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Fibroblast Growth Factor-2 and Epidermal Growth Factor Modulate Prolactin Responses to TRH and Dopamine in Primary Cultures

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Fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) are expressed in most tissues of the organism including pituitary. FGF-2 increases PRL levels and PRL mRNA in GH3 cells and primary cultures, and it has been involved in the lactotroph proliferation and hyperplasia. EGF also increases PRL levels in vitro. However, the effects of these two factors in the responses of lactotroph cells to TRH and dopamine (DA) remain to be clarified. In the present work we have studied the modulator activity of FGF-2 and EGF on in vitro PRL in responses to TRH and DA in primary cultures from in vivo vehicle- or estrogen (E2)-treated rats. We have found that FGF-2 (2×10^{-11} *M*) prevents the EGF-induced dose-dependent increase in PRL levels in control cells, and reversed the EGF-stimulating effects in cells from E2-treated rats. Both FGF-2 (2×10^{-11} *M*) and EGF (6.6×10^{-9} *M*) significantly increase (>30% and >120%, respectively) the PRL levels in response to TRH (10^{-6}, 10^{-5} *M*). FGF-2 blocked the inhibitory effects of low doses of DA (10^{-9} *M*). EGF was unable to do so, although markedly increased (>200%) the post-DA PRL rebound. In cells from in vivo E2-treated rats, FGF-2 increased (>50%) the PRL secretion in response to TRH, while EGF reduced responses to high doses of TRH (10^{-6}, 10^{-5} *M*). In addition, FGF-2 reversed and EGF increased the inhibitory effects of DA. Both FGF-2 and EGF completely blocked the post-DA PRL rebound, in these cells. Taken together our data suggest that FGF-2 and EGF are important regulators of lactotroph responsiveness to TRH and DA in vitro, although their actions are highly dependent on estrogenic milieu.

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Introduction

The basic form of the fibroblast growth factor (bFGF), also known as FGF-2, is a potent mitogen for mesoderm- and neuroectoderm-derived tissues. The pituitary produces the largest quantity of FGF-2 per gram of tissue of all the organs studied *(1)*. The major source of FGF-2 within the pituitary appears to be the folliculostellate cells *(2)*. The FGF receptors (FGFR) are also expressed in the pituitary *(3,4)*. Therefore, the FGF-2 system may be important in the regulatory functions of the hypothalamus and pituitary gland *(3)*. Previous reports have studied the effects of FGF-2 in the regulation of pituitary secretions *(5,6)*. On the basis of that research, it has been stated that FGF-2 acutely increases PRL and TSH levels in pituitary primary cultures after long incubations (48 h) in medium containing high concentrations of FGF-2 *(7)*, and also that FGF-2 and FGF-1 induced a marked increase in PRL mRNA expression in GH3 cells (a cell line derived from a rat pituitary tumor) *(8)*. We and other authors have also previously shown that FGF-2 induced a marked increase in PRL secretion in primary pituitary cells in a dose-dependent manner, and that heparin is capable of modulating the responses to FGF-2 *(9)*. The stimulatory effects on the secretion of the anterior pituitary seem to be independent of FGF-2 proliferative and differentiating activity, which was proven in rat pituitary cells *(5)* and also in human adenomas *(10)*.

Other growth factors, such as the epidermal growth factor (EGF), have also been shown to have important regulatory effects on the secretory functions of the anterior pituitary. EGF, a polypeptide isolated from the mouse submandibular gland *(11)*, was initially described as a mytogenic factor for normal and tumor tissues, as it induces differentiation and modulates proliferative functions *(12)*. EGF has also been found in considerable quantities in the anterior

pituitary and it appears to be secreted by some subpopulations of endocrine cells of the anterior pituitary gland *(13)*. It has been postulated that EGF may work as a regulatory factor for the anterior pituitary secretion, especially in lactotroph and somatotroph cells, as the EGF-binding sites appear to be restricted to these cell types *(14)*. It has been reported that EGF increases the PRL mRNA accumulation *(15)*, and PRL *(16)*, GH *(17)*, and ACTH *(18)* secretion.

The hypothalamic control of PRL secretion is mainly exerted by two factors: dopamine (DA), which tonically inhibits PRL secretion *(19,20)*, and thyrotropin releasing factor (TRH), which stimulates PRL secretion *(21)*, although many other neuropeptides, neurotransmitters, and hormones may modify the circulating PRL levels. TRH is the major PRL-releasing factor (PRF) for the lactotroph axis. TRH is produced in the paraventricular nucleus of the hypothalamus and released into the hypophyseal portal system *(22)*. The physiological role of TRH on PRL release remains disconcerting because under many physiological conditions there is a dissociation between the release of PRL and TSH. For example, TRH immunoneutralization has only very modest effects on PRL secretion from pituitary explants *(23)*. And TRH activity on PRL secretion is dependent on many other factors, such as substance P *(24)* or EGF *(25)*.

