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Heparin Increases Prolactin and Modifies the Effects of FGF-2 Upon Prolactin Accumulation in Pituitary Primary Cultures

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We have studied the effects of heparin on prolactin accumulation in the medium from primary pituitary cultures, and whether heparin interferes with the effects of fibroblast growth factor-2 (FGF-2) on PRL regulation in vitro. In the absence of exogenous FGF-2, heparin increased prolactin accumulation in the culture medium in a dose-dependent manner. FGF-2 also increased the prolactin levels of primary cells in a time- and dose-dependent manner. However, low doses of heparin reduced the effects of FGF-2, but higher doses of heparin increased the maximal FGF-2-induced prolactin secretion and ED50. In vivo estrogenization of rats resulted in the abolition of FGF-2 capability to promote prolactin release in vitro. However, heparin restored cell responsiveness to FGF-2.

Our results suggest that heparin, when present in the medium, binds FGF-2, therefore reducing its ability to interact with FGF receptors in a dose-dependent manner up to a critical molar concentration, at which heparin itself starts to activate the FGF receptor, and strengthens the activation induced by its proper ligand, FGF-2. Prolactin responses to FGF-2 are blocked by estrogen pretreatment, and it is probable that this introduces lactotroph cells in the proliferative stage. In conclusion, heparin modulates PRL secretion and PRL responses to FGF-2 in vitro.

Key Words: Heparin; fibroblast growth factor-2; FGF-2; bFGF; prolactin; primary cultures; pituitary.

Introduction

Fibroblast growth factors are a large family of peptides, comprised of at least 23 members. The FGF-2 or bFGF is one of the best-characterized members of the FGF family. FGF-2 acts through a high affinity membrane receptor (FGFR) that activates tyrosine kinases *(1)*. This receptor requires dimerization to start the intracellular signaling cascade *(2)*. In addition, the majority of target cells have low-affinity binding sites for FGF-2: glycosaminoglycans such as heparin or heparan sulfate proteoglycans (HSPGs). These low-affinity binding sites are located on the cell surface and in the extracellular matrix. FGF-2 lacks a signal peptide, which is usually present in all the peptides to be secreted. It has been proposed that FGF-2 may be released with the extracellular matrix of the tissues *(3)*, actually bound to heparan sulfate compounds. It is well known that FGFs bind HSPGs and heparin, which is why they are also known as heparin-binding growth factors *(4)*. The importance of FGF-2–heparin interactions has been demonstrated by various observations such as heparin protection for FGF-2 from denaturization and enzymatic degradation. HSPGs' high capacity for binding with FGF-2, together with the fact that many cell types possess heparin-like molecules on their surface nearby the high affinity receptor, suggest a physiological role for heparin-like low-affinity FGF binding sites *(5)*.

The anterior pituitary is able to synthesize FGF-2 *(6)*. In fact, FGF-2 is present in large amounts in rat pituitaries, being the organ that produces the greatest quantity of FGF-2 per gram of tissue. Folliculostellate cells seem to be the major source of FGF-2 within the pituitary *(7)*. Previous reports have studied the effects of the FGF-2 in the regulation of the pituitary secretions *(8,9)*. It has been stated that FGF-2 acutely increases PRL and TSH levels in pituitary primary cultures after being in medium containing high concentrations of FGF-2 for 48 h *(10)*. This stimulatory effect seems to be independent of the FGF-2-mitogenic and differentiating activity in the pituitary *(8)*.

In a previous study we reported that FGF-2 and FGF-1 induced a marked increase in the PRL mRNA expression levels in GH_3 cells (a rat cell line derived from a pituitary tumor) after a 24-h incubation period *(11)*. Other authors have also shown that FGF-2 may have a permissive effect on the action of some hypophysiotropic hypothalamic releasing factors, such as TRH-induced PRL and TSH secretion in vitro, which is enhanced by the pre-incubation of pituitary cells with FGF-2 *(10)*. Similarly, the effect of growth hor-

