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Effect of melatonin on androgen receptor and catalase mRNA

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SUMMARY

Among the various effects that have recently been demonstrated for melatonin is the regulation of several mRNAs, the control of at least some of them being mediated by the nuclear receptor for this hormone. The Harderian gland is a secondary sexual organ under androgenic control whose physiology is also regulated by melatonin, the action of melatonin being similar to that of androgens. This raises the question of whether melatonin regulates the mRNA for the androgen receptor. In the present work, using Northern blot techniques we found that melatonin regulates that mRNA when administered both acutely and chronically. When given chronically, melatonin displayed the androgenic actions previously reported by others to accompany androgen administration. We also report an effect of melatonin on catalase mRNA that parallels its effect on androgen receptor mRNA, suggesting that the action of melatonin on catalase mRNA might not be direct but, instead, an effect of the changes induced by this hormone in androgen receptor mRNA. It is known that nuclear receptors and their ligands in general interact with each other. We report the regulation of androgen receptor mRNA by melatonin and propose the hypothesis that this hormone would regulate steroid receptors in general or would interact with these receptors or their ligands. This hypothesis would explain both the classic studied effects of melatonin, mainly on the reproductive system, and some of the more recently found actions of this agent.

Key Words: Melatonin - androgen receptor - antioxidant enzymes.

INTRODUCTION

Melatonin is an agent synthesized in several tissues, the most important and classically known being the pineal gland. Although its effects are multiple (regulation of circadian and circannual rhythms, adaptation of the reproductive system to seasonal changes, immunostimulation, inhibition of cell proliferation, scavenging of free radicals, regulation of several mRNAs, etc.), little is known about its mechanisms of action. It appears that its free radical scavenging ability is due to its particular chemical structure, and to its high liposolubility, which facilitates its entrance into the cell (Reiter, 1995). Several authors are currently investigating the mechanisms used in the other effects described, although different hypothesis exist for the various actions. The regulation of several mRNAs has been reported and could be involved in some of these effects: the decrease in mRNA for aminolevulinte synthase and the increase in mRNA for some antioxidant enzymes could account for the cellular protection effect against the high level of porphyrin-induced oxidative stress in the female Harderian gland (Antolín et al., 1996); the increase in interleukin 2 could be involved in the immunostimulation produced in bone marrow after the administration of melatonin (Maestroni et al., 1994); a decrease in the mRNA for estrogen receptors has been proposed as the cause of the inhibition of cell proliferation in MCF-7 cells (Mollis et al., 1994), etc.

Two kinds of receptors have been characterized for melatonin. One of them is a membrane receptor G-protein coupled (Rivkees et al., 1989;

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Ebisawa et al., 1994) and the other one corresponds to a receptor of the nuclear receptor family: the RZR-α and the RZR-β receptors (Becker-Andre et al., 1994; Wiesenberg et al., 1995). Although both receptors could mediate the transcriptional effects of melatonin -the first one through a second messenger cascade and the second one directly acting, as the nuclear receptors family usually does- the nuclear receptor has already been reported to regulate the transcription of several genes (Steinhilber et al., 1995; Schrader et al., 1996).

The Harderian gland (HG) is a secondary sexual organ under androgenic control (Payne et al., 1977) that displays a marked sexual dimorphism and important biochemical differences between the sexes. Morphologically, the male HG has two cellular types: type I cells, with small lipid droplets, and type II cells, with large lipid droplets. The female gland show a single cell type (type I cells), which produces porphyrins and shows porphyrin deposits in the lumen of the gland (Bucana and Nadakavukaren, 1989). After castration, type II cells disappear from the gland of the males, which thereafter resembles the female gland. These morphological changes parallel other biochemical variations, such as a decrease in porphyrin concentration and deposits. Simultaneous administration of testosterone prevents all the alterations deriving from castration and the administration of melatonin is also able to prevent these effects of androgen deprivation (Buzzell et al., 1994).