Dopamine is the main regulatory inhibiting factor for PRL, and PRL release is tonically inhibited by hypothalamic DA *(7)*. DA accounts for the inhibitory activity exerted by the hypothalamus on the lactotroph axes *(26)*. The remaining third appears to be made up from GABA and somatostatin inhibitory effects. The inhibitory action of DA on PRL appears to be mediated by the activation of the type 2 dopaminergic receptor (D2r), present on the surface of the membrane of lactotroph cells. This receptor is coupled to Gi proteins and it is responsible not only for the suppression of PRL secretion but also for the inhibition of the expression of the PRL gene and lactotroph proliferation. Moreover, the knockout mice for this receptor develop lactotroph hyperplasia and prolactinoma *(20)*. Thus, the role of DA as a physiological PRL-inhibiting factor (PIF) seems to be firmly established.

However, the regulation of PRL secretion is in fact very complex, and many other factors may have a significant role in particular situations. The role of estrogens has to be emphasized, which have been described as a competence factor for lactotroph cells and as necessary for the expression of the PRL gene. Indeed, it is commonly known that in vivo treatment with estrogens leads to an increase in PRL secretion in humans *(27)* and rats *(28)*, in addition to increasing the PRL secretion of the pituitary culture cells *(29)*. The stimulatory effect of estrogens is likely to be due to action at the pituitary, on the one hand, increasing the transcription rate of the PRL gene *(30)*, and on the other hand, estrogens may also act at the hypothalamic level, facilitating the release and activity of stimulatory hypophysiotropic factors such as TRH and VIP *(31)*. The estrogens besides promoting the proliferation of lactotroph cells *(32)* also induce the expression of the PTTG (pituitary tumour transforming gene) and local production of FGF-2, playing an important role in the early development of lactotroph hyperplasia *(29)*.

Taken together, the data regarding the effects of FGF-2 and EGF on PRL secretion previously reported remain to be clarified as to whether these growth factors might affect the PRL secretion in response to TRH and DA, up to now considered to be the main regulatory factor for PRL secretion and lactotroph function. In this report we addressed this question in in vitro pituitary primary cultures. Considering the effects of estrogens as a main modulator of the responsiveness of lactotroph cells and their proven interaction with FGF-2 within the pituitary *(33)*, it becomes of the greatest interest to study whether the effects of in vivo treatment with estrogens (estradiol, E2) might somehow change the effects of FGF-2 and EGF on PRL responses to TRH and DA.

Results

We have previously reported that FGF-2 increases the PRL mRNA levels in GH3 cells *(8)* and it also induces the accumulation of the PRL peptide in the culture medium of primary pituitary cells *(9)*. These effects are time- and dose-dependent, and can be markedly modified by heparin, also in a dose-dependent manner *(9)*. In this work we first studied the interaction of the growth factor EGF on the PRL responses to FGF-2, on primary cultured pituitary cells at incubations of 24 and 48 h (Fig. 1). As expected, FGF-2 (2×10^{-11} *M*) increased basal PRL levels at both 24 and 48 h (Fig. 1), although more potently at 48 h (Fig. 1B). In the absence of FGF-2, increasing doses of EGF proportionally increased the PRL levels at both 24 and 48 h. However, FGF-2 (2×10^{-11} *M*) prevented EGF-induced PRL release after 24 h (Fig. 1A), and reverted the EGF-induced stimulation of PRL secretion to inhibitory, following 48 h of coincubation (Fig. 1B).

As was previously known *(34)*, and is shown in Fig. 2, TRH increases PRL levels in a time-dependent manner, in the culture medium of pituitary primary cells from normal (Fig. 2A) and previously in vivo E2-treated rats (Fig. 2B). The effects of TRH on PRL appear to occur after a few seconds of exposure of the cells to TRH, although the best responses were obtained at 1 and 3 h. Therefore, we have chosen 3 h as the preferred incubation time to study the possible effects of TRH-induced PRL increase, unless otherwise stated. We have studied the effect of both EGF and FGF-2 on the 3-h PRL responses to increasing doses of TRH (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} *M*) (Fig. 3). The FGF-2 did not modify the TRH-induced PRL increase, except for the higher doses of FGF-2 (10^{-5} *M*), in cells from vehicle-treated rats (Fig. 3A). However, FGF-2 significantly increased PRL accumulation in the medium of the pituitary primary cells from in vivo E2-treated rats at all doses of TRH (Fig. 3B).