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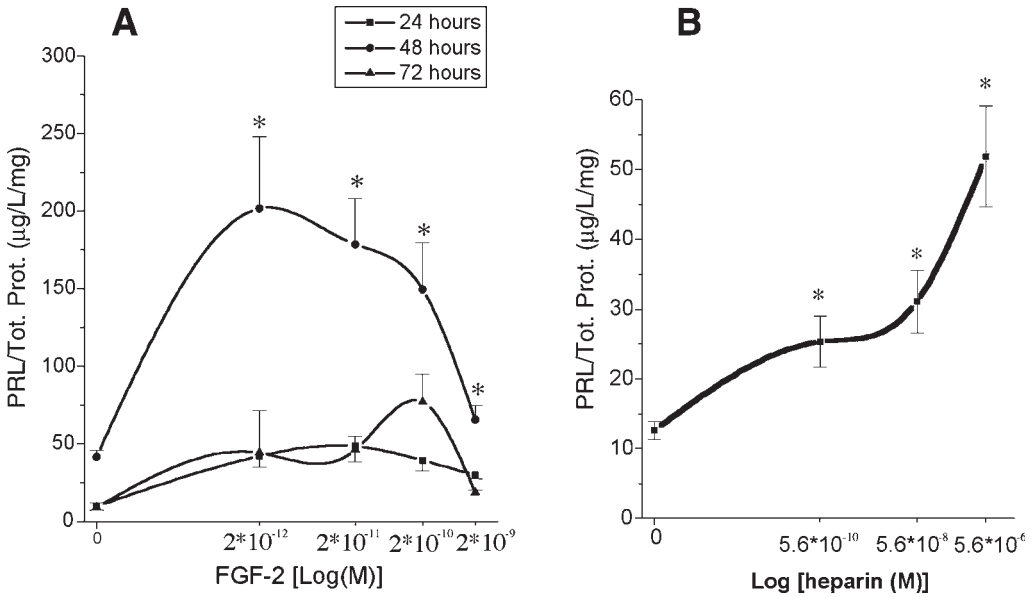

Fig. 1. PRL responses to FGF-2 in primary pituitary cultures. (**A**) Time-responses at different incubations. Mean ± SD of two different experiments, run in quadruplicate (6 pituitaries/experiment). Duncan test. * = $p < 0.05$ vs control (FGF-2 = 0). (**B**) Dose-dependent induction of PRL levels by HEP in primary cultures at 48 h. Mean ± SD of two different experiments, run in quadruplicate (2 pituitaries/experiment). Duncan test. * = $p < 0.05$ vs control (HEP = 0).

mone releasing hormone (GHRH) upon secretion of somatostatin and neurotensin from 44-2C cells (a cell line derived from the hypothalamus) is also enhanced by FGF-2 *(9,12)*.

On the other hand, chronic estrogenization of rats is known to result in the development of anterior pituitary tumors, which particularly affect lactotroph cell differentiation. After 2 weeks of treatment with estradiol (E_2) all rats developed pituitary tumors, associated with increased pituitary weight and serum PRL levels. Furthermore, estrogens cause an increase in vasoactive intestinal peptides (VIP), transforming growth factor (TGF)-β1, and vascular endothelial growth factor (VEGF) *(13,14)*, within the pituitary to induce proliferation of lactotroph cells. In addition, the recently isolated PTTG (pituitary tumor derived transforming gene) appears to be tumorogenic in vivo and to regulate FGF-2 secretion. PTTG is up-regulated by estrogens in vivo and in vitro, and FGF-2 expression is induced by PTTG and estrogens in pituitary tumor cells *(15)*.

In this report we study the effects of heparin on PRL levels in vitro, the effects of its interactions with FGF-2 on PRL release in pituitary primary cultures, and the effects of FGF-2 on lactotroph cells in vitro of anterior pituitary cells obtained from estrogen-induced hyperplasia.

Results

We carried out PRL dose-response to FGF-2 (0, 2.0×10^{-12}, 2.0×10^{-11}, 2.0×10^{-10}, and 2.0×10^{-9} *M*) after incubations of 24, 48, or 72 h. The best responses were obtained at 48 h (Fig. 1A). Further experiments were carried out for 48-h incubations, unless otherwise stated.

Maximal PRL peaks were obtained at a dose of 2.0×10^{-12} *M* (272.13 ± 18.26 µg/L/mg protein), but there was a dose-dependent increase in PRL levels up to 2.0×10^{-12} *M*, and a dose-dependent decrease in PRL levels at higher doses (Fig. 1A). Thus, FGF-2 induced a biphasic bell-shape response curve upon PRL levels in primary cultures.

As represented in Fig. 1B, heparin increases the PRL levels in the medium of primary cultures. In fact, heparin induced a dose-dependent increase in PRL levels, with a maximal response of up to 400% over the control value (Duncan test: $p < 0.05$, at 5.6×10^{-6} *M*).

