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Independence of Estrogen-Induced Pituitary Proliferation on Local IGF-I mRNA and EGF mRNA Expression. Modifying Effects of Tamoxifen and Terguride

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Summary

Pituitary hyperplasia as well as proliferation of the endometrium are typical responses to estrogen administration in rodents. Both insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF) have been implicated as paracrine mediators and amplifiers of estrogen action in the rodent uterus. The auto/paracrine role of IGF-I, EGF, their receptors and IGF binding proteins in pituitary proliferation has not yet been solved. Here we have used a semi-quantitative reverse transcription polymerase chain reaction (RT PCR) assay to demonstrate the changes in IGF-I mRNA and EGF mRNA abundance in the proliferating male rat pituitary in response to estradiol benzoate (EB; 1 mg/kg b.w. twice weekly i.m. for 3 weeks) and modifying effect of drugs antagonizing the pituitary enlargement – antiestrogen tamoxifen (TAM, 5 mg/kg b.w. daily) and also the dopaminergic agonist terguride (TER, 0.66 mg/kg b.w. daily, routinely used for the treatment of prolactinomas). In three separate experiments, EB induced a 2.2–2.5 fold increase in pituitary weight. The abundance of IGF-I and EGF mRNAs in pituitaries of EB-treated animals did not differ from the controls in two experiments and in the third series with the most marked pituitary hyperplasia mRNAs of both growth factors were even significantly decreased. Antiestrogen TAM administered with EB partially blocked the EB-induced proliferation and significantly stimulated IGF-I mRNA ($p=0.003$) and EGF mRNA ($p=0.023$) expression, while EB or TAM alone did not stimulate mRNAs of the studied growth factors. Significant antiproliferative effect of dopaminergic agonist TER on EB-induced pituitary proliferation ($p=0.006$) was accompanied with decreased IGF-I mRNA ($p=0.025$), but not EGF mRNA abundance. Our results suggest that the estrogen-induced pituitary proliferation is independent of the local expression of IGF-I and EGF mRNAs.

Key words

Estrogen induced pituitary proliferation – IGF-I mRNA – EGF mRNA – Tamoxifen – Terguride – RT PCR

Introduction

Estrogens exert a strong proliferative effect in rodents especially in two target organs – the uterus and pituitary. Estrogen-induced uterine proliferation is associated with induction of the expression of a variety of growth factors and cytokines which are thought to function in an autocrine or paracrine fashion to amplify the estrogen-induced proliferative signal and mediate the interaction of different cell types (Murphy and Ghahary 1990). The best studied growth factors in the uterine response to estradiol are IGF-I and EGF (DiAugustine *et al.* 1988, Murphy and Ghahary 1990, Nelson *et al.* 1990, Ignar-Trowbridge *et al.* 1992, Hána and Murphy 1994). The presence of these growth factors IGF-I and EGF and their receptors and the insulin-like growth factor binding proteins in the hypothalamo-hypophyseal system is well recognized (Bach and Bondy 1992, Michels *et al.* 1993, Fan and Childs 1995, Ray and Melmed 1997).

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The production of EGF is localised mainly in somatotropes, gonadotropes and thyrotropes while EGF receptors are to be found in all subsets of pituitary cells and mainly in lactotropes and somatotropes (Chabot *et al.* 1986, Fan and Childs 1995). *In situ* hybridization localised IGF-I mRNA in the rat pituitary mainly to non-endocrine folliculo-stellate cells scattered throughout the gland and IGF-I receptor mRNA was homogeneously distributed throughout the anterior and intermediate lobe of the pituitary (Bach and Bondy 1992). IGF-BP2 production appears to occur in folliculo-stellate cells (Michels *et al.* 1993).

The physiological role of IGF-I and EGF produced in the pituitary, regulation of their expression and the possible participation in pathological processes in pituitary is still not fully understood. A possible mitogenic role of growth factors in growth stimulation of pituitary adenomas is suggested by some authors. The production of growth factors IGF-I and EGF, bFGF, TGFα and their possible role in the growth regulation of human nonfunctioning adenomas is suggested by *in vitro* studies (Renner *et al.* 1993). An overexpression of EGF receptor in some types of pituitary adenomas was found by LeRiche *et al.* (1996) and Chaidarun *et al.* (1994). Proliferation of pituitary cells with adenoma formation is induced by estrogen administration in rodents. Estrogen-induced pituitary proliferation is represented mainly by proliferation of lactotropes resulting in adenoma formation with an increased prolactin expression and secretion (Holtzman *et al.* 1979, Yamamoto *et al.* 1986). A possible role of auto/paracrine action of EGF and IGF-I in this process is suggested. Some studies on estrogen-induced pituitary proliferation utilizing *in situ* hybridization suggested an increased expression of IGF-I mRNA and IGF-BP2 mRNA in response to estradiol (Michels *et al.* 1993). Increased release of EGF from rat pituitary mixed cells *in vitro* in response to estrogen status was also demonstrated (Mouihate and Lestage 1995). In contrast to the previous results, estradiol-induced a decrease of EGF protein content per mg of anterior pituitary tissue in experiments of Nedvídková and Schreiber (1993).