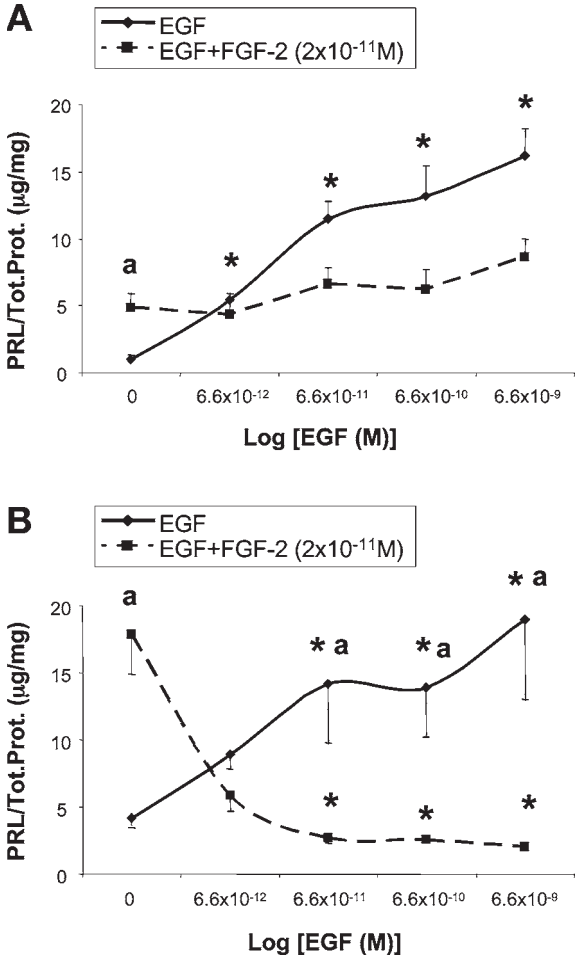

Fig. 1. Effects of FGF-2 in PRL responses to EGF in primary pituitary culture cells. Mean ± SD of two different experiments, run in quadruplicate (6 pituitaries/experiment). Mann–Whitney test. **a** $p < 0.05$ control vs FGF-2 (2×10^{-11} *M*). Duncan test, $*p < 0.05$, EGF vs EGF + FGF-2 (**A**) dose-response curve to EGF and EGF + FGF-2 at 24 h; (**B**) dose-response curve to EGF and EGF + FGF-2 at 48 h.

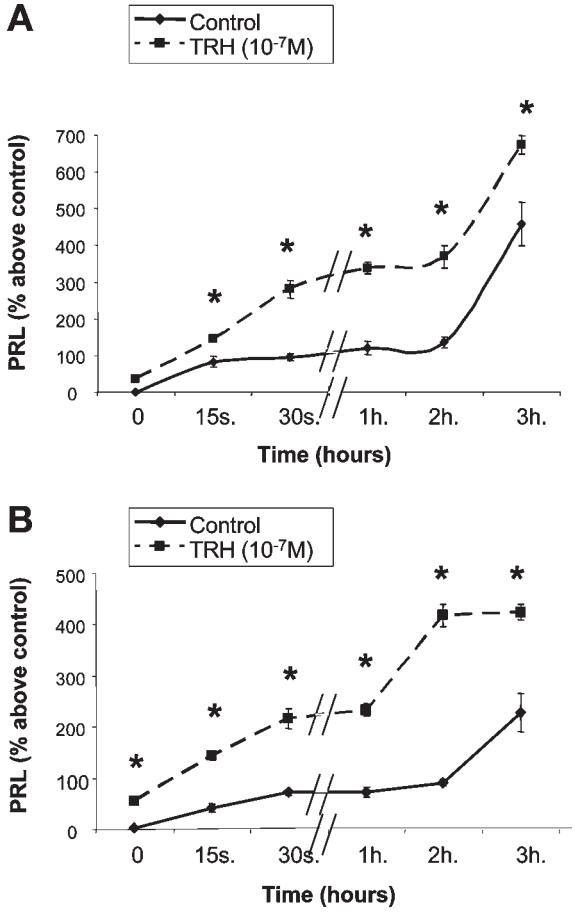

Fig. 2. PRL responses to TRH in primary pituitary culture cells. Mean ± SD of two different experiments, run in quadruplicate (6 pituitaries/experiment). Duncan test, $*p < 0.05$, TRH vs control. (**A**) Time-response curve to TRH, in cells from in vivo vehicle-treated rats. (**B**) Time-response curve to TRH, in cells from in vivo estrogenized rats.

In contrast, EGF increased the PRL levels in response to the higher doses of TRH (10^{-6}, 10^{-5} *M*) in pituitary primary cells from in vivo vehicle-treated rats and significantly reduced the PRL levels in response to the same doses of TRH in cells from in vivo E2-treated rats (Figs. 3C,D).

We have also studied the effects of FGF-2 and EGF on the inhibitory responses to DA. Dopamine efficiently reduced the PRL levels in a dose-dependent manner after 2 h incubations, in pituitary primary cultures from vehicle-treated rats, and FGF-2 was able to markedly reduce the inhibitory effect of the lowest doses of DA used (10^{-9} *M*: 96.20 ± 1.87% vs 53.02 ± 10.38%) (Fig. 4A). Moreover, prior in vivo E2 administration to rats resulted in DA, although conserving some ability to reduce the accumulated PRL levels in the culture medium, was much less effective (Fig. 4B). Then, the addition of FGF-2 (2×10^{-11} *M*) completely reversed the inhibitory effects of DA, even inducing a clear increase in PRL levels at some of the doses employed (10^{-8}, 10^{-7}, and 10^{-6} *M*) (Fig. 4B). Interestingly, under the same conditions, EGF did not change the inhibitory effect of DA on PRL levels of pituitary cells of vehicle-treated rats (Fig. 4C). But EGF clearly improved the inhibitory effects of DA on PRL accumulation in the culture medium of cells from in vivo E2-treated rats (Fig. 4D).