We also studied the effects of different doses of heparin (0, 5.6×10^{-10}, 5.6×10^{-8}, and 5.6×10^{-6} *M*) on the FGF-2-induced PRL levels. The co-administration of heparin to the primary cultures challenged by FGF-2 resulted in dose-dependent changes in PRL levels (Fig. 2). The lower doses of heparin used (5.6×10^{-10} *M*) reduced the effects of all doses of FGF-2, producing a displacement of the dose-response curve to the right, as revealed by the shift of ED_{50} from 2.41×10^{-12} *M* to 1.1×10^{-11} *M*. A reduction of approx 60% in maximal PRL levels was also observed [161.1 ± 1.17 (FGF-2+heparin) vs 272.13 ± 18.26 µg/L/mg protein (FGF-2), Duncan test: $p < 0.05$]). Second, medium doses of heparin (5.6×10^{-8} *M*) reduced the effect of lower doses and caused an increase in the responses to higher doses of FGF-2, but no further alteration to ED_{50} (1.1×10^{-11} *M* vs 1.25×10^{-11} *M*). In this case the maximal PRL secretion increased by 60% in relation to the control value (435.25 ± 46.38 vs. 272.13 ± 18.26 µg/L/mg protein; Duncan test: $p < 0.05$). Third, higher doses of heparin (5.6×10^{-6} *M*) produced a further right-displacement of the FGF-2 dose-

A

Heparin dose	=	$5.6x10^{-10}$	$5.6x10^{-8}$	$5.6x10^{-6}$
ED50 (FGF-2)	$2.4x10^{-12}$	$1.1x10^{-11}$	$1.25x10^{-11}$	$3.4x10^{-11}$
Maximal PRL level (μg/L/mg prot.)	272.1±18.3	161.1±11.7	435.3±46.4	235.8±15.9