The stimulatory effect of estrogen on pituitary proliferation can be mimicked by EGF administration (Murdoch *et al.* 1982). EGF also induces differentiation of lactotropes in both GH3 and pituitary cultures from neonatal rats and this is accompanied by high basal secretion of prolactin (Felix *et al.* 1995). Mitogenesis of GH3 cells producing prolactin can be stimulated by IGF-I or insulin through the stimulation of estrogen receptors and this can be blocked by pure antiestrogens ICI 164384 and ICI 182780 (Newton *et al.* 1994). These results suggest a possible effect of IGF-I on the estrogen receptor pathway.

IGF-I exerts its effects in an auto/paracrine and also endocrine fashion. The main source of IGF-I in the systemic circulation is the liver. Considering that estrogens suppress the liver IGF-I production, we do not expect the endocrine role of IGF-I to be important in our experiments. Analogically to the suggested auto/paracrine role of IGF-I and EGF expressed in estradiol-induced uterine proliferation, these growth factors might play an important role in the estradiol-induced pituitary proliferation. Here, we have used a sensitive reverse transcription polymerase chain reaction (RT-PCR) assay to examine the pituitary IGF-I and EGF gene expression in response to the estradiol stimulus and the modifying effects of antiestrogen tamoxifen and D_2 agonist terguride.

Materials and Methods

Materials

MMLV-reverse transcriptase was purchased from Gibco BRL Life Technologies, Inc., whereas Taq DNA polymerase was purchased from Promega, Madison, WI, USA.

Animals and experimental protocols

Male Wistar rats (Velaz, Prague) with body weight of 170–270 g (animals of similar weight in each experiment) were administered i.m. estradiol benzoate (Agofollin-depot, Biotika, Czech Rep., EB) in a microcrystalline suspension 1 mg/kg b.w., twice a week, for 3 weeks. Controls (C) received saline injection in the same regimen. Antiestrogen tamoxifen (Zitazonium, Egis, Hungary, TAM) 5 mg/kg b.w. daily and dopaminergic agonist terguride (Mysalfon, Léčiva, Czech Rep., TER) 0.66 mg/kg b.w. daily were administered in the diet for 3 weeks. Groups of animals thus treated were examined: EB, EB+TAM, EB+TER, TAM, TER, saline (C). Animals were decapitated after three weeks of treatment, their blood was collected and anterior pituitaries were isolated, weighed and quickly frozen on dry ice (in some experiments also the liver). Ten animals were used in each control or treated group. Experiments with EB were repeated three times.

All experiments were performed in accordance with protocols approved by the Animal Care Committee of the Faculty of Medicine, Charles University, Prague.

RNA extraction and RT-PCR assay

RNA was extracted from the pituitaries and liver using the single-step method described by Chomczynski and Sacchi (1987). For reverse transcription (RT), 2 μg of total RNA in 9 μl of water were denaturated for 10 min at 65 °C and then placed on ice. To this were added 10 μl 5X RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM

$MgCl_2$), 20 μl of water, 5 μl of 0.1 M DTT, 2.5 μl of 10 mM dNTPs, 100 ng of oligo dT primer and 500 U MMLV-RT. The reaction mixture was incubated at 37 °C for 75 min. The reaction was terminated by heating at 90 °C for 5 min and then placed on ice. For the amplification step, 4 μl of the RT product were amplified in a volume of 50 μl containing 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1 % Triton X-100, 2.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM each primer 1 unit of Taq DNA polymerase. The thermal profile was 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1.5 min for IGF-I and mouse β-actin amplification. The amplification protocol for EGF was 94 °C for 1 min, 67 °C for 1 min, 72 °C for 1.5 min for EGF amplification. As a final extension step, the reaction was heated to 72 °C for 5 min and then cooled. IGF-I and β-actin mRNAs were amplified in one tube as described previously (Hána and Murphy 1994). A set of primers, 5' GGC TGC TTT TGT AGG CTT CAG TGG 3' and 5' GGA CCA GAG ACC CTT TGC GGG G 3' was used for amplification of IGF-I mRNA. This primer set resulted in amplification of 210 bp fragment corresponding to the nucleotide sequence of 219 to 428 (Bell et al. 1986). The PCR product was verified by Southern blot with a rat IGF-I cDNA probe (Murphy *et al.* 1987). As internal control for the RT-PCR reaction, a 540 bp fragment of mouse β-actin mRNA was also amplified using commercially available primers (Clontech, Laboratories, Inc., Palo Alto, CA). To avoid inter-tube differences, the amplification of β-actin and IGF-I mRNAs was performed in the same tube. No amplification products were apparent for either β-actin or IGF-I when the reverse transcriptase was deleted. As discussed below, the amount of input RNA and the number of amplification cycles was optimized to ensure that the PCR products were quantified during the exponential phase of the amplification. A 203 bp fragment corresponding to nucleotides 3241 to 3424 of rat EGF (Saggi *et al.* 1992) was amplified, using the following designed primers: 5' TGG ACT GCC TTG CCC TGA CT 3' and 5' CTC GGT GCT GAC ATC GTT CT 3'. The identity of the EGF fragment of 203 bp was verified by sequencing (see acknowledgements). EGF and β-actin (as an internal control) were amplified in the same tube. All primer sets used were tested for their ability to amplify rat genomic DNA and none of them gave the expected product. As a control for contamination, reverse transcriptase and/or the template RT product were omitted from some tubes. In initial experiments to optimize the PCR reaction, samples were taken after different numbers of cycles to establish the exponential phase of the amplification. Having established the exponential phase of the amplification reaction, samples were routinely collected at two points in the amplification protocol to verify that the reaction had not reached a plateau. Routinely, a sample of 15 μl was withdrawn after 25 cycles for the reaction where IGF-I and β-actin were amplified in the same tube and after 27 or 23 cycles, where EGF and β-actin was amplified for EGF quantification. In each case, the reaction was allowed to continue for further 5 cycles to ensure that amplification had not reached a plateau. Aliquots of the PCR products were resolved by electrophoresis on 2 % agarose gel. The samples of rats treated with drugs and their controls were analyzed in the same RT-PCR reaction and on the same gel. For quantitation, the photograph of the ethidium bromide stained gel was analyzed using a densitometer. The optical density (O. D.) area of the IGF-I or EGF band (expressed in arbitrary units) was divided by the O. D. area of the β-actin band of the corresponding sample. The value obtained in this fashion was expressed as a percentage of the mean value of the corresponding control pituitaries. Only samples analyzed in the same reaction and on the same gel were compared to avoid possible differences in the whole process of the reaction and measurement.

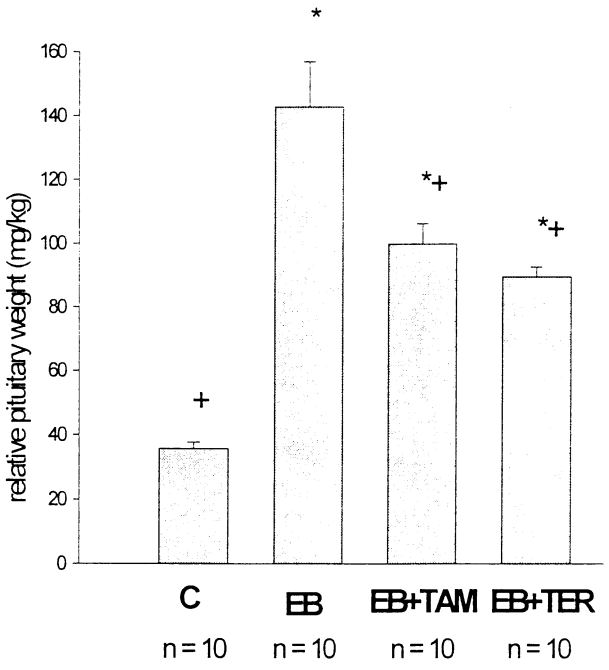

Fig. 1. *The relative pituitary weight (pituitary/body weight in mg/kg) of controls (C), estradiol benzoate (EB), EB + TAM and EB + TER treated rats. Expressed as mean ± S.E.M. * significant difference from C group (Kruskal-Wallis test $p < 0.001$, Neményi test $p < 0.05$) + significant difference from EB-treated group (Kruskal-Wallis test $p < 0.001$, Neményi test $p < 0.05$).*