Finally, both FGF-2 and EGF also modified the increase in the PRL levels after the dopaminergic inhibition (post-DA PRL rebound) as shown in Fig. 5. FGF-2 did not change the magnitude of the post-DA PRL rebound in cells from vehicle-treated rats (Fig. 5A). On the contrary, EGF enhanced the post-DA PRL rebound at all doses used in cells from vehicle-treated rats (Fig. 5C). On the other hand, in cells from in vivo E2-treated rats both FGF-2 and EGF markedly reduced the rebounded PRL secretion to the higher doses of DA (10^{-6}, 10^{-5} *M*) (Figs. 5B,D, respectively). Unexpectedly, EGF at doses of 10^{-7} *M* increased the post-DA PRL rebound in cells from E2-treated rats.

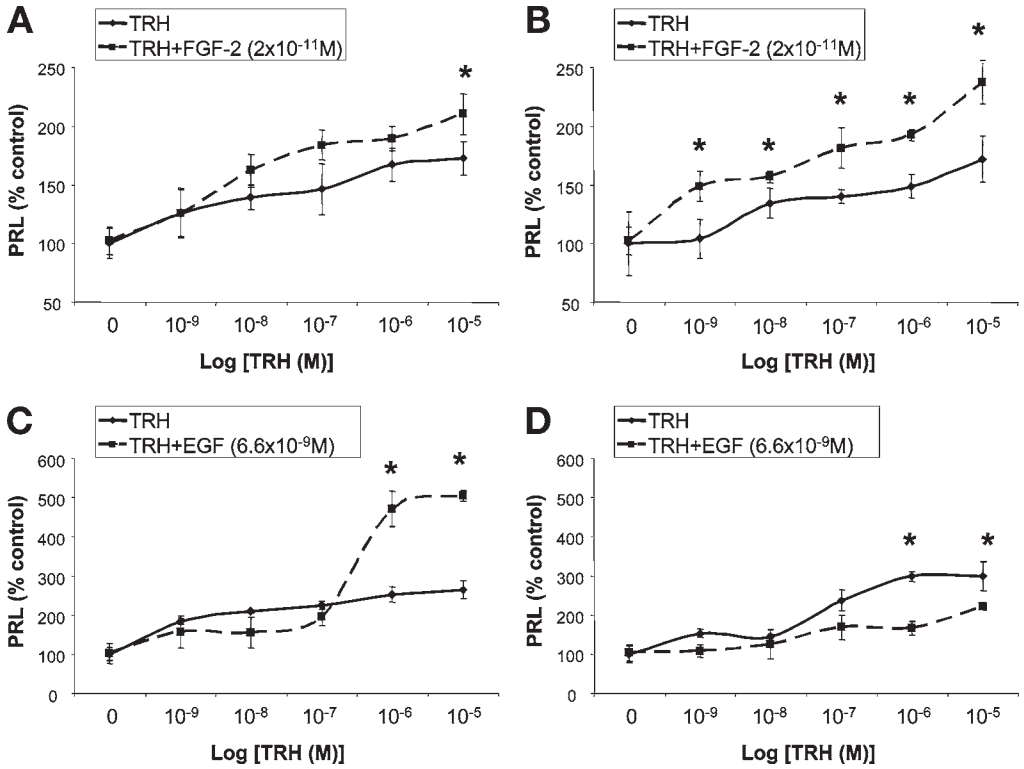

Fig. 3. Effects of FGF-2 or EGF in PRL responses to TRH in primary pituitary culture cells. Mean ± SD of two different experiments, run in quadruplicate (6 pituitaries/experiment). Mann–Whitney test. *$p < 0.05$, TRH vs TRH + FGF-2 (2×10^{-11} *M*) or TRH + EGF (6.6×10^{-9} *M*), respectively. (**A**) Dose-response curve to TRH and TRH + FGF-2, in cells from in vivo vehicle-treated rats. (**B**) Dose-response curve to TRH and TRH + FGF-2, in cells from in vivo estrogenized animal. (**C**) Dose-response curve to TRH and TRH + EGF, in cells from in vivo vehicle-treated rats. (**D**) Dose-response curve to TRH and TRH + EGF, in cells from in vivo estrogenized animal.