B

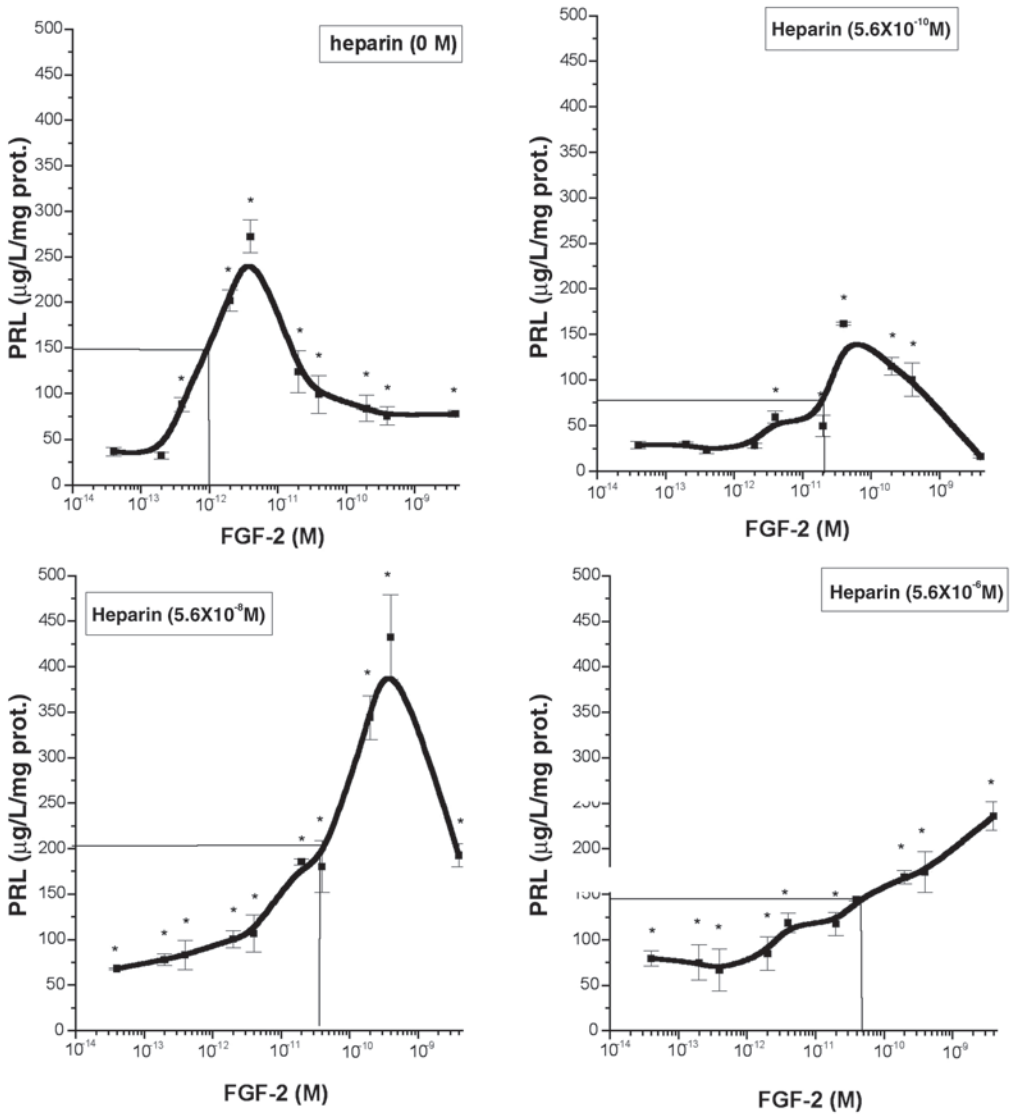

Fig. 2. Dose-dependent effect of HEP on FGF-2-induced PRL levels. PRL responses to FGF in primary cultures at 48 h. Effect of HEP administration at different doses. Mean ± SD of two different experiments, run in quadruplicate (4 pituitaries/experiment). Duncan test. * = $p < 0.05$ vs control (FGF-2 = 0). (**A**) HEP increases EF_{50} (shift to the right), then reduces FGF-2 potency on PRL release. (**B**) HEP reduces FGF-2-induced maximal PRL levels at low doses, but increases the whole secretion at medium doses.

response curve, represented by an ED_{50}= 3.4×10^{-11}. At these doses of heparin, the typical bell-shape of the dose-response curve to FGF-2 disappeared (Fig 2B).

Finally, we developed PRL dose-response experiments to FGF-2 (0, 2.0×10^{-14}, 2.0×10^{-13}, 2.0×10^{-12}, 2.0×10^{-11}, and 2.0×10^{-10} *M*) in primary cultures of anterior pituitary cells obtained from estrogen-in vivo treated rats at incubations of 24 and 48 h. In these cells we did not obtain any response to FGF-2 (Fig 3A). Co-administrating of higher doses of heparin (5.6×10^{-6} *M*) to the cultures, restored the

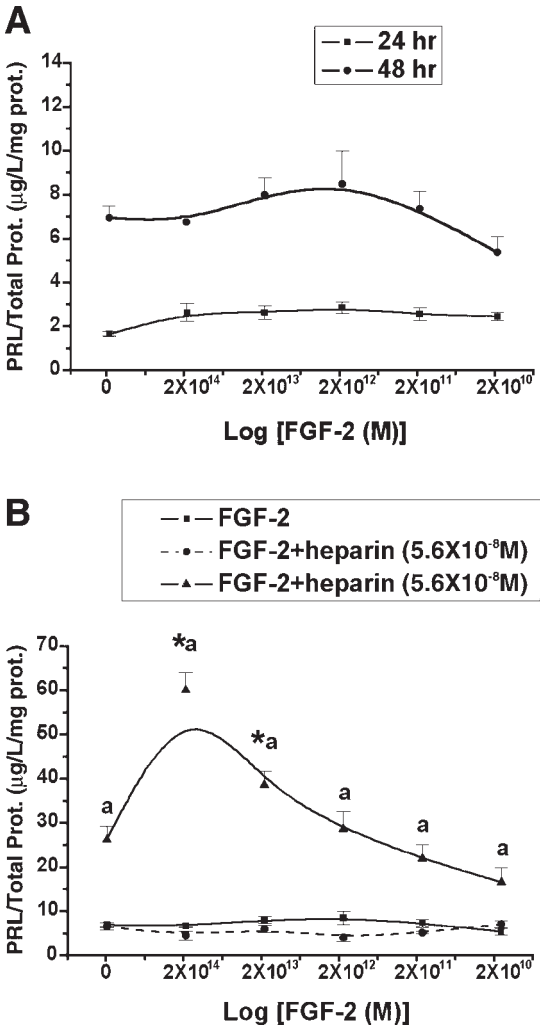

Fig. 3. (**A**) PRL responses to FGF-2 in primary pituitary cultures from estrogenized animal. Dose-responses at 24 and 48 h. Mean ± SD of two different experiments run in quadruplicate (4 pituitaries/experiment). Duncan test. * = $p < 0.05$ vs control (FGF-2 = 0). (**B**) PRL responses to FGF in primary cultures from in vivo estrogenized rats, at 48 h. Effect of HEP administration at different doses. Mean ± SD of two different experiments run in quadruplicate (6 pituitaries/experiment). Duncan test. * = $p < 0.05$ vs control (FGF-2 = 0). HEP shifted PRL responses to FGF-2 to the left, thus increased the responsiveness of cells. HEP increased also FGF-2-induced maximal PRL levels.