Statistical analysis

Nonparametric Kruskal-Wallis test was used to determine the significance of differences in the pituitary weight after different regimens. The Neményi test was used for comparing the relative pituitary weights in individual groups with each other. The

Wilcoxon's two sample rank sum test was used to determine the significance of differences between two groups of animals after different regimens of treatment. Only two groups of samples analysed in the same RT PCR reaction were compared. The results of relative mRNA expression were never used for multiple testing of the same data (the methodology of mRNA semiquantitative measurements does not enable this; see above in Methods).

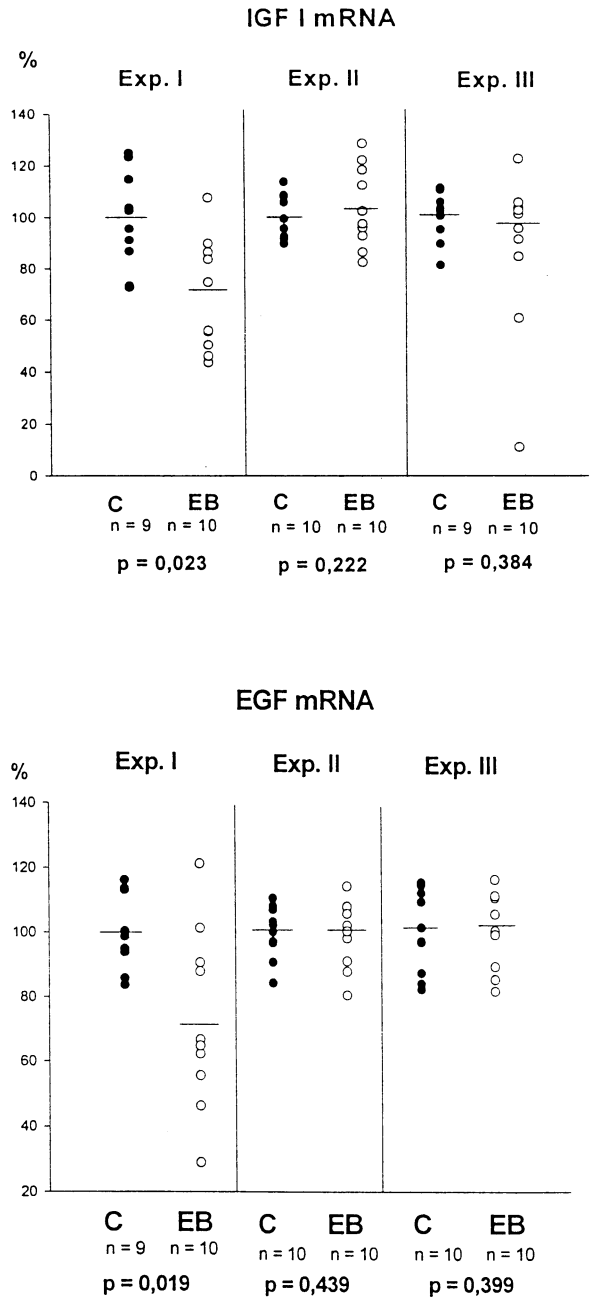

Fig. 2. *IGF-I mRNA and EGF mRNA pituitary abundance in estradiol benzoate (EB) treated rats expressed in % of controls (C). The horizontal bars indicate the mean.*

Results

EB induced significant growth of the anterior pituitary in all three experiments. A 2.2 to 2.5 fold increase in pituitary weight was seen in EB-treated animals when compared with the saline controls. The relative enlargement of pituitaries was even more pronounced when expressed in relation to the body weight (3.4–4.0 fold), because the EB rats had a smaller increase in body weight during the experiment than the controls. As can be seen in Figure 1, the antiestrogen TAM, when administered with EB, significantly blocked the increase of pituitary weight ($p<0.05$). The dopaminergic agonist TER suppressed the effect of EB on pituitary weight ($p<0.05$) even more markedly than TAM (Fig. 1).