Given that 1) melatonin has a nuclear receptor, 2) it regulates several mRNAs and 3) several interregulations have been described for this kind of receptor and their ligands (Mangelsdorf et al., 1995), it could be speculated that melatonin might also regulate the mRNA for androgen receptor. Also, the question arises as to whether melatonin directly regulates all of the mRNAs described above or only some of them (probably nuclear receptors, i.e. steroid receptors) that, in turn, could regulate others. In support of this, it has recently been found in our laboratory that melatonin regulates some antioxidant enzyme mRNAs in thymocytes; such regulation is not direct but is rather mediated by the effect of melatonin on the mRNA for the glucocorticoid receptor (Sainz et al., unpublished results). In view of the previously reported increase in some antioxidant enzyme mRNAs in the HG after chronic administration of melatonin (Antolin et al., 1996) and the androgenic regulation of the physiology of this gland, we wondered whether the ability of melatonin to increase such antioxidant enzyme mRNAs could be the consequence of its regulation of androgen receptor gene expression.

The aims of the present work were: 1) to study if melatonin regulates the mRNA for androgen receptors and 2) if this were the case, to see whether the variations in androgen receptor mRNA are accompanied by parallel variations in the mRNA for antioxidant enzymes.

Materials and Methods

Animals

56 male and 16 female Syrian hamsters of 30 days of age were used in this study. Animals were kept in the animal room of the University of Oviedo on a 14:10 light regimen, lights on at 07:00. Food and water were provided ad libitum.

Experimental design

Experiment 1

In order to study whether melatonin regulates the mRNA for androgen receptors when acutely administered, and to see if this hypothetical regulation is related to its regulation of the antioxidant enzymes, low (50 μg/kg bw) or high (500 μg/kg bw) doses of melatonin were injected intraperitoneally to male Syrian hamsters at 17:00 h, each group being formed by 8 animals. An additional group (n=8) was injected with vehicle. After 2 h of treatment, animals were sacrificed by decapitation and the HG were carefully extracted and immersed in liquid nitrogen.

Experiment 2

To study if the androgenic effects of melatonin in the HG are mediated by the regulation of the androgen receptors by this agent, 6 groups of 30-day old Syrian hamsters were formed (n=8): two groups of intact males; two groups of castrated males and two groups of intact females. Each day, one group of each was injected intraperitoneally with a high dose of melatonin (500 μg/kg bw) and the other was injected in the same way with vehicle. After 40 days of melatonin administration, animals were sacrificed by decapitation and the HG were carefully removed and immersed in liquid nitrogen.

Drugs

All drugs were obtained from Sigma (Spain) unless otherwise indicated. Melatonin was dissolved in a minimal amount of ethanol. Then, saline (9% NaCl) was added to obtain the final desired concentration.

Morphological studies

Half of one HG from each animal used in experiment two was fixed by immersion for 10 min in a solution containing 1.5% paraformaldehyde (Merck, Darmstadt, Germany) and 2.5% glutaraldehyde (TAAB Lab. Equipment, Reading, U.K.) in 0.1 M phosphate buffer (pH 7.3). The tissue was then cut into smaller pieces (1mm thick) and fixed for a further 12 h. After three washes (10 min each) in 0.1 M phosphate buffer (pH 7.3), tissue was immersed in a mixture of 1% OsO_4 and 1.25% potassium ferrocyanide for 2 h for postfixation purposes. After dehydratation in graded acetone series, the pieces were embedded in SPURR Resin (EMS, Fort Washington, Pa).

Semithin sections were obtained from two blocks from each animal and stained with toluidine blue for observation under a LABOFOT microscope (Nikon, Tokyo, Japan).

Immunohistochemical studies

Paraffin sections (6-8 μm) were used after deparaffination in xylene and hydration. Endogenous peroxidase activity was quenched by incubation in aqueous 3% H_2O_2 for 30 min. After a brief wash in water, sections were equilibrated in TBS buffer (pH 7.4). Nonspecific binding was blocked by incubation with 3% normal rabbit serum for 30 min. Rabbit anti-rat polyclonal androgen receptor antibody was used at a 1:20 dilution. Incubation in the primary antibody was performed at 20° for 18 h. Then, sections were washed in TBS and treated with goat anti-rabbit IgG (Vector Labs., Burlingame, USA) diluted 1:500 in TBS for 1 h. They were then washed in TBS, treated for 45 min with ABC-Elite (Vector Labs., Burlingame, USA) prepared in 0.1 M Tris-HCl, 5 M NaCl and 0.01% Tween-20 (pH 8.5), and washed again in 0.1 M Tris HCl (pH 7.0) and reacted in 0.5 mg/ml diaminobenzidine and 0.01 M imidazole (pH 7.0). Sections were counterstained with hematoxylin.