PRL responses to lower doses of FGF-2 (2×10^{-14} and 2×10^{-13} *M*) (Fig 3B).

Discussion

The pituitary is the organ that produces and secretes the largest amount of FGF-2 per gram of tissue. It is uncertain which is the major source of FGF-2 within this gland, but it has been reported that folliculostellate cells produce and distribute FGF-2 throughout the pituitary *(16,17)*. Some types of endocrine-secreting cells may also contain FGF-2, as shown by Schechter and co-workers, who were able to prove that gonadotroph cells store FGF-2 in large amounts *(18)*.

It has been suggested that FGF-2 may play an important role in tumor progression, perhaps by favoring angiogenesis and lactotroph differentiation *(20–22)*. Other reports have shown that FGF-2 is regulated in many pituitary tumors, and also in cell lines *(19,23–25)*. Further to proliferation and differentiation actions, FGF-2 seems to have important paracrine regulatory actions within the pituitary. Baird and co-workers have shown that FGF-2, acutely administered, induces PRL and TSH secretion in rat pituitary cells in primary culture, in a dose-dependent manner, but only when cells are pre-incubated in vitro with high amount of FGF-2 for at least 48 h *(8)*. On the other hand, Larson and co-workers have shown that the acute administration of FGF-2 reduced PRL secretion and blocked TRH-induced PRL release *(26)*. Our group has also reported that FGF-2 and FGF-1 increase PRL-mRNA levels as well as PRL levels in culture medium, in GH_3 cells *(11)*. Under further analysis all these data suggest that FGF-2 could be an important specific paracrine regulator of lactotroph cells.

The present study shows that the effects of FGF-2 upon in vitro PRL secretion from pituitary cells are time-dependent; the maximal response occurs at 48 h, and the responses after shorter and longer times decrease (24 and 72 h). However, our results confirm that FGF-2 needs long incubation periods in order to modulate PRL secretion in vitro, but pre-incubation with FGF-2 was not necessary.

It could be observed that PRL levels vary in response to FGF-2 in a biphasic dose-dependent manner. In our case, a first phase of dose-dependent increase in PRL levels up to a maximal response at 2.0×10^{-11} *M* can clearly be seen; and also a second phase of dose-dependent decrease in PRL levels onwards. The response profile for FGF-2 is compatible with the dimerization models that explain the FGF-2 and FGF-receptor interaction, and its later activation, as extensively described in the literature *(27–32)*.

On the other hand, heparin—a strong anionic glycosaminoglycan—has been identified as a low-affinity-binding molecule for FGF, present in the membrane surface nearby the proper FGF high-affinity low-capacity receptor. One of the explaining hypotheses for the activation of FGF receptor assumes that heparin works as a complementary molecule necessary for the binding of FGF-2 *(5)*, being a regulatory factor of the FGF–FGFR interaction. Heparin or heparan sulfate can facilitate the oligomerization of two or more FGF molecules, and it appears that a specific side-by-side heparin–FGF-2 interaction is required for the activation of the FGFRs *(31)*, starting the intracellular signalling. In fact, FGF-2/FGFR binding depends on the presence of heparin *(33)*, and the alteration of the heparin-binding domain of FGF-2 alters its biological activity *(34,35)*.

Our results show that heparin can stimulate the PRL secretion in the absence of FGF-2, in primary cultures, and increase the PRL mRNA levels in GH_3 cells in a dose-dependent manner (data not shown). Regarding the FGF-2/heparin/FGFR interaction hypothesis, there are two major expla-

nations for these results. First, heparin may become bound to the FGF-2 produced by the folliculostellate cells of the culture, after which the FGF-2–heparin complex would activate the FGF receptor. In this case heparin would increase the effect of the endogenous concentration of FGF-2 in the culture. However, this possibility is not convincing enough, because the spontaneous production of FGF-2 in primary cultures is very low in relation to the doses necessary for stimulation of PRL secretion, and because GH_3 cells do not produce FGF-2 naturally, although they do respond to heparin, increasing PRL mRNA levels.