Abundance of IGF-I mRNA in EB-treated pituitaries was compared with the controls in three separate experiments. It did not differ significantly in two of them (mean±S.E.M.; 103.75±5.00 % in EB vs. 100±2.66 % in the controls, $p=0.222$ and 97.77±5.29 % vs. 100±3.34 % respectively, $p=0.384$) and the abundance of IGF-I mRNA after EB was even significantly decreased in the experiment, in which the growth of pituitaries after EB was the most pronounced (69.41±6.89 % after EB vs. 100±5.40 % in the controls, $p=0.023$) (Fig. 2). EGF mRNA was also decreased after EB in this experiment (72.54±8.75 % after EB vs. 100±3.96 % of controls, $p=0.019$), while it was not changed after EB in other experiments (99.56±3.28 % vs. 100±2.51 % in the controls, $p=0.439$, 101.51±3.99 % vs. 100±3.98 % in controls, $p=0.399$), where also IGF-I mRNA was not different from controls (Fig. 2). IGF-I mRNA in the liver tissue in the experiment, in which IGF-I and EGF mRNA were suppressed after EB, also decreased after EB (69.80±6.96 % after EB vs. 100±9.53 in the controls, $p=0.006$).

IGF-I and EGF mRNAs in the pituitary of EB+TAM-treated animals were surprisingly more abundant in comparison with EB-treated animals (129.06±3.14 % vs. 100±3.82 %, $p=0.003$ for IGF-I mRNA and 126.84±9.4 % vs. 100±18.12 %, $p=0.023$ for EGF mRNA) (Fig. 3), while EB or TAM alone did not stimulate mRNAs of the studied growth factors. A similar increase in mRNAs after administration of the studied growth factors was obtained when the EB+TAM group was compared with saline controls.

IGF-I mRNA in EB+TER-treated pituitaries was significantly less abundant than in animals only treated with EB (73.70±9.07 % vs. 100±9.29 %, $p=0.025$). The EGF mRNA content did not differ in these groups (109.06±7.93 % in EB+TER vs. 100±9.39 % in EB, $p=0.121$).

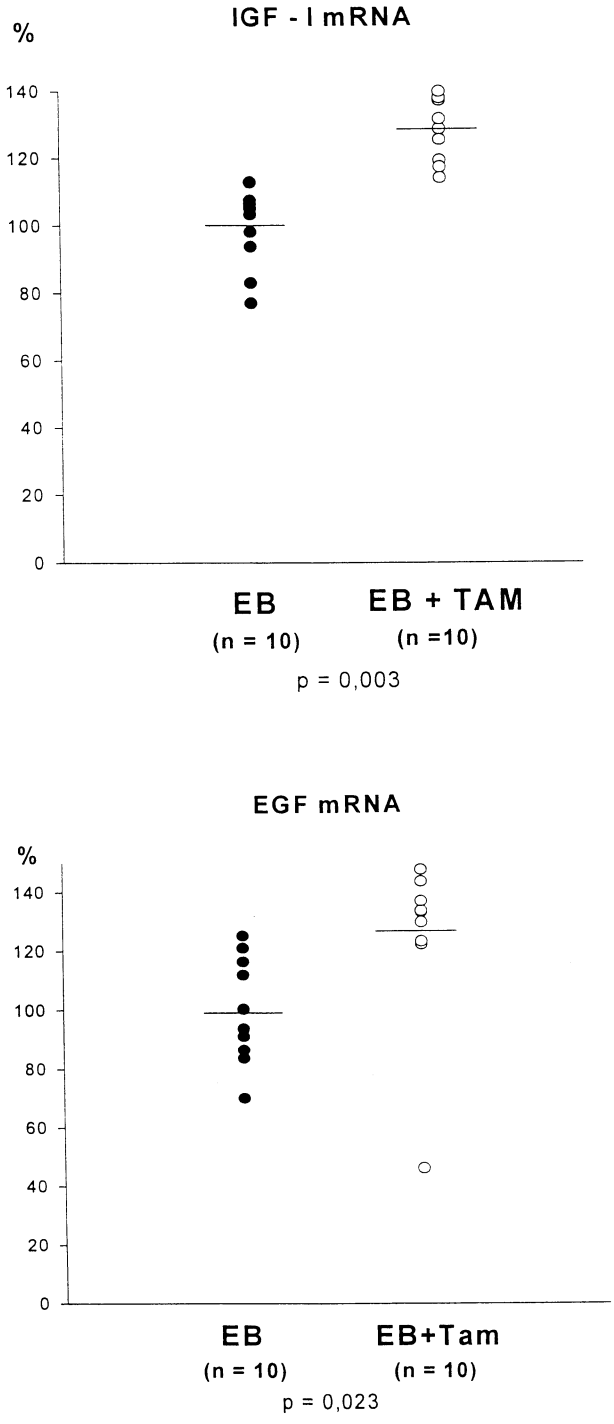

Fig. 3. *IGF-I mRNA and EGF mRNA pituitary abundance in EB vs. EB+TAM treated rats in % related to rats treated with EB only. The horizontal bars indicate the mean.*

