Morphometric evidence of the regulation of the activity of LH-immunoreactive cells by corticosterone. An in vivo and in vitro study

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Summary. The present study was performed to investigate the morphometric effects of the administration of corticosterone on LH-immunoreactive cells of pituitary monolayer cultures. In addition, the effect of bilateral adrenalectomy on the LH-adenohypophyseal cells was explored. In vitro administration of corticosterone induced a marked decrease in the morphometric parameters considered (cellular, cytoplasmic and nuclear areas). In vivo, bilateral adrenalectomy produced a significant increase in these parameters when compared to those obtained in normal and sham-operated animals. However, when the bilaterally adrenalectomized rats received a daily dose of corticosterone no significant changes in the morphometric parameters were found. Morphometrically, these combined in vivo-in vitro findings confirm a corticosterone-induced hypoactivity of LH-cells together with a direct effect of this glucocorticoid on LH-adenohypophyseal cells.

Key words: Luteinizing hormone, Corticosterone, Morphometry, Culture, Adrenalectomy, Rat

Introduction

The anterior pituitary gland secretes follicle stimulating (FSH) and luteinizing hormone (LH) in multiple forms. Gonadotropes in the anterior pituitary are controlled by GnRH produced in the hypothalamus, whose action is modulated by gonadal and adrenal steroids. In fact, the above forms can be altered by changing the hormonal milieu (see McAndrews et al., 1994).

Stress hormones have an important effect on the reproductive capacity of many mammalian species (see D'Agostino et al., 1990). In rodents, the effects of stress on reproductive process are well documented. In the rat, prolonged elevations in plasma concentrations of gluco-corticoids of either endogenous or exogenous origin have been consistently correlated with a decline in the reproductive endocrine function (Vreeburg et al., 1984). Chronically stressed rats exhibit elevated plasma levels of the adrenal glucocorticoid hormone corticosterone and a decrease in the secretion of LH and testosterone (Tache et al., 1976, 1979).

Glucocorticoid receptors have been shown to be present in the pituitary (De Kloet et al., 1975) as well as within species brain sites, including the rat hypo-thalamus (Fuxe et al., 1985; Reul and DeKloet, 1985, 1986) and LHRH neurons (Ahima and Harlan, 1992). Glucocorticoids inhibit LHRH-induced LH secretion in dispersed anterior pituitary cells obtained from intact adult male rats (Tibolt and Childs, 1985; Kamel and Kubajak, 1987). Nevertheless, at present, it is not well established whether or not glucocorticoids exert their effects directly upon the cells and, indeed, there are some discrepancies about the response of LH-cells induced by corticosterone, especially in vitro.

Since morphometry is a suitable method for determining the activity of adenohypophyseal cells identified by immunohistochemistry (Carretero et al., 1992, 1995; Rubio et al., 1992, 1994; Sánchez et al., 1993), in order to elucidate the possible direct effect of corticosterone on this type of cell an in vivo and in vitro morphometric and immunohistochemical study of LH cells was carried out following adrenalectomy with or without treatment with corticosterone and after treating monolayer pituitary cultures from male adult rats with the glucocorticoid.

Materials and methods

In vivo experiments

Animals

Twenty-five adult male Wistar rats (180-200 g b.w.) housed under standard conditions (t^a: 22±2 ºC, r.h.: 50±5%, light time: 8.00 to 20.00 h, balanced diet and water ad libitum) were used.

Sham operated animals

Similar protocol to that described in adrenalec-tomized animals but the adrenal glands were left in their positions after visualization.

Bilaterally adrenalectomized rats

Under ketamine anaesthesia (50 mg/Kg b.w.), five rats were adrenalectomized according to the protocol described by Sánchez et al. (1990) and sacrificed on the 14th day after adrenalectomy.

Adrenalectomized and corticosterone-treated rats

Five adrenalectomized rats were intramuscularly treated with 5 mg/100 g b.w/day of corticosterone over 14 days following adrenalectomy and sacrificed on the 14th day. The completeness of adrenalectomy was verified by necropsy. Animals were sacrificed by decapitation and the pituitary glands were carefully dissected, fixed by immersion in Bouin fluid for 5 days, embedded in paraffin, and frontal serial 5 μm thick sections were obtained for immunohistochemical studies.