Discussion

It is well known that both inhibitory and stimulatory factors control PRL secretion. It is commonly accepted that DA is the main prolactin inhibiting factor (PIF), and its secretion from the hypothalamus is considered the major regulatory issue for the lactotroph cells of the anterior pituitary. On the other hand, many hormones, neuropeptides, and neurotransmitters have been identified as having stimulatory effects on PRL gene expression and PRL release from the pituitary, in vivo and in vitro. As previously described, FGF-2 increases the PRL levels in the culture medium *(5,8, 9,35,36)*, like other growth factors, EGF *(37)*, HB-EGF *(15,16,38)*, or VIP *(39,40)*, while TGFβ1 downregulated PRL secretion *(41,42)*.

FGF-2 has been identified as having important paracrine actions within the pituitary. FGF-2, acutely administered, induces PRL and TSH secretion in rat pituitary cells in primary culture, in a dose-dependent manner, but only when the cells are pre-incubated in vitro with a large amount of FGF-2 for at least 48 h *(5)*. In a previous study, we showed that FGF-2 induces a marked increase in PRL secretion in primary culture lactotroph cells in long-term incubations *(9)*, and that FGF-2 and FGF-1 markedly increase PRL mRNA levels as well as PRL levels in culture media, in GH3 cells *(8)*. We here show that EGF and FGF-2 alone promote PRL secretion in vitro, but when both growth factors are combined, FGF-2 prevents EGF-induced PRL release after 24 h, and reverts the EGF-induced stimulation of PRL secretion to inhibitory, following 48 h of co-incubation. So it appears that FGF-2 and EGF functionally antagonize one to each other the effects on PRL release in pituitary cells.

TRH potently increases PRL release in cells from both in vivo vehicle- or E2-treated rats (Fig. 2). We here show both FGF-2 and EGF enhance TRH-induced PRL secretion in vitro, although the differences in estrogenized rats is very small, in contrast to a previous report showing that the acute administration of FGF-2 reduced PRL secretion and blocked TRH-induced PRL release in cells from in vivo E2-treated rats *(35)*. However, in that report the authors carried out treatments over short time periods (30–120 min) with very high doses of FGF-2 ($>10^{-6}$ *M*), which may explain the differences with our results.

On the other hand, physiological PRL secretion is under the tonic inhibitory control of DA released from the tuberoinfundibular dopaminergic neurons to the hypophyseal portal blood. PRL release is inhibited by DA by means of D2 receptors present in the lactotroph cells *(20)*. However, when lower doses of DA than those required for inhibition are