RNA isolation and Northern analyses

Total RNA was purified from a pool of HG from each group according to the method of Chomczynski and Sacchi (1987). (Poly A)$^+$-RNA was obtained from total RNA with a purification mRNA kit (Pharmacia Biotech). After electrophoresis in a 1% agarose gel, the Poly A$^+$ was transferred to a nylon membrane (HYBON^{TM}-N$^+$, Amersham LIFE SCIENCES) and hybridized in a conventional buffer containing 40 % formamide at 42°C for 16 h with the following ^{32}P-labelled probes: a Bam HI fragment for the rat androgen receptor cDNA clone pcDNA I (He et al., 1990); a 1.6 kb Hind III/ECO RI fragment from the rat catalase cDNA clone pTZCTL (Furuta et al., 1980); a 0.8 kb SalI fragment from the rat glutathione peroxidase (GPx) cDNA clone, LK 440 (Yoshimura et al., 1988), and a 2.1 kb fragment from the human -actin cDNA clone, pHFBA-1 (Gunning et al., 1983), which was used to normalize the remaining mRNA values.

Statistical analysis

Data are the results of three independent experiments. Results are expressed as mean± standard errors. Statistical analyses were performed with ANOVA followed by a Student Newman-Keuls test. The autoradiographies shown belong to a representative experiment.

Results

Melatonin regulates the mRNA for androgen receptors

Two hours after administration, acute injection of a low dose of melatonin (50 μg/kg bw) induced an increase in the mRNA for androgen receptors of 40% (Fig. 1). When the dose administered was high (500 μg/kg bw), melatonin decreased the mRNA for this receptor by 40% (Fig. 1).

