Neuro
-endocrinology

Neuroendocrinology 2001;74:367–374

Received: June 12, 2001
Accepted after revision: September 5, 2001

Effect of Long-Term Treatment with the Antiestrogen EM-652.HCl on Pituitary Estrogen Receptor Alpha and Prolactin mRNA Expression in Intact, Ovariectomized and Gonadotropin-Releasing Hormone-Treated Female Rats

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Key Words

Gonadal steroids · Receptors · Prolactin · Antiestrogens · Gonadotropin-releasing hormone · Ovary · In situ hybridization

Abstract

Estrogen receptor α (ERα) is the predominant estrogen receptor subtype in the anterior pituitary gland. In order to assess the influence of the pure antiestrogen EM-652.HCl on ERα gene transcription, we have studied the effect of long-term administration of the antiestrogen in ovariectomized rats as well as in intact female rats treated or not with the GnRH-agonist D-trp^6, des-Gly-NH_2^{10} GnRH ethylamide (GnRH-A), a treatment which induces pharmacological castration. To evaluate the degree of pituitary responsiveness to changes in estrogen exposure, prolactin (PRL) mRNA levels were also measured. ERα and PRL mRNA levels were evaluated by quantitative in situ hybridization. It was found that, 49 weeks after ovariectomy (OVX), pituitary ERα mRNA levels were decreased by 55%. Long-term administration (49 weeks) of EM-652.HCl to OVX animals resulted in a further 41% decrease in ERα mRNA. On the other hand, ovariectomy induced an 82% decrease in PRL mRNA levels while the administration of EM-652.HCl to OVX animals did not further decrease PRL mRNA. The administration of EM.652.HCl or GnRH-A alone to intact rats during 52 weeks did not significantly modify pituitary ERα mRNA levels. Concomitant administration of both GnRH-A and EM-652.HCl induced 41 and 47% decreases in ERα mRNA levels, when compared to the levels measured in vehicle-treated and GnRH-treated animals respectively. Combined administration of EM.652.HCl and GnRH-A induced 56 and 65% decreases in PRL mRNA, respectively. When EM-652.HCl was administered concomitantly with GnRH-A, the inhibitory effect on PRL mRNA levels was more marked than that observed in GnRH-A-treated animals. The present data demonstrate that when circulating estrogens are absent or maintained at very low levels by GnRH administration, EM-652.HCl can still depress ERα gene transcription. It is suggested that estrogens can positively regulate pituitary ERα gene transcription and that the antiestrogen EM-652.HCl can downregulate by itself pituitary ERα gene transcription.

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Introduction

The secretion of anterior pituitary hormones is finely regulated by a complex interaction between hypothalamic factors as well as by peripheral hormones including sex steroids [1]. Estrogens are directly involved in the regula-

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0028–3835/01/0746–0367$17.50/0

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tion of anterior pituitary hormone secretion [2]. One classic pituitary target for estrogens is the lactotrope cell in which 17β-estradiol (E_2) stimulates prolactin synthesis and secretion [3–9]. The action of estradiol is mediated through an intracellular estrogen receptor (ER), a member of the steroid/thyroid hormone receptor superfamily, which regulates gene expression via estrogen-responsive elements [10]. Recently, a second ER, called ERβ, has been cloned from a rat prostate library, which led to rename the original one ERα [11].

It has been shown that in rat pituitary, ERα sequences are present at much higher levels than ERβ [12–15]. In situ hybridization and immunocytochemical studies have demonstrated the presence of ERα in several pituitary cell types, including gonadotropes, lactotropes and corticotropes [12, 16]. The estrogenic regulation of ER expression in the rat pituitary has been recently investigated. It has been reported that administration of estradiol to ovariectomized rats induced an increase in ER mRNA expression [17, 18]. On the other hand, Schreihofer et al. [19] found that, in castrated male or female rats, administration of estradiol during 3 days suppressed ERβ mRNA expression but did not modify ERα mRNA levels.

Estrogen is considered the most potent stimulator of prolactin (PRL) gene transcription [20]. In the rat pituitary, ligand-bound ER has been shown to activate PRL transcription via direct interaction with the PRL gene distal enhancer [21, 22]. In ERα-disrupted mice, a marked reduction in PRL mRNA expression has been reported [23], thus providing evidence that ERα plays a critical role in PRL gene transcription.

EM-652.HCl is a pure nonsteroidal antiestrogen which has been developed in our laboratories [for review, see 24]. This compound has a very high affinity for ER and, in fact, is more potent than estradiol in displacing [^{3}H]estradiol from the rat uterine receptor [24]. EM-652.HCl exerts a potent inhibitory activity on both ERα and ERβ [25]. In nude mice, treatment with the antiestrogen induced an almost complete disappearance of ER in the uterus and vagina [26]. The efffect of EM-652.HCl on pituitary ERα has not been investigated.