Discussion

There is a typical proliferative response to estrogen administration both in the uterus and the pituitary. The effect of estrogens on uterine tissues is most probably mediated and potentiated by the auto/paracrine action of growth factors EGF and IGF-I (Murphy and Ghahary 1990). An increased expression of several growth factors in the pituitary in relation to increased estrogen levels has also been described – TGFα (Borgundvaag *et al.* 1992), IGF-I (Michels *et al.* 1993) and EGF (Mouihate and Lestage 1995). Their auto/paracrine role in the pituitary proliferation was implicated. A very impressive effect of estradiol on TGFα expression and its relation to pituitary growth was shown by Borgundvaag *et al.* (1992). True growth factor-mediated pituitary cell transformation has been best exemplified by studies of TGFα overexpression (McAndrew *et al.* 1995). On the basis of these premises, we examined the abundance of IGF-I mRNA and EGF mRNA in the proliferating pituitary. In contrast to the above mentioned studies, we did not find any clear relation between estradiol-induced pituitary proliferation and the pituitary amount of IGF-I and EGF mRNAs. IGF-I and EGF mRNA were not more abundant in estrogen-stimulated pituitaries in our experiments as compared to the controls and the most markedly stimulated pituitary growth in one experiment was accompanied by an even lower abundance of mRNAs than in the controls. Our results are in agreement with the observed decrease of EGF protein content per mg of anterior pituitary tissue in the experiments of Nedvídková and Schreiber (1993). The effect of different doses of estrogens might be the factor influencing the expression of growth factors, the lower levels of estrogens having a stimulating effect and the higher levels acting suppressively. The enhancing effect of endogenous estrogens on EGF production in different phases of the cycle has been described (Mouihate and Lestage 1995) and the same effect was found with lower dose in estrogen pellets (Michels *et al.* (1993). Our experiments with the antiestrogen TAM might be in agreement with this hypothesis. Attenuation of the effect of estrogens on pituitary cell proliferation by coadministration of TAM leading in our experiments to the increased pituitary IGF-I and EGF mRNA abundance might be the factor influencing the growth factors expression. However, this could also be explained by the described different interaction of estradiol and TAM with estrogen receptor (Sasson and Notides 1988), where the simultaneous effect of both drugs on receptor and postreceptor events could differ from the separate effects of these drugs.

Altogether, our results of EB and EB+TAM treatment on the proliferation of pituitary cells and on IGF-I and EGF mRNA abundance in the pituitary speak against the causal auto/paracrine role of IGF-I and EGF in the estrogen-induced pituitary growth. Considering the suppressive effect of estrogens on the liver IGF-I mRNA expression the endocrine action of systemic IGF-I is also unlikely.

The participation of changes in the dopaminergic system in the process of lactotroph

proliferation is suggested (Sarkar *et al.* 1982). The antiproliferative effect of D_2 agonists in prolactinomas is well known from experiments and clinical practice. A suppressive effect of D_2 agonist on pituitary proliferation and TGFα expression was demonstrated (Borgundvaag *et al.* 1992). We detected a similar effect of TER on estradiol-induced pituitary growth and on IGF-I mRNA but not on EGF mRNA abundance in the pituitary. Suppressive effect of these drugs on protein kinase C activity, which mediates PRL gene expression, as the explaining mechanism, is suggested (Matsuno *et al.* 1995). Changes in the number of D_2 receptors in prolactin-producing GH3 cells were not found under the influence of estradiol or EGF (Zhang *et al.* 1993).

Our results lead us to the conclusion that estrogen-induced pituitary proliferation is independent of the local IGF-I and EGF expression. Although the suppressive effect of the D_2 agonist TER on pituitary proliferation is accompanied by a significant decrease of IGF-I mRNA abundance in the pituitary, we do not expect a causal relation between its decrease and reduced size of the pituitary. An interesting finding seems to be the increase of both IGF-I and EGF mRNAs in response to agonist plus antagonist-partial agonist, EB+TAM coadministration, while no increase is elicited when the drugs are administered alone.

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