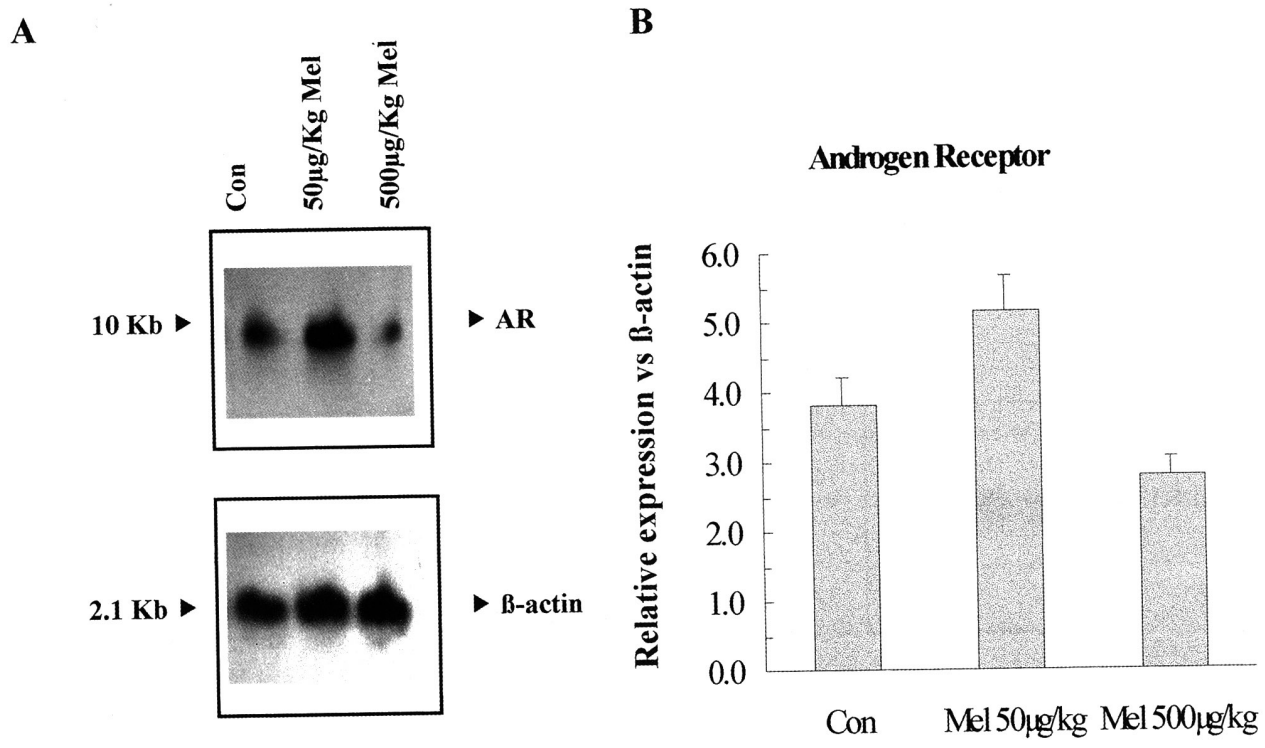

Fig. 1.– Effect of a single injection of a low (50 μg/kg bw) or a high (500 μg/kg bw) dose of melatonin on the mRNA levels for the androgen receptor after 2 hours of the treatment of adult males Syrian hamster. **A:** Northern blot. **B:** Graph of the densitometric analysis of the autoradiography after normalization with the β-actin.

The regulation by melatonin of the mRNA for some antioxidant enzymes parallels the control of the mRNA for androgen receptors by this agent

In the same experiment in which we studied the regulation by melatonin of the mRNA for androgen receptors (experiment 1), we found that melatonin administered at the low dose induced a 31% rise in the mRNA for catalase. When administered at the high dose, it induced a 29% decrease in the mRNA for this antioxidant enzyme (Fig. 2, A and C). This result parallels that found for the androgen receptor.

Melatonin did not have any effect on the mRNA for GPx, either when injected at the low or the high dose (Fig. 2, A and B).

Melatonin decreases the mRNA for androgen receptors after castration

The presence of androgen receptors in the HG of Syrian hamsters was confirmed by immunohistochemical techniques. This receptor was present in the cellular nucleus of the HG in both males and females, the gland of the males showing staining in both type I and type II cells (Fig. 3).