In order to fully investigate the effect of EM-652.HCl on pituitary ERα mRNA expression, we studied the effect of long-term administration of EM-652.HCl in ovariectomized rats as well as in intact female rats treated or not with a GnRH agonist, a treatment which markedly depresses ovarian functions [27, 28]. To evaluate the changes of pituitary responsiveness to various levels of estrogens, we also evaluated variations in PRL mRNA levels in the same experimental conditions.

Materials and Methods

Animals and Treatments

Ten- to 12-week-old female Sprague-Dawley rats (Crl:CD(SD)br) (Charles River Laboratory, St Constant, Canada) weighing approximately 210–240 g at start of treatment were used. The animals were acclimatized to the environmental conditions (temperature: 22 ± 3°C; humidity: 50 ± 20%; 12-hour light/12-hour dark cycles, lights on at 7:15 h) for 1 week before starting the experiment. The animals were housed individually and were allowed free access to tap water and a pelleted certified rodent feed (Lab Diet 5002, Ralston Purina, St Louis, Mo., USA). The experiments were conducted in a Canadian Council on Animal Care approved facility in accordance with the CCAC Guide for Care and Use of Experimental Animals.

In the first experiment, 45 rats were randomly distributed between 3 groups of 15 animals each as follows: (1) intact controls; (2) ovariectomized (OVX) controls; (3) OVX + EM-652.HCl (2.5 mg/kg BW rats). On day 1 of the study, the animals of the appropriate groups were bilaterally OVX under isoflurane anesthesia. Treatment with EM-652.HCl suspended in 0.4% methylcellulose or vehicle alone (control groups) was initiated on day 2 of the study and was given once daily by oral gavage (0.5 ml/rat) for 49 weeks. The daily dose of EM-652.HCl was selected on the basis of data from previous experiments indicating that at 2.5 mg/kg there was a maximal effect of estrogen-sensitive parameters [24, 26].

In the second experiment, animals were randomly assigned to 4 groups of 15 animals per group as follows: (1) intact controls; (2) intact + EM-652.HCl (2.5 mg/kg BW) rats; (3) intact + GnRH-A (0.008 mg/kg BW) rats; (4) intact + GnRH-4 + EM-652.HCl rats. EM-652.HCl was administered once daily described above while GnRH-A (D-trp^6, des-Gly-NH_2^{10} GnRH ethylamide diacetate) was injected subcutaneously once daily as solution in 0.3% NaCl, 0.25% $NaH_2PO_4H_2O$, pH 5.6–6.2. Animals were treated for 52 weeks. The daily dose of GnRH-A was chosen on the basis of previous reports showing that at 0.008/mg/kg BW there was marked inhibition of ovarian steroidogenesis [27, 28].

In situ Hybridization

ERα Probe Preparation. Specific ERα cRNA probe was prepared as previously described [15, 29]. Briefly, this probe was generated from its linearized rat ER cDNA subcloned into a pBluescript II KS(+) plasmid vector. ERα cDNA templates were linearized with BamHI and HindIII for antisense and sense, respectively. Radioactive cRNA copies were synthesized by incubation of 250 ng linearized plasmid in 6 m*M* $MgCl_2$, 40 m*M* Tris (pH 7.9), 2 m*M* spermidine, 10 m*M* NaCl, 10 m*M* dithiothreitol, 0.2 m*M* ATP/GTP/CTP, 200 μCi [α-^{35}S] UTP (Dupont NEN, Boston, Mass., USA), 40 U RNAsin (Promega, Madison, Wisc., USA) and 20 U of either T3 (antisense probe) or T7 (sense probe) RNA polymerase for 60 min at 37°C.

PRL Probe Preparation. The plasmid containing the rat PRL cDNA cloned in the *Eco*RI site of pSP64 was generously provided by Dr. R.A. Maurer [30]. The 717-base pair fragment was purified on a 5% polyacrylamide gel and electro-eluted with an electro-eluter (Bio-Rad, Richmond, Calif., USA, model 422) as described [9]. The fragment was then labeled with [α-^{35}S]dCTP ([α-^{35}S]deoxycytidine 5′-triphosphate) (1,000 Ci/mmol) (Amersham, Arlington Heights, Ill., USA) by the random primer method [31] to a specific activity ranging between 0.8 and 1.2 × 10^9 dpm/μg. As control, sections were treated with RNAse (100 mg/ml; Pharmacia Inc.) for 45 min at 37°C prior to hybridization.