The other possible mechanism depends on the ability of heparin to activate the FGF receptor or another unknown receptor by itself. It has been described that heparin is able to bind to FGFR independently of previous binding to FGF-2 *(36,37)*, and it could produce changes in the activity of the receptor.

We have also shown that heparin modulates, in a dose-dependent manner, the FGF-2–induced PRL increase. So, heparin clearly increases the stimulatory effects of FGF-2 when administered at medium doses (5.6×10^{-8} *M*), but these effects decrease when heparin is administered at lower doses (5.6×10^{-10} *M*)—probably reducing the binding of FGF-2 to its receptors—and also at higher doses (5.6×10^{-6} *M*), at which it probably interferes with the dimerization process needed to activate FGF-2 receptors (Fig 2B).

Finally, in vivo estrogenization of rats results in the abolition of FGF-2 capability to favor PRL increase in the culture medium in vitro. We did not measure FGF-2 levels in the pituitary culture cells of rats prior to estrogenization, but it is likely that there was a high level of FGF-2s in this system, because estrogen induces FGF-2 and VEGF expression in the pituitary *(15)*. It seems rather reasonable that in a high FGF-2 milleu, exogenous FGF-2 had no effects on PRL secretion in vitro. Interestingly, when heparin was added to the pituitary cultures, PRL responses to FGF-2 have been restored.

In conclusion, we have observed that FGF-2 induces a marked increase of PRL secretion in primary lactotroph cells in long incubations. We have also shown that heparin by itself induces a PRL secretion in pituitary primary cells in a dose-dependent manner. Also, heparin is able to modulate PRL secretory responses to FGF-2 and restores responses in estrogenized cells.

Materials and Methods

Adult female Sprague-Dawley rats weighting 150-200 g were housed in a constant dark–light cycle (12:12 h). Standard pelleted diet (Panlab S:L:, Barcelona, SP) and tap water were available *ad libitum*. Animal manipulations were carried out following the conventions and ethical rules collected in the 86/609/CEE Directive of the European Union. Some 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. After this period, the rat pituitaries increased in size and seemed hyperplastic *(38)*.

The anterior pituitaries were collected, after rat decapitation under pentobarbital anesthesia (50 mg/kg in 0.1 *M* CO_3Na_2), in EBSS (Sigma-Aldrich Química SL, Alcobendas, SP) in sterile conditions. Pituitaries were washed with fresh EBSS, minced, and incubated in EBSS containing 0.1% trypsin (Sigma-Aldrich Química SL, Alcobendas, SP) at 37°C, for 30 min. The cell suspension was centrifuged for 5 min at 720*g*, and then washed three times in EBSS. Finally, pellets were resuspended in DMEM (Seromed) containing antibiotics, and cells were mechanically dispersed, yielding a viability greater than 95%. The culture medium was DMEM supplemented with 10% FBS (Gibco BRL) and 1% antibiotics: 20 mg/mL ampicilin (Sigma-Aldrich Química SL, Alcobendas, SP) 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 *(39)*.

Experiments were carried out in serum-free medium containing FGF-2 (Sigma-Aldrich Quimica SL, Alcobendas, SP) at $0, 2 \times 10^{-12}, 2 \times 10^{-11}, 2 \times 10^{-10}$, and 2×10^{-9} *M* doses, at three times—24, 48 and 72 h—for the time-response study. In the dose-response studies the following doses of FGF-2 were administered: $0, 2 \times 10^{-14}, 2 \times 10^{-13}, 1 \times 10^{-13}, 2 \times 10^{-12}, 1 \times 10^{-12}, 2 \times 10^{-11}, 2 \times 10^{-10}$, and 2×10^{-9} *M*; with/without heparin (Sigma-Aldrich Quimica SL, Alcobendas, SP) at $0, 5.6 \times 10^{-10}, 5.6 \times 10^{-8}$, and 5.6×10^{-6} *M*. In all cases the best responses to FGF-2 were obtained at 48 h; for this reason, the following studies were carried out at 48 h, unless otherwise stated. The medium was collected and stored at –20°C until measurement by specific RIA for PRL (Dr. A. F. Parlow NPH-NIH, USA). Intraassay coefficients of variation were lower than 8%, and assay sensitivity was 1.35 ng/mL.

Total protein in culture medium was measured by the Bradford method *(40)*. Hormone levels are expressed by micrograms of total protein in the culture medium. Thus, units of the graphs are ng (PRL) by μg/mL (total protein in each well).

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

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