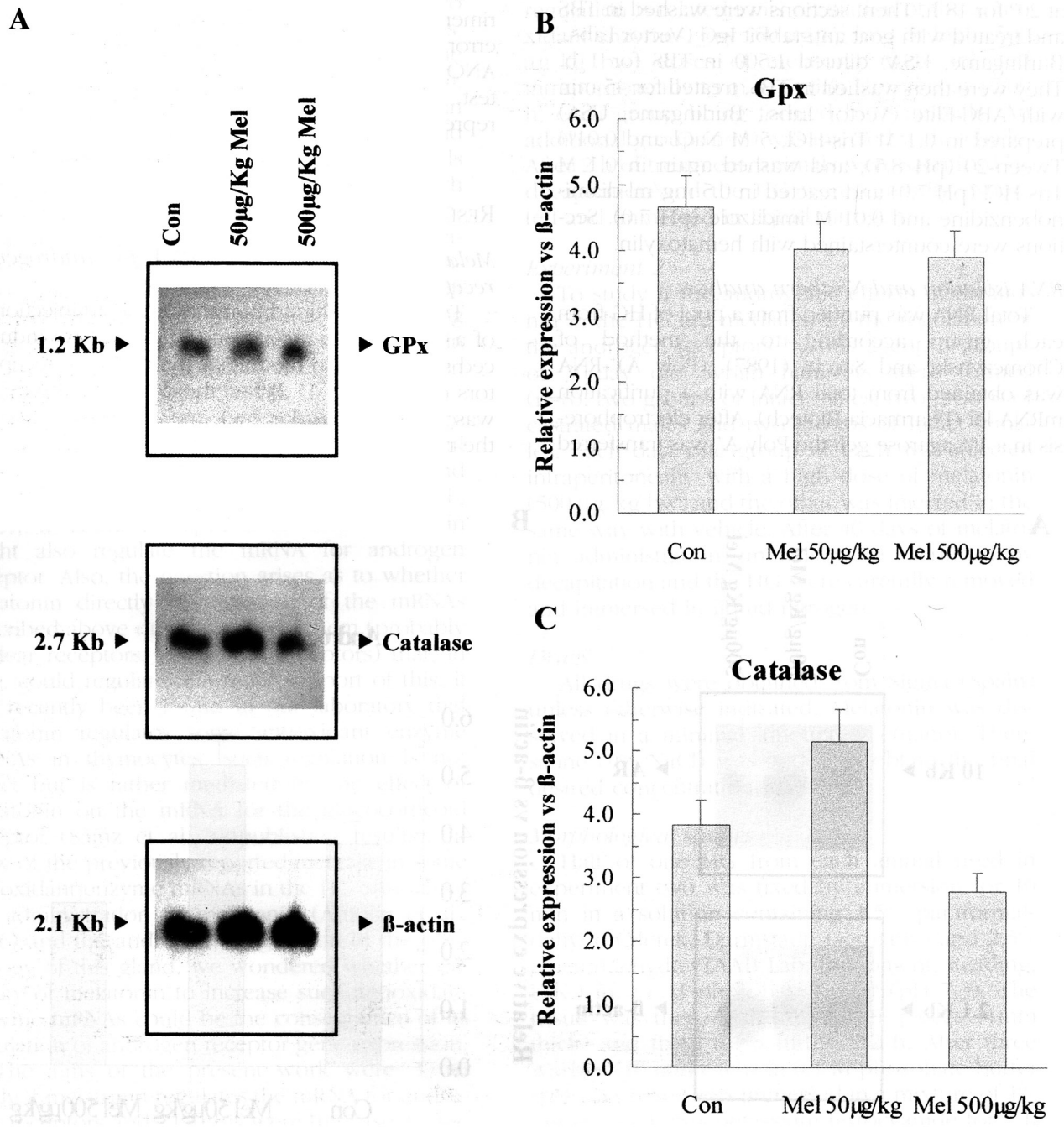

Fig. 2.– Effect of a single injection of a low (50 µg/kg bw) or a high (500 µg/kg bw) dose of melatonin on the mRNA for glutathion peroxidase (GPx) and catalase after 2 hours of administration to adult male Syrian hamster. **A:** Northern Blot; **B** and **C:** Graphed data of the densitometric analysis of the autoradiography after normalization with β-actin.