In vitro experiments

Pituitary cultures

Following anaesthesia with isoflurane, male Wistar rats (175-200 g, b.w.) were killed by decapitation and the anterior pituitaries were removed and washed in Earle's balanced salt solution. Enzymatic dispersion was carried out by incubation in Hank's solution augmented by 0.15% of $MgCl_2$, 0.1% of papain, 0.01% of DNase and 0.1% of neutral protease, for 30 minutes at 37 °C. Mechanical dispersion was achieved by passing the pituitaries through Pasteur pipettes and 20 to 22 gauge needles. After centrifugation, the supernatant was removed and the cells were resuspended in an appropriate volume of Dubelcco's modified Eagle's medium, supplemented with 10% calf serum, 2.5% fetal calf serum, 2% L-glutamine, 100 IU/ml of penicillin and 1000 IU/ml of streptomycine (Sánchez et al., 1991). The cells were seeded on 50 culture dishes (30 x 15 mm) at a final concentration of 5×10^5 cells/dish and incubated at 37 °C in a 5% CO_2/95% air atmosphere for 3 days. On the 3rd day, the medium was removed and replaced by fresh medium containing 1% ethanol (10 control dishes) or fresh medium supplemented with 10^{-6}M cortico-sterone, previously diluted in 1% ethanol (10 treated dishes). After 3 hours of incubation, the medium was removed and the dishes were fixed in Somogyi fluid for 1 hour and processed for immunocytochemistry.

Immunocytochemistry

Endogenous peroxidase was blocked with methanol and H_2O_2 and nonspecific reactions of the secondary antibody were blocked by incubation in normal swine serum (1:30) in TBS (Tris-saline buffer 0.05M, pH 7.6). Cells were incubated at 4 °C overnight, with rabbit anti-LH (Dako, diluted 1:1200), followed by swine anti-rabbit IgG (Dako, diluted 1:100) and rabbit peroxidase anti-peroxidase complex (Dako, diluted 1:100) at room temperature. The reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Pre-absorption tests were performed with LH and other pituitary hormones, and negative controls (substitution of the primary antibody with normal rabbit serum or TBS) were also obtained.

Morphometry and statistics

Using a MIP-2 automatic image analyzer (IMCO-10, Kontron), the cellular, nuclear and cytoplasmic areas of 100 LH-immunopositive cells per animal (500 per group) or 50 LH-immunopositive cells from each dish (500 cells per group) were calculated in an interactive fashion. Cells were randomly selected from different zones of the dishes. For this purpose, the surface of the dish was subdivided into 10 similar areas and 5 immuno-reactive cells per area were measured. The results obtained were processed statistically and the differences observed were compared using an ANOVA test, accepting $p < 0.05$ as significant for the Fisher-PLSD and Scheffé F tests jointly. Results are expressed as arithmetical mean±standard error of the mean.

Results

A) In vivo experiments

A.1. Morphological characteristics

Pituitary glands of untreated and sham-operated males showed immunoreactive LH-cells located throughout the pars distalis without any special situation (Fig. 1). Most cells were round or oval in shape with well defined nuclei, sometimes arranged eccentrically (Fig. 1). Deposits of the chromogen occupied the cytoplasm including the processes, homogeneously. However, according to the intensity of the staining, two types of LH-immunoreactive cells could be visualized: strong and weak immunoreactive cells (with no specific location within the gland) (Fig. 1). Following bilateral adrenalectomy, an increase in the intensity of the immunoreactivity was observed (Fig. 2). Although the presence of ring-seal immunoreactive cells was scarce, occasionally three or four of them were observed (Fig. 2). When bilaterally adrenalectomized animals received a daily dose of corticosterone the morphological characteristics were similar to those described for the untreated animals (Fig. 3).

A.2. Morphometric data

In untreated animals, the mean cellular size was 149.61±4.01 μm^2. The nuclei occupied 28.00±0.54 μm^2

and the cytoplasm 121.61 ± 3.84 μm^2. Similar results were observed in sham-operated animals (p>0.05). Following bilateral adrenalectomy, a significant increase in all the parameters considered was detected (Table 1). The data on bilaterally adrenalectomized animals receiving a daily dose of corticosterone were similar to those described for the untreated animals, with the exception of a slight decrease in the cellular and cytoplasmic area (see Table 1).

B) In vitro experiments

In control dishes, LH-immunoreactive cells displayed irregular shapes, mainly oval or polygonal (Fig. 4). The immunoreactivity was located throughout the cytoplasm, although close to the nucleus it was possible to observe some small areas free of chromogen

Table 1. Morphometric results obtained in the different experimental groups.