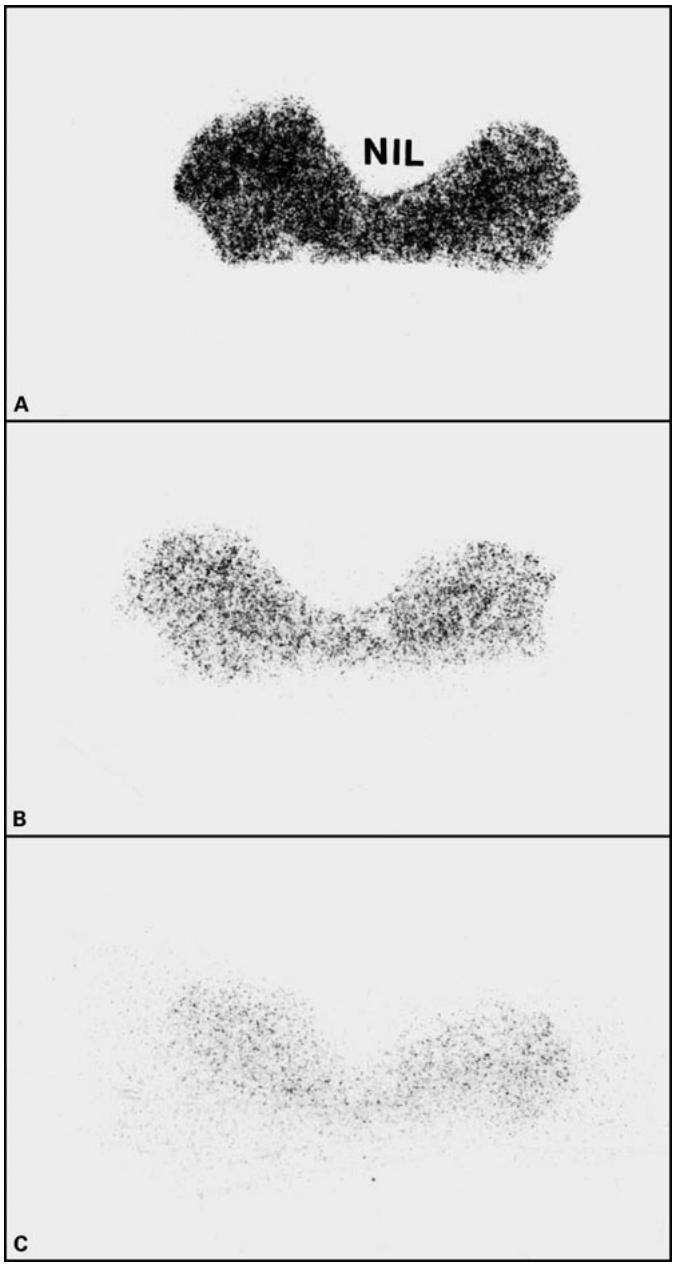

Fig. 1. Typical X-ray autoradiographs showing the labelling obtained in the anterior pituitary lobe following in situ hybridization with a labelled ERα cRNA probe. The neurointermediate lobe (NIL) is devoid of reaction. **A** Intact rats. **B** Ovariectomized rats. **C** Ovariectomized animals treated with EM-652.HCl.

Histological Procedure. Approximately 24 h after the last dosing, animals fasted overnight (5 animals/group) were perfused transcardially with 200 ml 4% (w/v) paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4). Pituitaries were excised and post-fixed in the same fixative for 48 h at 4°C. They were then placed in 15% sucrose in 0.1 *M* phosphate buffer before being quickly frozen in isopentane chilled in liquid nitrogen.

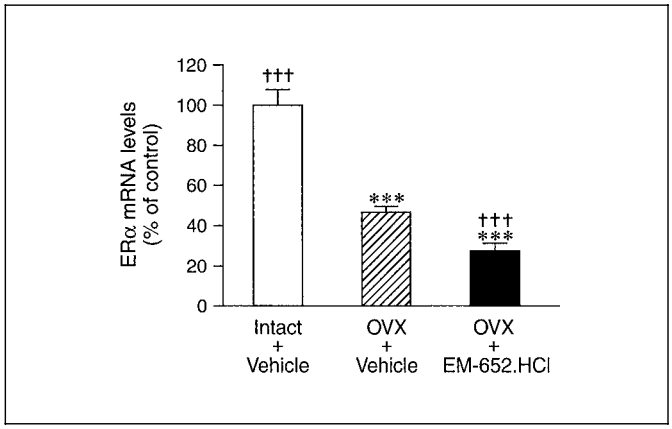

Fig. 2. Effects of ovariectomy and treatment of ovariectomized animals with EM.652.HCl on ERα mRNA levels. Results are expressed as a percentage of the control value (vehicle-treated intact animals). *** $p < 0.001$, intact rats vs. the other experimental groups. ††† $p < 0.001$ ovariectomized rats vs. the other groups.