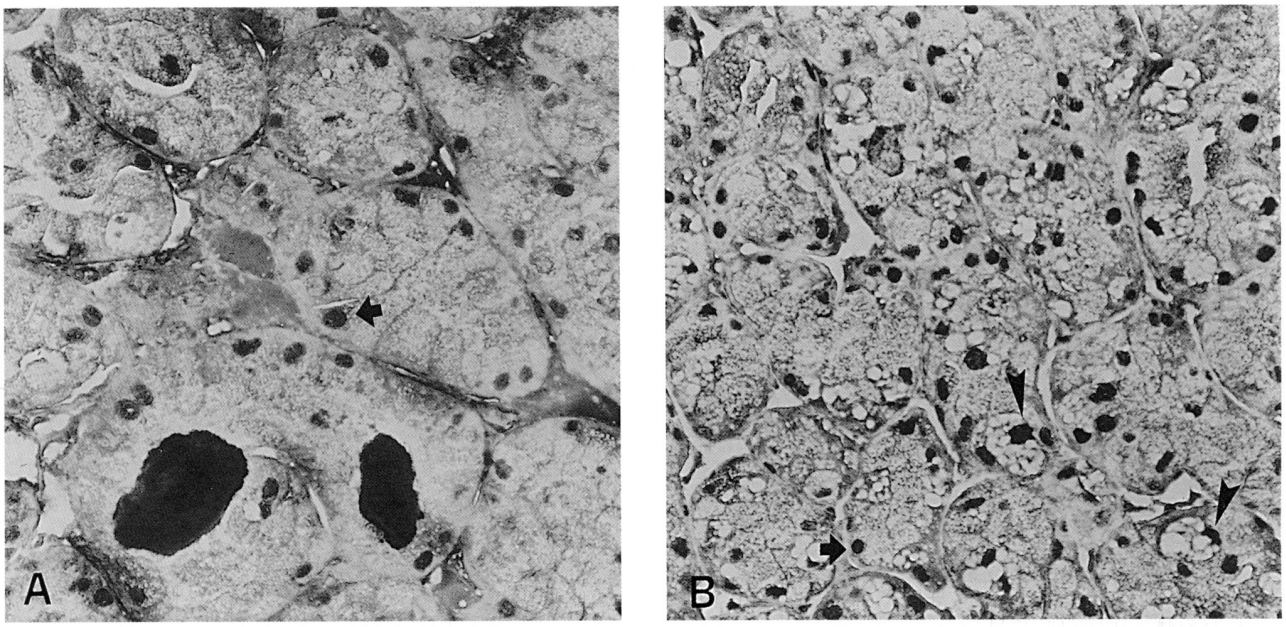

Fig. 3.– Immunolocalization of the androgen receptor in the Harderian gland of both intact males and females. The staining appears in the nuleus of the single type (type I) of cells of the female **(A)** (large black areas: luminal porphyrins) and in the nucleus of both type I (arrows) and type II (head arrows) cells in the male **(B)**. **A:** x 300; **B:** x 250.

When hamsters were chronically injected with melatonin, the mRNA for androgen receptors was not modified either in intact males or females. However, melatonin prevented the upregulation of the mRNA for androgen receptors caused by testosterone deprivation in castrated males (Fig. 4).

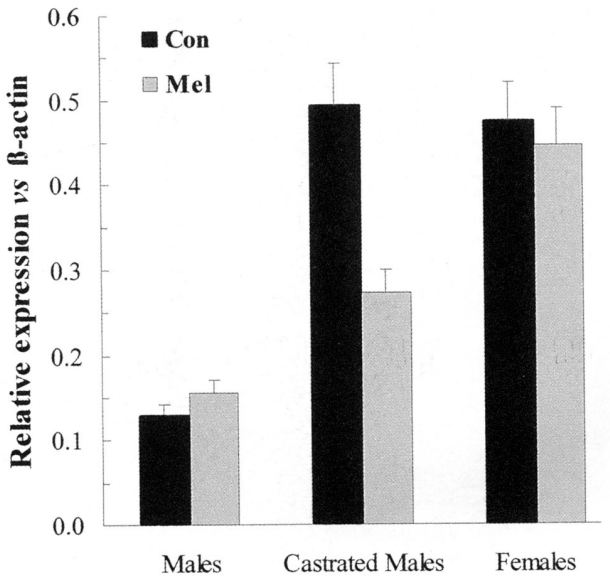

Fig. 4.– Effect of the chronic administration (8 weeks) of a high dose (500 µg/kg bw) of melatonin to males, castrated males and females adult Syrian hamsters on the mRNA for androgen receptor in the Harderian gland.

Chronic administration of melatonin induced an increase in type II cells in the glands of the females (Fig. 5B) and did not alter the morphological aspect of the glands of the males (Fig. 5D). When melatonin was administered chronically to castrated males (Fig. 5E), beginning treatment immediately after gonadectomy, it partially prevented the appearance of the morphological changes induced by castration (Fig. 5F).

Discussion

The literature has consistently shown that melatonin has androgenic effects in the HG. Castration of male hamsters induces a significant decrease in the number of type II cells and an increase in porphyrin deposits while melatonin administration prevents these changes (Rodríguez-Colunga et al., 1991) as well as the biochemical alterations induced by androgen deprivation (Menéndez-Peláez et al., 1991; Buzzell et al., 1994). The administration of melatonin to female hamsters induces a marked increase in type II cells, accompanied by an important decrease in intraluminal porphyrin deposits (Rodríguez-Colunga et al., 1992; Antolín et al., 1996). Also, in the HG melatonin concentrations and melatonin binding sites seem to be under androgenic regulation (Hoffman et al., 1989; Menéndez-Peláez et al., 1993).

The androgen receptor has been shown to be down-regulated by testosterone in the HG of the