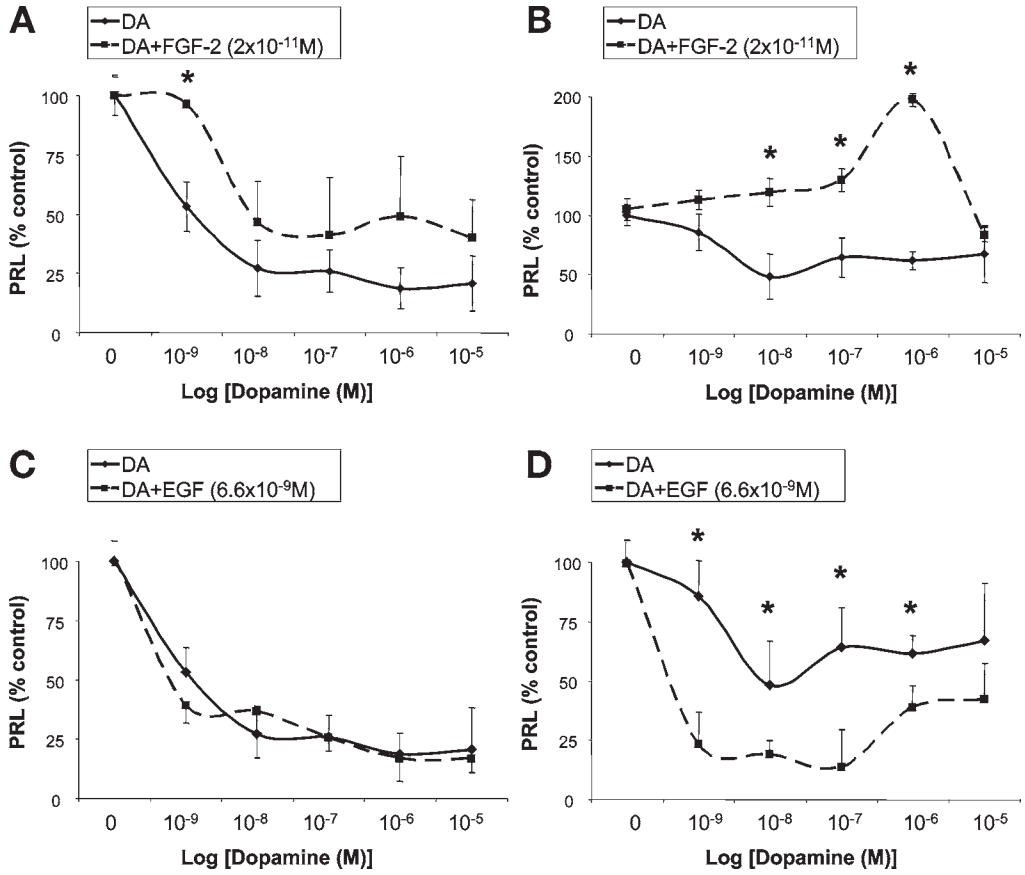

Fig. 4. Effects of FGF-2 or EGF in PRL responses to DA in primary pituitary culture cells. Mean ± SD of two different experiments, run in quadruplicate (6 pituitaries/experiment). Mann–Whitney test. *$p < 0.05$, DA vs DA + FGF-2 (2×10^{-11} *M*) or DA + EGF (6.6×10^{-9} *M*), respectively. (**A**) Dose-response curve to DA and DA + FGF-2, in cells from in vivo vehicle-treated rats. (**B**) Dose-response curve to DA and DA + FGF-2, in cells from in vivo estrogenized rats. (**C**) Dose-response curve to DA and DA + EGF, in cells from in vivo vehicle-treated rats. (**D**) Dose-response curve to DA and DA+ EGF, in cells from in vivo estrogenized rats.

used, DA can stimulate PRL secretion in vitro, presumably by the activation of D1 receptors *(43,44)*. We have found and here report that FGF-2 can modulate the action of DA, impeding the inhibition of PRL secretion by DA in vitro (Fig. 4), while EGF was unable to do so, according to previous reports *(35)*. Nevertheless, when we study the post-DA PRL rebound in cells from vehicle-treated rats, we found that EGF increased it of significant form. This information indicated us that that the lactotroph cells could rise to basal state more quickly with EGF, although it did not modify the inhibition of PRL secretion by DA.

Interestingly, the in vivo estrogenization of rats resulted in a change in the FGF-2 responses to PRL secretion induced by TRH, and inhibition by DA. In the presence of FGF-2, DA was not only unable to reduce the PRL levels in the culture medium, but even induced a marked dose-dependent increase in the in vitro PRL levels of cells from in vivo E2-treated rats. An explanation of this effect might be that in a high-estrogen milieu, exogenous FGF-2 might increase the expression of D1 receptors in the lactrotoph cells *(46)*. On the other hand, EGF did not show these effects. In fact, EGF appears to increase the dopaminergic inhibition of PRL in cells from in vivo E2-treatred rats. Interestingly, both growth factors blocked the post-DA PRL rebound in cells from in vivo E2-treated rats. The effect of FGF-2 occurs with doses close to those FGF-2 circulating levels found in vivo *(32,45)*, and, given the magnitude of the effect, it indicates that FGF-2 is important in the responses of lactotroph cells to DA.

Explanation for all these data appears to be complex. TRH, DA, FGF-2, and EGF receptors signal primarily via activation of phospholipase and phosphoinositide-PKC pathways, through initial stimulation of G proteins and tyrosine kinase receptors in pituitary cells. The activation of all these receptors converge in up/downregulation of MAPK and up/downregulation of different transcription factors, promoting or diminishing of PRL gene transcription *(47–51)*. With our information only we postulated that FGF-2 and EGF could easily modulate different aspects of TRH and DA signaling, modifying the PRL responses in pituitary cells.

When we introduced the estrogenic effects in our experiments, we increased the complexity of the system. In our results we found that estrogens were able to change the gen-