In situ hybridization was performed in frozen sections as previously described for the localization of PRL mRNA [9] and ERα mRNA [16]. Following hybridization, the sections were exposed to Kodak X-Omat films for 6 and 48 h for PRL and ERα probes, respectively.

Quantitative analysis of hybridization signals was carried out on autoradiographic films using a Sony CCD XC-77 video camera with high resolution (570(H) × 485(V) TV lines) coupled to a Macintosh computer (Power PC 7500/100) and Image Software (version 1.60 non-FPU, W. Rasband, NIH, Bethesda, Md., USA) as described [32]. The optical density (OD) of the hybridization signals was measured under bright-field illumination. The images of the pituitaries were digitized and subjected to densitometric analysis, yielding measurements of integrated OD (area of anterior lobes × average OD).

Statistical Analysis

Quantitative data are presented as means ± SEM. The mean and SEM were calculated from 6 to 8 measurements per animal (5 animals/group) performed in serial sections of respective pituitaries. Comparison of the mRNA levels between treatment groups was performed by ANOVA (Statview 4.5 ANOVA); $p < 0.05$ was considered as statistically significant.

Results

ERα mRNA

Following hybridization with the labelled ERα cRNA probe, a diffuse autoradiographic reaction was observed over the anterior lobe of the pituitary (fig. 1). No labelling could be detected over the intermediate and posterior lobes. Hybridization with the labelled sense probe did not generate any specific labelling (not shown). As illustrated in figure 1 and 2, the hybridization signal was decreased