	CA	CytA	NA
In vivo			
Untreated group	149.61±4.01	121.61±3.84	28.00±0.54
Sham-operated group	146.97±4.17	120.82±3.96	26.15±0.71
ADX	171.12±3.52*	131.11±3.52*	40.01±0.64*
ADX+C	138.90±2.55	110.65±3.58	28.25±0.59
In vitro			
Untreated group	363.10±17.88	303.12±16.60	59.98±1.82
C	226.30±13.92*	182.32±12.64*	44.04±1.72*

ADX: bilaterally adrenalectomized group; ADX+C: bilaterally adrenalectomized group treated with corticosterone; C: corticosterone treated dishes; CA: cellula area; CytA: cytoplasmic area; NA: nuclear area; *: p<0.05 when compared to untreated or sham groups.
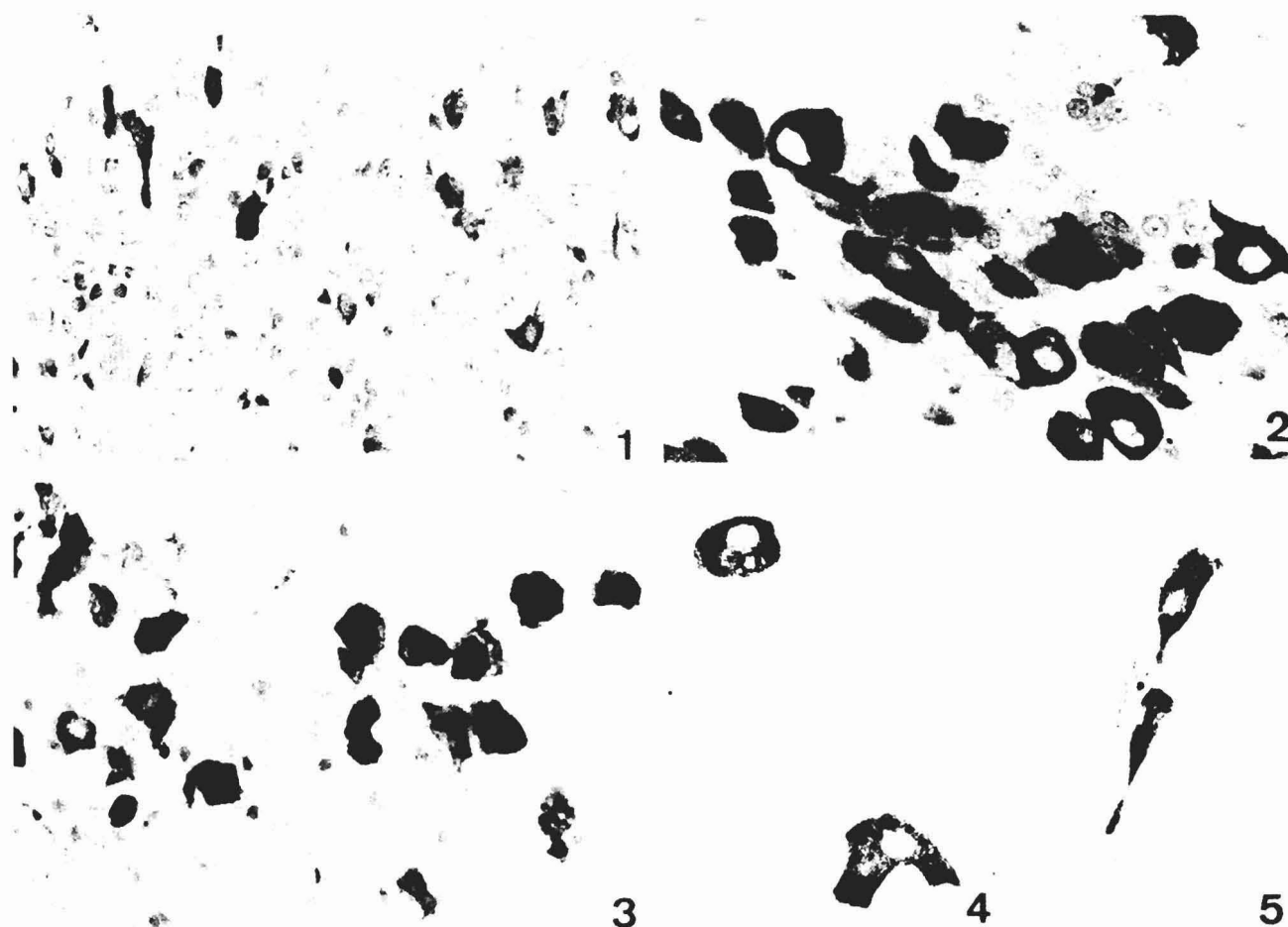