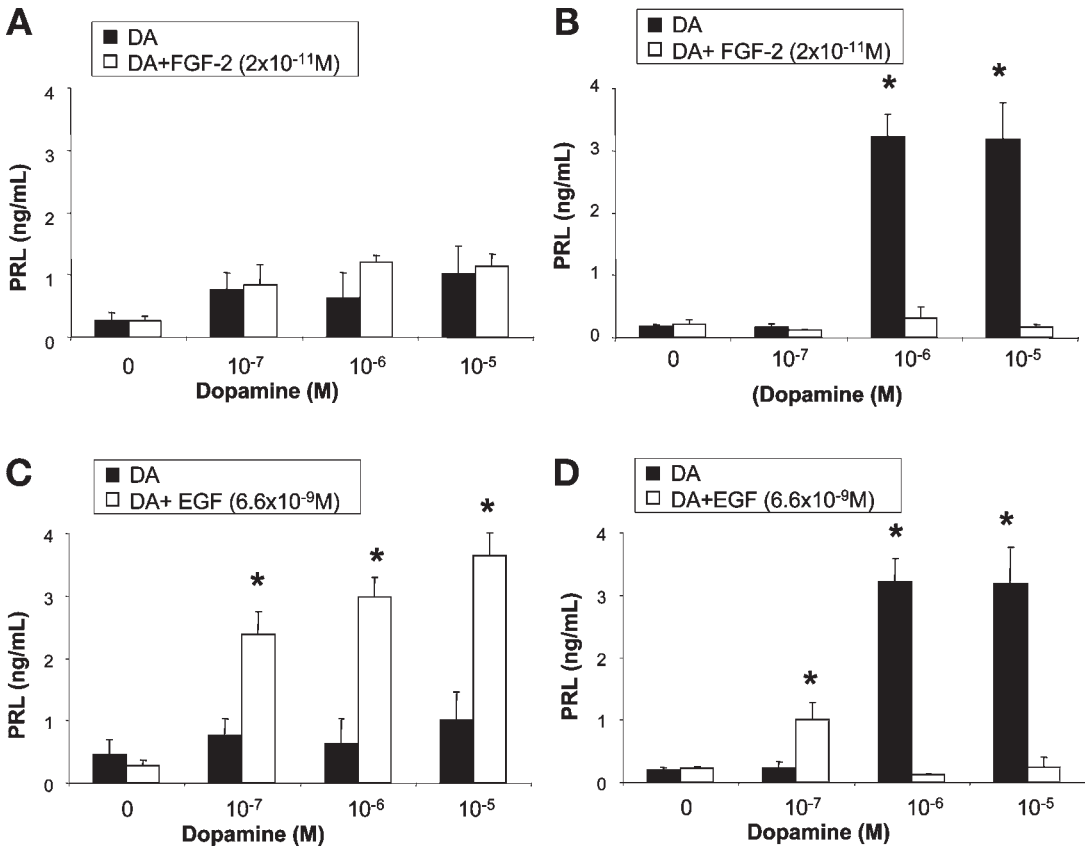

Fig. 5. Effects of FGF-2 or EGF in the post-DA PRL rebound in primary pituitary cells in vitro. DA alone or DA+FGF-2/EGF were added to medium and incubated for 2 h; later, the medium was removed and fresh medium added (without DA, FGF-2 or EGF), and cultures was incubated for 1 h, until collection of medium. Mean ± SD of two different experiments, run in quadruplicate (6 pituitaries/experiment). Mann–Whitney test. *$p < 0.05$, DA vs DA + FGF-2 (2×10^{-11} *M*) or DA + EGF (6.6×10^{-9} *M*), respectively. (**A**) Dose-response to DA and DA+ FGF-2 (2×10^{-11} *M*) in cells from *in vivo* vehicle-treated rats. (**B**) Dose-response to DA and DA + FGF-2 (2×10^{-11} *M*) in cells from in vivo estrogenized rats. (**C**) Dose-response to DA and DA + EGF (6.6×10^{-9} *M*) in cells from in vivo vehicle-treated rats. (**D**) Dose-response to DA and DA + EGF (6.6×10^{-9} *M*) in primary culture cells from in vivo estrogenized rats.

eral regulation of PRL secretion induced by TRH or DA when we administered FGF-2 and EGF. The estrogens were able to modulate many physiological aspects of pituitary cells. On the one hand, we were able to induce FGF-2 and VEGF expression in the pituitary *(29)*, on the other hand, the estrogens through second messenger pathways could also have the capability to globally change the responsiveness of lactotroph cells to the mentioned regulatory factors. With our information we could not know where the estrogens modify the PRL regulation; for these, specific studies should be needed to clarify this point.

In summary, TRH and DA are recognized as the primary regulators for PRL secretion. DA inhibits basal PRL secretion while TRH stimulates it. However, there are paracrine factors such as FGF-2 and EGF that modulate the response of PRL to TRH and DA, as well as inducing PRL secretion alone. Indeed, FGF-2 and EGF increases the PRL secretion induced by TRH and the inhibitory effect of DA on PRL sercretion (Fig. 6). Nevertheless, when we increase the estrogens in the milieu, there is a large modification of PRL secretion induced by TRH and DA.

In conclusion, we show here that FGF-2 and EGF increase the PRL levels in the medium of primary cultures in vitro. Interestingly, both growth factors behave as functional antagonists to each other, when both are present at the same time in the culture medium. FGF-2 and EGF modulate the responses of the lactotroph cells to TRH and DA, although with particular actions each one and their effects on in vitro PRL secretion are highly dependent on the estrogenic milieu.

Materials and Methods

Adult female Sprague–Dawley rats weighing 150–200 g were housed in a constant dark–light cycle (12:12 h). Standard pellet chow (A04, Panlab S.L., Barcelona, SP) and tap water were available *ad libitum*. Animal manipulations were carried out following the conventions and ethical rules included in Directive 86/609/CEE of the European Union. Half of randomly cycling rats were administered with 3-benzoate 17β-estradiol (Sigma-Aldrich Quimica SL, Alcobendas, SP), 5 mg/kg every 3–4 d, sc, during 2 wk and the