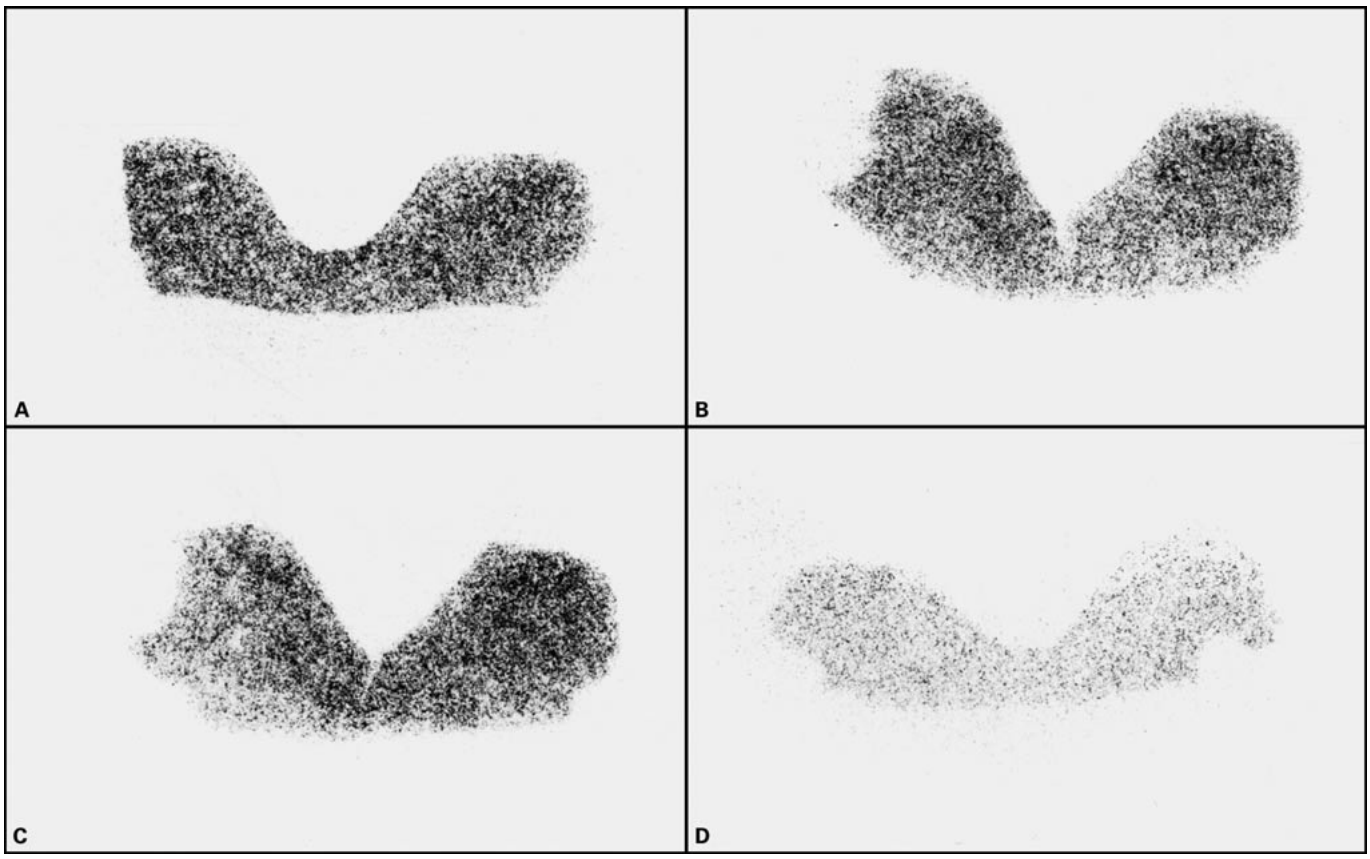

Fig. 3. Typical X-ray autoradiographs illustrating the hybridization signal following in situ hybridization with the labelled ERα probe in the anterior pituitary of intact animals. **A** Vehicle-treated rats. **B** EM-652.HCl-treated rats. **C** GnRH-A-treated rats. **D** Animals treated with both GnRH-A and EM-652.HCl.

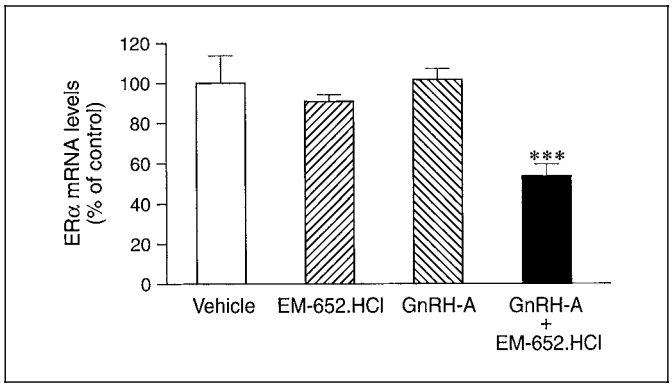

Fig. 4. Effects of EM-652.HCl and GnRH-A administered alone or in combination on ERα mRNA levels. Results are expressed as a percentage of the control value (vehicle-treated rats). *** $p < 0.0001$, vehicle-treated animals vs. all the other experimental groups. The group treated with both GnRH-A and EM-652.HCl is significantly different ($p < 0.001$) from the groups treated with GnRH-A or EM-652.HCl alone.

by 55% ($p < 0.001$) 48 weeks following ovariectomy. The administration of EM-652.HCl to ovariectomized rats during 48 weeks resulted in a further 41% decrease ($p < 0.001$) in ERα mRNA levels as compared to the values obtained in ovariectomized animals.

In the second series of experiments, we evaluated the long-term effect of the antiestrogen in intact rats and in female rats with a decreased ovarian function induced by the chronic administration of a GnRH-A. The administration of EM-652.HCl during 52 weeks did not significantly modify the hybridization signal (fig. 3, 4). Similarly, the amounts of ERα mRNA were not modified by chronic treatment with the GnRH-A alone. The concomitant administration of both GnRH-A and EM-652.HCl resulted in 41 and 47% decreases ($p < 0.001$) in ERα mRNA levels when compared to the levels measured in vehicle-treated and GnRH-treated animals, respectively (fig. 3, 4).