Fig. 1. Untreated rat. Note the presence of LH-immunoreactive cells throughout the adenohypophysis. x 200

Fig. 2. Bilaterally adrenalectomized rat. An increase in the intensity of the staining is observed when compared to untreated animals. x 400

Fig. 3. Bilaterally adrenalectomized rat treated with corticosterone. Similar characteristics to those observed in the untreated groups. x 400

Fig. 4. LH-immunoreactive cells from an untreated dish. x 400

Fig. 5. Corticosterone-treated culture. Note the decrease in the size of the cells. x 400

deposits (Fig. 4). Following administration of corticosterone to the culture medium, no important morphological changes were observed, although most cells showed a more irregular shape when compared to the untreated dishes (Fig. 5).

B.2. Morphometric data

All the parameters considered were significantly higher in vitro than in vivo. In this sense, the mean cellular area was 363.10 ± 17.88 μm^2; the mean cytoplasmic area was 303.12 ± 16.60 μm^2, and the mean nuclear area was 59.88 ± 1.82 μm^2. Following corticosterone incubation of the dishes a marked and statistically significant decrease was observed in all the parameters (Table 1).

Discussion

Exposure to different stressors, both natural and experimental, may disrupt reproductive functions in many species (Moberg, 1987). At present it is well-known that a variety of states of endogenous and exogenous glucocorticoid excess are associated with the development of hypogonadotropic hypogonadism in animals and humans, and that glucocorticoids affect the hypothalamic-pituitary gonadal axis under different experimental conditions (Rosen et al., 1988; Brann et al., 1990; Park et al., 1993). The effects of glucocorticoids on LH and FSH secretion are different, depending, among other experimental conditions, on the stage of the cycle, the sex of the animal and the time of exposure to the glucocorticoid (D'Agostino et al., 1990; Brisky and Sylvester, 1991; McAndrews et al., 1994). In general, it has been shown that glucocorticoids inhibit LH-secretion, although their effects on FSH secretion are more variable (see Kamel and Kubajak, 1987).

The present in vivo findings, obtained using a morphometric protocol, confirm an inhibitory effect of glucocorticoids on the cellular activity of LH-cells. Adrenalectomy induced morphometric changes, suggesting hyperactivity of the LH-immunoreactive anterior pituitary cells, reflected in a significant increase in the cellular, cytoplasmic and nuclear areas. Additionally, these morphometric changes were reversed by chronic treatment with corticosterone, indicating that the effect is caused by this glucocorticoid. These results are in good agreement with previous reports indicating that adrenalectomy induces an increase in the release of LH (Vreeburg et al., 1984; Park et al., 1993).

Whether glucocorticoid treatment in vivo exerts its adverse effect on LH-cells entirely at the level of the pituitary gland or additionally through another site is still not clear (Hwan and Freeman, 1987; Rosen et al., 1988; McAndrews et al., 1994). However, taking into account the in vitro findings of the present study and previous information (D'Agostino et al., 1990; Baldwin et al., 1991; McAndrews et al., 1994) we believe that the idea of a direct action can be entertained.

The present in vitro findings are in complete agreement with the in vivo results since with the morphometric protocol used, an inhibitory effect of corticosterone is evident ton the LH-immunoreactive cells. In fact, after administration of the glucocorticoids significant decreases in all the morphometric parameters were observed, suggesting a state of hypoactivity of the LH-cells.

Discrepancies about a direct in vitro effect of glucocorticoids on anterior pituitary cells have been reported. Suter and Schwartz (1985) described the absence of response of the rat pituitary LH-cells in culture following incubation with glucocorticoids. However, there is also evidence supporting a decrease in LH secretion in vitro after administration of glucocorticoids, suggesting that this effect may be exerted directly at pituitary level (Padmanabhan et al., 1983; Hwan and Freeman, 1987; Kamel and Kubajak, 1987; D'Agostino et al., 1990; Baldwin et al., 1991; McAndrews et al., 1994).

The results of the present study are consistent with the above-mentioned in vitro inhibitory effects of glucocorticoids on LH-cell and it is the first report to demonstrate, by means of a combined in vivo-in vitro immunocytochemical-morphometric method, that glucocorticoids exert their inhibitory effect on LH-immuno-reactive cells at the level of the anterior pituitary in the male rat (regardless of the fact that in vivo there also exist other extra pituitary sites of action). However, further studies should be carried out to elucidate whether this pituitary effect is direct on the LH-cell or occurs through some mediating pituitary factor.

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