the possibility that there was some effect. A representative section stained in the absence of primary antibody is shown for comparison as a negative control (Fig. 6D). These data suggest a specific effect of GH on fat cell ER.

**Discussion**

The development of the mammary gland is a complex, dynamic process, which requires stromal and epithelial elements (Kratochwil 1969, Cunha 1994) and appropriate hormones, including GH, IGF-I and E2. We have demonstrated the importance of synergy between E2 and GH or IGF-I during pubertal mammary development in the rat (Kleinberg et al. 1990, Feldman et al. 1993, Ruan et al. 1995). We have found that GH can induce IGF-I mRNA in the mammary fat pad, and IGF-I can then substitute for GH in stimulating TEB development in the presence of E2 (Ruan et al. 1992, 1995). E2 alone is totally ineffective in stimulating TEBs in the absence of GH or IGF-I, while GH or IGF-I are only partially stimulatory in the absence of E2.
Since hormonal effects are mediated through specific receptor proteins, changes in the levels of receptors can play a major role in hormone action. A number of publications have shown that GH can stimulate ER production in a variety of tissues, including liver (Norstedt et al. 1981, Freyschuss et al. 1991, 1994), isolated hepatocytes (Ignatenko et al. 1992, Freyschuss et al. 1993, Stavreus-Evers et al. 1997), and uterus (Bezecny et al. 1992). Similarly, E_2_ has been shown to affect GH receptor levels (Bennett et al. 1996, Slootweg et al. 1997). E_2_ is also known to stimulate GH secretion (Friend et al. 1996), indicative of another level of interaction between these hormones. Results presented here demonstrate that GH can increase ER levels in the mammary gland, and indicate that the effect is at the level of ER transcription as demonstrated by RT-PCR. Since the values obtained with the DCC and ER–EIA assays were similar, it is unlikely that the GH effect also occurred through activation of preexisting ER. If that were the case, one might expect that the DCC assay, which measures binding activity, would give a higher percentage increase in ER in response to GH than what is measured by immunoreactivity (ER–EIA assay), which would not distinguish between active and inactive ER.

Our data also show that this effect of GH on ER is primarily in stromal tissue, since gland-free fat pads showed increases in ER levels equivalent to those in total mammary tissue, and immunocytochemical analysis showed that GH induced ER–positivity in fat cells but not in the epithelial compartment. In the rodent, ER has been found in both the epithelial and stromal compartments of the mammary gland (Haslam & Shyama 1981, Haslam & Nummy 1992), and E_2_ has been shown to act directly on the gland (Silberstein et al. 1994). In non-treated hypophysectomized and ovariectomized animals we were only able to discern ER–positivity in the epithelial cells. Since mammary glands from these animals were composed predominantly of stromal elements, the low levels of ER that we observe in cytosolic homogenates might be reflective of the low amount of glandular material. It is conceivable, therefore, that even if GH (or perhaps IGF-I) had an effect on epithelial ER, it might be obscured by the high percentage of stromal cells.

The same might hold true for PRL. While we were unable to demonstrate an effect of PRL on ER levels, earlier studies using mouse mammary glands (Muldoon 1981) and isolated rat mammary epithelial cells in culture (Edery et al. 1984) have presented data showing that PRL can increase ER. This discrepancy might thus be attributable to the fact that the amount of epithelial elements in hypophysectomized and ovariectomized animals is quite sparse relative to stroma, and that if PRL is affecting ER in the epithelium, we might not be able to measure it.

GH receptors have been identified in the mammary gland stroma (Lincoln et al. 1995). A recent paper by Ilkbahar et al. (1999), has demonstrated expression of GH receptor in both epithelium and stroma of mouse mammary gland, with significantly higher levels in the stromal component. Moreover, the expression varied with the stage of mammary development; the highest levels were found in virgin animals, declined during late pregnancy, and were lowest during lactation. It is interesting to speculate that the role of GH in the mammary fat cell might be at least partly a permissive one, by increasing the levels of ER and thus allowing E_2_ to act. IGF-I receptors are not believed to be present in fat cells (DiGirolamo et al. 1986, Bolinder et al. 1987, Berneis & Keller 1996), suggesting that this effect of GH is IGF-I independent. The relative roles of GH and IGF-I on other aspects of adipocyte differentiation and fat metabolism are far from clear (Richelsen 1997). E_2_ also has pronounced effects on fat distribution and metabolism (Pedersen et al. 1992, Westerveld et al. 1995) which may or may not be related to those of GH and/or IGF-I.

One must be cautious in interpreting the possible physiologic importance of our observations. IGF-I plus E_2_ can cause full experimental mammary gland development without GH, bringing into question the necessity for the stimulation of ER in stromal tissue in the process of mammary development. In that case the only importance of GH might be induction of IGF-I. The observed effect of GH on induction of ER could merely be an effect on fat tissue which is unrelated to mammary gland development. In contrast, synergy between E_2_ and GH or IGF-I might be multileveled with synergy between GH and E_2_, occurring in the stromal compartment and synergy between IGF-I and E_2_ in the epithelial compartment, both by different mechanisms. In this regard, IGF-I has been shown to activate ER transcription in mammary and pituitary tumor cells (Newton et al. 1994, Lee et al. 1997).

Our hypothesis for mechanisms of mammary development is that GH stimulates IGF-I in the mammary fat pad, which in turn, acts by paracrine means to induce formation of terminal end buds and ductal morphogenesis from pluripotent epithelial stem cells (Kleinberg 1997). The mechanism or mechanisms by which estrogens interact in this process are largely unknown. Our data show that GH induces ER in the stromal compartment of the mammary gland, and provide at least one potentially important mechanism to explain the interaction between GH and E_2_.

Acknowledgements

This work was supported by a Department of Veterans Affairs Merit Review and a grant from the NIH (R01 CA64709).

References

Bennett PA, Levy A, Carmignac DF, Robinson IC & Lightman SL. 1996 Differential regulation of the growth hormone receptor gene:
effects of dexamethasone and estradiol. *Endocrinology* 137 3891–3896.


Feldman M, Ruan W-F, Cunningham BC, Wells JA & Kleinberg DL 1993 Evidence that the growth hormone receptor mediates differentiation and development of the mammary gland. *Endocrinology* 133 1602–1608.


Ignatenko LL, Mataradze GD & Rozen VB 1992 Endocrine mechanisms for the formation of sex-related differences in hepatic estrogen receptor content and their significance for the realization of an estrogen effect on angiotensinogen blood level in rats. *Hepatology* 15 1092–1098.


Muldoon TG 1981 Interplay between estradiol and prolactin in the regulation of steroid hormone receptor levels, nature, and functionality in normal mouse mammary tissue. *Endocrinology* 109 1339–1346.


Received 26 May 1999
Accepted 19 July 1999