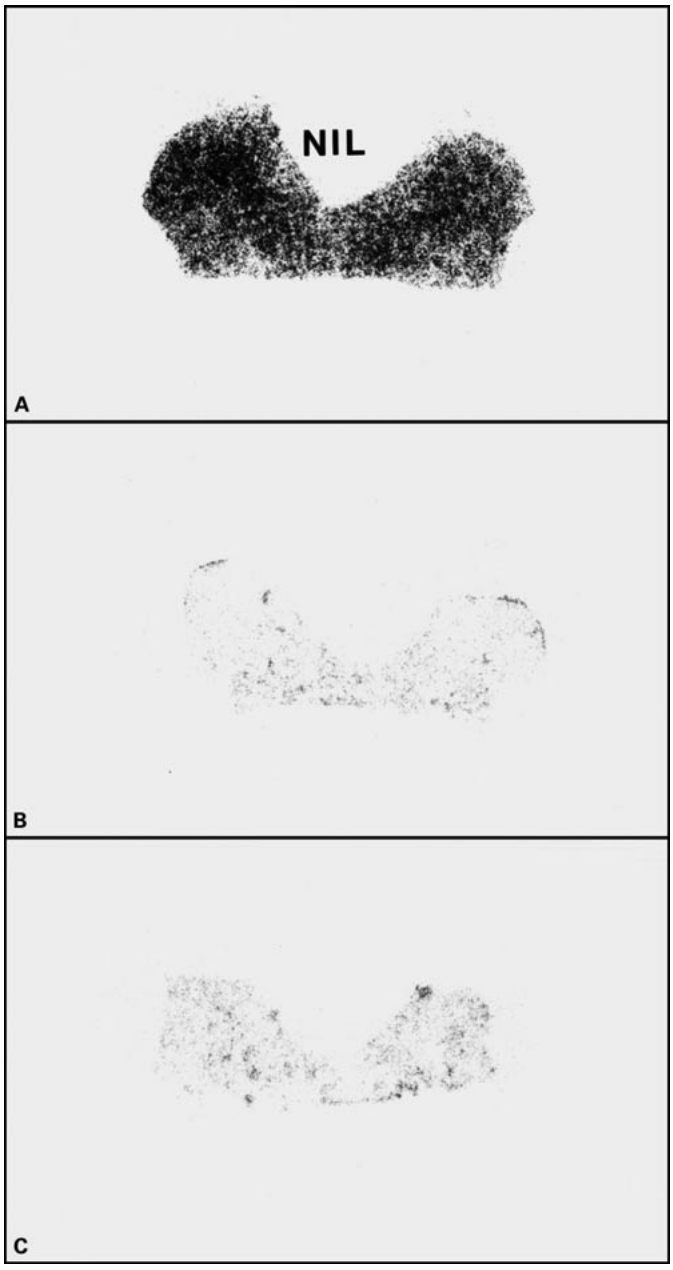

Fig. 5. Typical X-ray autoradiographs showing labelling in the rat anterior pituitary following in situ hybridization with a labelled PRL probe. The neurointermediate lobe (NIL) is devoid of labelling. **A** Intact rats. **B** Ovariectomized rats. **C** Ovariectomized rats treated with EM-652.HCl.

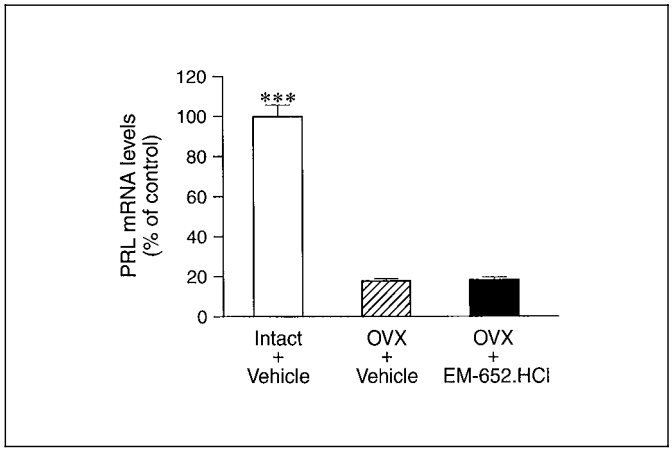

Fig. 6. Effect of ovariectomy and chronic administration of EM-652.HCl to ovariectomized rats on PRL mRNA levels. *** $p < 0.001$, ovariectomized rats vs. the other groups.

PRL mRNA

In intact control animals, the autoradiographic signal obtained following hybridization with the [^{35}S]-labelled PRL cDNA probe was rather uniform throughout the anterior lobe, with the neurointermediate lobe being completely unlabelled (fig. 5A). Pre-treatment of sections with RNase completely prevented any labelling (not shown). We have also previously demonstrated that the signal was completely abolished when the hybridization was performed in the presence of an excess of unlabelled probe [9]. Ovariectomy induced a 82% decrease in the levels of PRL mRNA while chronic administration of EM-652.HCl (49 weeks) to ovariectomized rats did not further modify PRL mRNA levels (fig. 5, 6).

The administration of the antiestrogen to intact animals caused a 56% decrease ($p < 0.001$) in PRL mRNA (fig. 7, 8) while chronic treatment with the GnRH-A induced a 65% decrease ($p < 0.001$) in PRL mRNA expression, an effect which is significantly different from that induced by EM-652.HCl. When EM-652.HCl was administered concomitantly with the GnRH-A, a further 15% decrease in PRL mRNA levels ($p < 0.001$) was observed compared to the effect seen in animals which received only the GnRH-A.