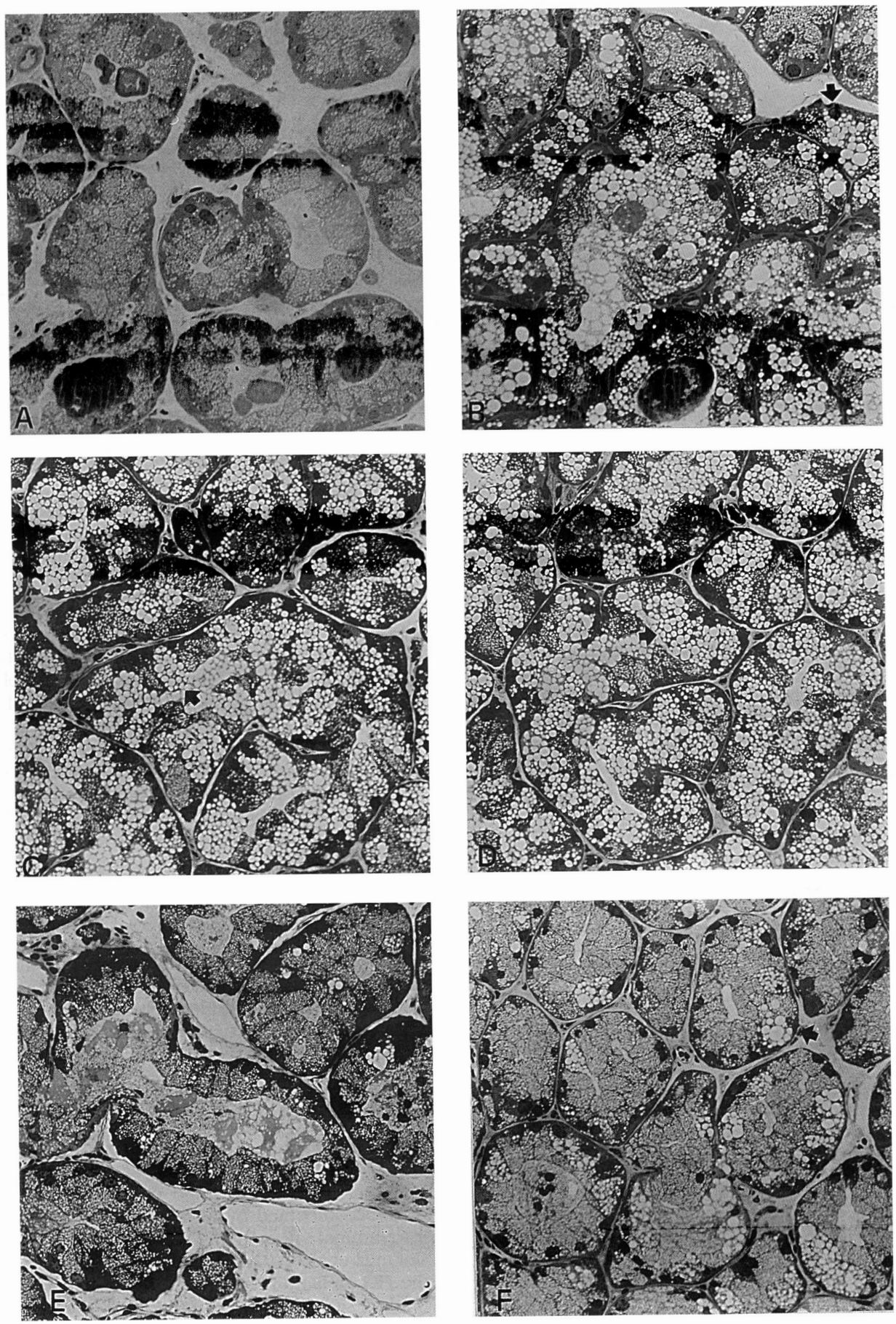

Fig. 5.– Effect of the chronic administration (8 weeks) of a high dose (500 µg/kg bw) of melatonin to males, castrated males and females hamsters in the morphology of the Harderian gland. Notice an increase of type II cells in the Harderian glands of castrated males and females. **A:** females; **B:** melatonin-treated females; **C:** males; **D:** melatonin-treated males; **E:** castrated males; **F:** melatonin-treated castrated males. Arrows: type II cells. **A-D:** x 250; **E-F:** x 300.

hamster (McBlain et al., 1994) and rat (Domínguez et al., 1994). Here we propose that this receptor is also under the regulation of melatonin, this control being exerted directly on the receptor expression or as consequence of an interrelationship among the several known nuclear receptors and their ligands, melatonin showing a final effect parallel to that of testosterone.

The rapid effects, at two hours after administration of both doses of melatonin, on the mRNA for androgen receptors indicates that this agent could well be regulating the expression of the androgen receptor gene, although further confirmation is needed. The consequences of this change at morphological level were not evaluated after the acute administration of melatonin, since morphological changes can only be detected at long term.