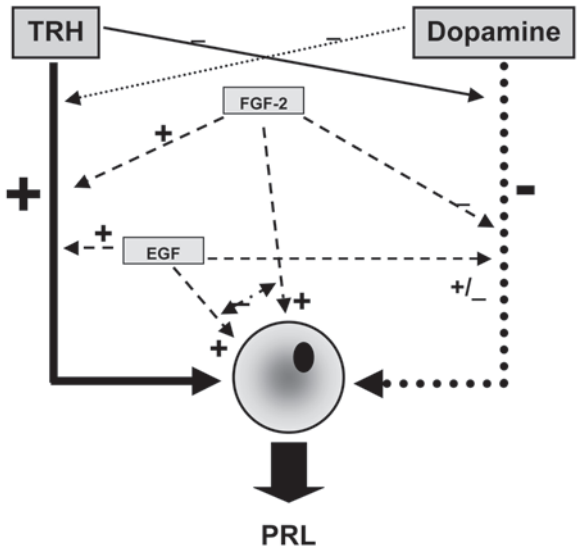

Fig. 6. PRL responses to TRH, DA, and modulation by FGF-2 and EGF in the normal pituitary cells cultured in vitro. TRH and DA is recognized as the primary regulator factors for PRL secretion. Dopamine inhibits basal PRL secretion while TRH stimulates the same. However, there are paracrine factors such as FGF-2 and EGF that may modulate the response of PRL to TRH and DA, as well as inducing PRL secretion alone. Indeed, FGF-2 and EGF increase the PRL secretion induced by TRH and the inhibitory effect of DA on PRL secretion, depending on estrogenic milieu. FGF-2 increases PRL secretion induced by TRH and reduced DA inhibition. While EGF also increases TRH effects, do not modify the inhibitory responses to DA, but increases the post-DA PRL rebound. However, in lactotroph cells from in vivo estrogenized rats, FGF-2 increase and EGF decrease the PRL responses to TRH, FGF-2 reversed and EGF boosts the inhibitory action of DA on PRL secretion, and both blocked the post-DA PRL rebound.

other half were administered oil (control group) After this period, the estrogenized rat pituitaries had increased in size and appeared to be hyperplasic *(52)*.

The anterior pituitaries were collected, by rat decapitation under pentobarbital anaesthesia (50 mg/kg in 0.1 *M* CO_3Na_2), in EBSS (Sigma-Aldrich Quimica SL). Pituitaries were washed with fresh EBSS, minced, and incubated in EBSS containing 0.1% trypsin (Sigma-Aldrich Quimica SL) at 37°C, for 30 min. The cell suspension was centrifuged for 5 min at 720*g*, and then washed three times in EBSS. Pellets were resuspended in DMEM (Seromed) containing antibiotics, and cells were mechanically dispersed, yielding viability greater than 95%. The culture medium was DMEM supplemented with 10% FBS (Gibco BRL) and antibiotics: 20 mg/mL ampicillin (Sigma-Aldrich Quimica SL) plus 0.6 mg/mL streptomycin (ICN). Cells were seeded in 24-well plastic culture dishes (Corning) at a density $5–10 \times 10^5$ cells/well (2–3 pituitaries/dish) and incubated at 37°C in a humidity-saturated atmosphere containing 5% CO_2 for 4 d. On the fifth day the medium was replaced with fresh serum-free DMEM plus 1% antibiotics, and exposed to the test substances *(53)*. Primary cultures of dispersed pituitary cells contained approx 30% of lactotroph cells, and with pituitaries of estrogenized rats there was an increase in the lactotroph population, reaching as much as 50–60%.

Experiments were carried out in serum-free medium containing TRH (Sigma-Aldrich Quimica SL) at 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} *M* doses, with/without FGF-2 (Sigma-Aldrich Quimica SL) at 2×10^{-11} *M*, and with/without EGF (Sigma-Aldrich Quimica SL) at 6.6×10^{-9} *M* for the dose-response studies. The study of DA (Sigma-Aldrich Quimica SL) effects was carried out in an independent experiment using 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} *M* doses, with/without FGF-2 at 2×10^{-11} *M*, and with/without EGF at 6.6×10^{-9} *M*. In all cases the best responses to TRH and DA were obtained at 3 h; for this reason, the following studies were carried out at 3 h, unless otherwise stated. To study whether FGF-2 or EGF modify the post-DA PRL rebound, we carried out co-incubations of DA with FGF-2 or EGF. The control was DA without FGF-2 or EGF. The doses for DA were 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} *M* for FGF-2 2×10^{-11} *M*, and for EGF 6.6×10^{-9} *M*. Dopamine alone or DA + FGF-2/EGF were added to medium and incubated for 2 h; subsequently, the medium was removed, fresh serum-free medium was added, without DA, FGF-2, or EGF, and cultures incubated for 1 h, until collection of the medium. The medium was stored at –20°C until measurements of PRL by specific RIA (reagents gently supplied by Dr. A. F. Parlow NHPP-NIH, USA). Intrassay coefficients of variation were lower than 8%, and assay sensitivity was 1.35 ng/mL.

Total protein in culture medium was used as loading control for each well, measured by the Bradford method *(54)*. Hormone levels are expressed by micrograms of total protein in the culture medium. Thus, units of the graphs are percentage over control.

Statistical analyses were carried out using the Mann–Whitney non-parametric test for comparison between groups, and the Duncan test for comparison between the doses effects respect to the control value. Significance was considered at $p < 0.05$.

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