Treatment of intact animals with EM-652.HCl alone induced a marked increase in serum levels of estradiol (50.1 ± 8.49 vs. 8.07 ± 2.52 pg/ml for control animals; $p < 0.001$). In the two groups of animals which received GnRH-A, no serum estradiol could be detected (sensitivity threshold: 5 pg/ml).

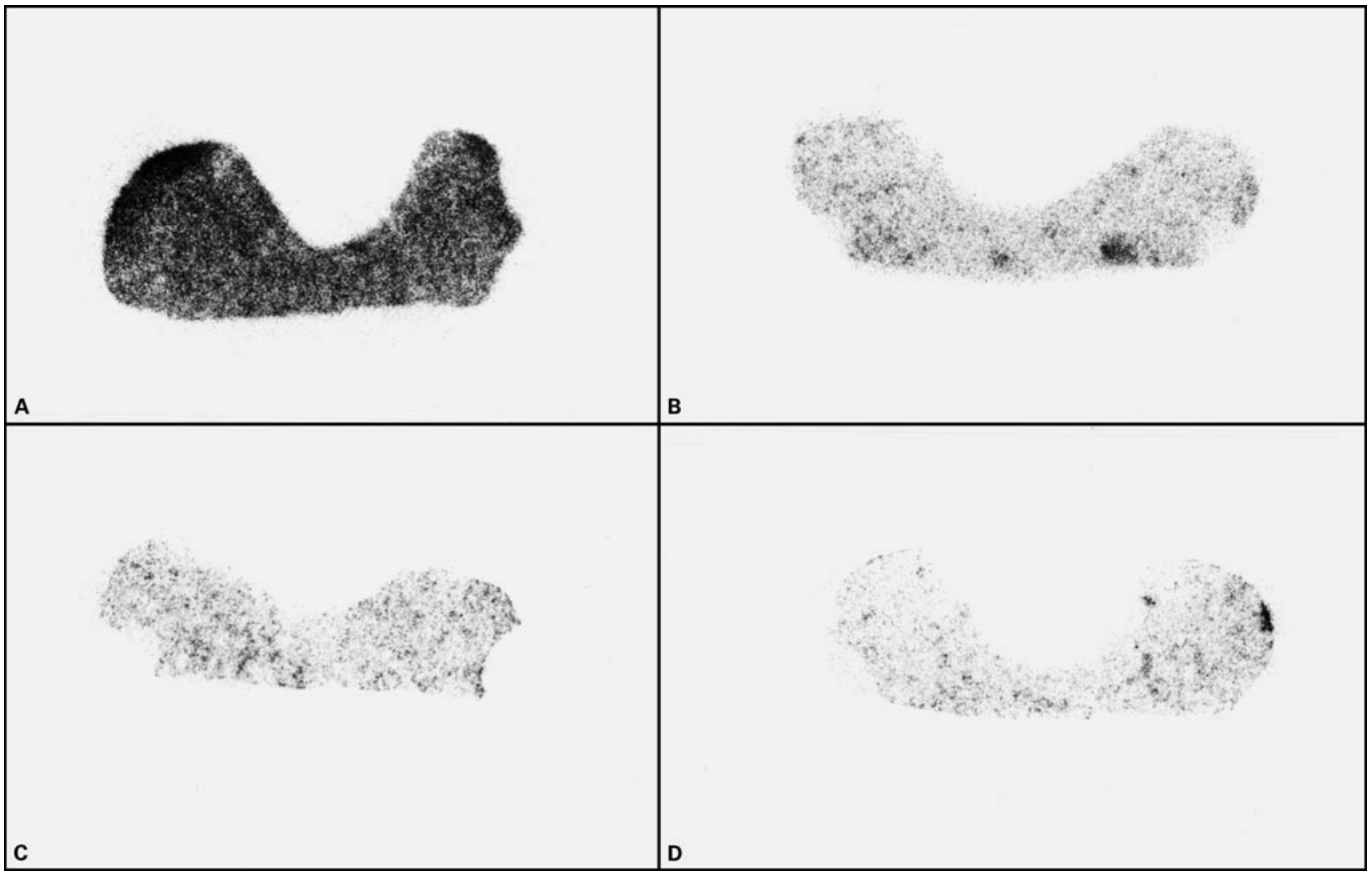

Fig. 7. X-ray autoradiographs illustrating the effect of EM-652.HCl (**B**), GnRH-A (**C**) and the combination of both GnRH-A and EM-652.HCl (**D**) on PRL mRNA expression in the anterior lobe of the pituitary. **A** Intact vehicle-treated animals.