No effect was observed on the mRNA for androgen receptors after chronic daily administration of melatonin for 8 weeks in male hamsters, with the expected lack of morphological changes. The absence of effects of chronic melatonin administration in the HG of intact males is consistent with the fact of this agent having androgenic effects; since androgens are present at very high levels in males, an agent hypothetically regulating androgen receptors with the finality of causing androgenic responses (showing a final effect similar to that seen after the administration of testosterone) should not be noticed.

Chronic administration of melatonin to castrated males prevented the increase in the mRNA for androgen receptors produced by androgen deprivation. Observation of the morphology of the glands of castrated male hamsters treated with melatonin revealed that this was similar to the situation in the intact male group, in contrast to the structure of the glands of castrated males, which showed low numbers of type II cells, being similar to the morphology of the females. Once again, these results support the hypothesis of the regulation by melatonin of the androgen receptor mRNA as being the way how this agent regulates the physiology of the HG, although further investigation is needed in order to elucidate whether this regulation is direct at the level of transcription or whether it depends on other interrelationships between both nuclear receptors and their ligands.

The high expression of androgen receptors in females is in agreement with the results previously published by others (Domínguez et al., 1994). It may be interpreted as the consequence of the lack of or very low amount of androgens in the glands of the females. The lack of regulation by chronic administration of melatonin of androgen receptors in the HG of females agrees with the results of Rodríguez Colunga et al. (1991), who found that the effects of melatonin on the porphyrin deposits in the HG of females are only observed after three months of administration, no variations being found after 8 weeks of treatment. So far, we do not have a feasible explanation for this finding, although we do not discard a different type of regulation in the females due to the existence of other important steroids, the estrogens and estrogen receptors, which have also been reported to be regulated by melatonin in other systems. This could originate a complex interrelationship of all the three nuclear receptors and their ligands. Although morphological changes were found in this work as well as in previous studies (Antolín et al., 1996), it is very likely that the regulation of both the biochemical and morphological status of the HG of females depends more on estrogens and their receptors than on androgens. The possible regulation of this receptor by melatonin in the HG should be further studied.

Previous work (Sainz et al., 1999) suggests that glucocorticoids control the expression of antioxidant enzymes in the thymus, the effect of melatonin on the mRNA for these enzymes being mediated by its regulation of the glucocorticoid receptor. Antolín et al. (1996) found that chronic administration of melatonin to female Syrian hamsters increases the expression of the Cu-Zn and Mn SOD genes in the HG, with no changes in the mRNA for GPx. At that time, catalase mRNA could not be detected. In the present work, we found that catalase expression after acute administration of melatonin at both high and low doses follows a pattern similar to that of androgen receptor expression in male hamsters. This could be due to a direct influence of melatonin on the expression of catalase. However, it also suggests a possible regulation by melatonin of catalase mRNA through the regulation of androgen receptors by this hormone, just as glucocorticoid receptor regulation by melatonin regulates the expression of antioxidant enzymes in the thymus. This is not surprising, since other authors have demonstrated that androgens regulate the activity of several antioxidant enzymes (Chainy et al., 1997; Ripple et al., 1997). The differences in terms of catalase detection with the results of Antolín et al. (1996) could be due to the fact that we used males and those authors used females or, more likely, to the fact that they used total RNA while we used $(polyA)^+$-RNA. Similar to their results, we did not detect variations in GPx mRNA after melatonin administration, indicating that this gene is possibly not under either melatonin or steroid receptor control.

Interrelationships between the several nuclear receptors or their ligands have recently been reported by several authors (Mangelsdorf et al., 1995; Tsai et al., 1994). The present results, together with previous findings about the effect

of melatonin on estrogen and glucocorticoid receptors, suggest that this hormone may be interacting with other nuclear (probably steroid) receptors or their ligands, leading as a final result to a regulation of steroid receptors by melatonin (Mollis et al., 1994; Sainz et al., 1999) and a regulation of the melatonin receptor by other nuclear receptors or their ligands (Menéndez-Peláez et al., 1993). Although further studies are needed, these results also support the hypothesis that the regulation of antioxidant enzyme gene expression by melatonin could be mediated by its effects on steroid receptors. The fact that melatonin regulates steroid receptors may explain both the classically studied effects of melatonin on the reproductive system and some of the new findings for this hormone.

Acknowledgements

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