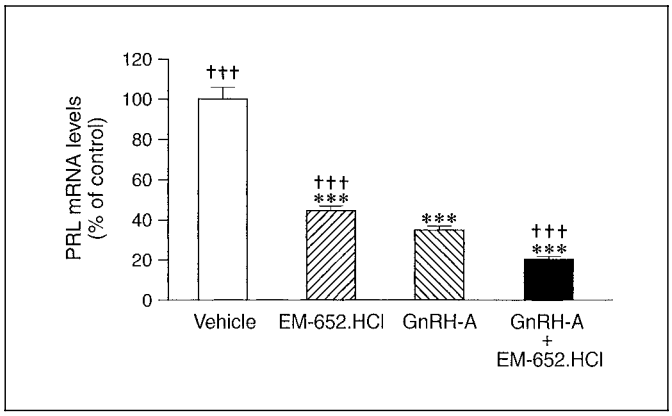

Fig. 8. Effects of EM-652.HCl administration alone and in combination with GnRH-A on PRL mRNA levels. Results are expressed as a percentage of the control value (intact vehicle-treated animals). *** $p < 0.001$, vehicle-treated rats vs. all the other experimental groups. ††† $p < 0.001$, GnRH-A-treated rats vs. all the other experimental groups.

Discussion

The present results clearly indicate that pituitary ER mRNA decreased by more than 50% after long-term ovariectomy. Such results are in agreement with a previous report indicating that in the rat pituitary ERα mRNA was decreased 3 weeks following ovariectomy and that administration of estradiol during 24 h restored ERα mRNA levels to those observed in intact controls [18]. Friend et al. [17] have also shown that 3-day treatment of rats ovariectomized 2 weeks previously with estradiol increased the levels of the pituitary full-length ER mRNA 2- to 3-fold. Altogether, these observations suggest that estrogens exert a positive regulation of pituitary ERα gene transcription.

In ovariectomized animals, EM-652.HCl has been shown to depress ERα mRNA levels. Since similar findings have been obtained in other tissues such as uterus,

vagina and mammary glands in ovariectomized rats [unpubl. data], it is likely that EM-652.HCl is decreasing ERα mRNA levels by a direct effect on estrogen-responsive cells. The mechanism of action of EM-652.HCl in the down-regulation of ERα mRNA levels is currently unknown. The antiestrogen might act on transcription factors to decrease ERα gene transcription and/or decrease the stability of the ERα mRNA. Such a direct effect of EM-652.HCl on ERα mRNA levels might contribute to counteract estrogen action at the anterior pituitary level. On the other hand, it cannot be totally excluded that EM-652.HCl could exert some indirect influence on pituitary cell ERα via the hypothalamus. We have recently shown, that in ovariectomized rats, EM-652.HCl was able to depress GnRH mRNA levels in preoptic area neurons [33].

In agreement with previous data [6–8], a marked depression of PRL mRNA level was observed following ovariectomy. A major decrease in PRL mRNA was observed in the ERα gene-disrupted mice, thus suggesting that this ER subtype is involved in the influence of estrogens on PRL gene transcription [23]. The present results also indicate that EM-652.HCl is acting as a pure antagonist in PRL expression regulation, since it is ineffective in the absence of circulating estrogens.

In contrast with the results obtained in ovariectomized rats, the long-term administration of the antiestrogen to intact animals did not modify ERα mRNA levels. This absence of effect of EM-652.HCl is possibly related to the marked increase in plasma levels of estradiol. At the dose of EM-652.HCl used, it might be possible that the antiestrogen could not completely block the estrogenic effect on ER mRNA expression. Moreover, the decrease of PRL mRNA levels following treatment with the antiestrogen was less than that induced by ovariectomy.

When estradiol levels were depressed by chronic administration of the GnRH-A, then the ERα mRNA levels were significantly further reduced by EM-652.HCl. Moreover, the decrease in PRL gene transcription induced by GnRH-A administration was not as striking as that observed in ovariectomized animals, indicating that the levels of estradiol were not completely suppressed by the GnRH-A. In fact, when EM-652.HCl was administered concomitantly with the GnRH-A, the levels of PRL mRNA were further reduced, reaching the levels observed in ovariectomized animals. On the other hand, the reduction in PRL mRNA levels caused by EM-652.HCl in the intact animals clearly indicate that a decrease in ERα mRNA expression is not required for the negative regulation of PRL mRNA expression by the antiestrogen.

The present data show that estrogens are positively regulating pituitary ERα gene transcription and that the antiestrogen EM-652.HCl can induce by itself downregulation of pituitary ERα mRNA levels.

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