Pergamon

0960-0760(93)E0018-3

J. Steroid Biochem. Molec. Biol. Vol. 48, No. 2/3, pp. 165–170, 1994

0960-0760/94 $6.00 + 0.00

Androgen-dependent Expression of Cystatin-related Protein (CRP) in the Exorbital Lacrimal Gland of the Rat

Joris Winderickx, Inge Vercaeren, Guido Verhoeven and Walter Heyns*

Laboratorium voor Experimentele Geneeskunde en Endocrinologie, Faculteit Geneeskunde, Catholic University of Leuven, Leuven, Belgium

Cystatin-related protein (CRP), also known as 20 (22)-kDa glycoprotein is expressed not only in the ventral prostate, but also in the lacrimal gland of adult male rats. In this study the expression of CRP in androgen-treated female animals is studied. CRP mRNA is absent in the lacrimal gland of untreated adult female rats, but can be induced by androgens, although this induction is slower than in castrated male rats. Estradiol, progesterone or glucocorticoids have no effect. In testicular feminized rats, however, CRP mRNA is not induced in the lacrimal gland by androgens. At the protein level, the presence of CRP in tears of adult male rats is demonstrated. In female animals or castrated male animals CRP can be induced by androgens in a dose-dependent way. Here also the induction is slower in female rats, even during secondary induction after previous full stimulation by androgens. These results indicate that androgens and a functionally normal androgen receptor are essential for the expression of CRP in the lacrimal gland. The time course of induction depends on the dose of androgens, the previous contact with androgens, the duration of the androgen-free interval and the sex of the animals.

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INTRODUCTION

Androgens control the synthesis of a wide variety of proteins in diverse target tissues. The mechanisms responsible for these hormone- and tissue-specific effects are a topic of intensive investigation. Previous work on the rat ventral prostate, a classical target tissue for androgens revealed the presence of several relatively abundant androgen-regulated proteins [1], such as prostatic binding protein (PBP) [2], a kallikrein-related protease [3], the proline-rich polypeptides (PRPs) [4] and a cystatin-related protein (CRP) [5], also described as a 20 or 22-kDa glycoprotein [6, 7]. In fact, several sequence variants of CRP mRNA [5, 8] are found in the rat ventral prostate, encoding respectively CRP1 and CRP2, which are slightly different forms of this protein. CRP2 like PBP and PRP is a specific secretion product of the rat ventral prostate, but CRP1 is also expressed and androgen-regulated in the exorbital lacrimal gland of male rats [5]. Unlike the prostate this organ can also be studied in female rats. For that reason we investigated whether CRP1 mRNA can also be demonstrated in the lacrimal gland of intact or androgen-treated female rats and of genetically male rats affected by testicular feminization due to the presence of a functionally abnormal androgen receptor. In addition, since the presence of a signal peptide indicates that CRP is secreted by the epithelial cells of the lacrimal gland [5], we also studied whether CRP is present and androgen-regulated in rat tears.

*Correspondence to W. Heyns at Legendo, K. U. Leuven, Onderwijs en Navorsing, Gasthuisberg, 3000 Leuven, Belgium.
Received 30 June 1993; accepted 14 Oct. 1993.

EXPERIMENTAL

Materials

Analytical grade reagents were used throughout this study. The CRP1-specific oligonucleotide (oligo 317) [8] used for hybridization was synthetized on a Cyclone DNA synthesizer. Nitrocellulose membranes (Schleicher and Schuell) and nylon membranes (Hybond, Amersham) were used respectively for protein and RNA blotting. The Coomassie protein assay from Pierce was used for the measurement of protein.

Animals

Wistar albino rats (3 month old) were supplied by the breeding center of the University and housed under normal conditions. Genetically male rats with testicular feminization (Tfm rats) were obtained from Dr K. W.

Chung (University of Oklahoma). If required, gonadectomy was performed under ether anaesthesia. Collection of tears was also performed under ether anaesthesia by means of a glass capillary, as described by Sullivan *et al.* [9]. Steroid hormones were given as subcutaneous injections in 0.2 ml of olive oil: androgens (equal amounts of testosterone and testosterone propionate) were given in various doses (50 to 1250 μg). For progesterone the daily dose was 1 mg, for estradiol 1 μg and for dexamethasone 50 μg. Before removal of organs, rats were anaesthetized by ether and bled to death via the carotid artery. Immediately thereafter, organs were removed and frozen in liquid nitrogen.

RNA preparation and Northern blotting

Total cellular RNA was extracted as described by Chirgwin *et al.* [10]. Northern blotting was performed according to Meinkoth and Wahl [11] using a specific oligonucleotide probe for CRP1 mRNA [5, 8], which is found both in the ventral prostate and the lacrimal gland of the male rat. This oligonucleotide was endlabeled by means of T4 polynucleotide kinase (BRL) as recommended by the supplier.

Polyacrylamide gel electrophoresis and immunoblotting

SDS polyacrylamide gel electrophoresis (PAGE) was performed on 15% separating gels as described by Laemmli [12]. Immunoblotting was done as described by Towbin *et al.* [13] using a CRP antiserum at a 1/50 dilution as primary antibody [5] and peroxidase-labeled goat antirabbit IgG at a 1/2000 dilution as second antibody.

RESULTS

Expression of CRP mRNA in the exorbital lacrimal gland of androgen-treated female rats

In male rats CRP1 mRNA is not only present and androgen-regulated in the ventral prostate, but also in the exorbital lacrimal gland. Since this gland has a similar size in female rats, we could also study the presence of CRP mRNA in the exorbital lacrimal gland of untreated or androgen-treated female rats. CRP mRNA was non-detectable on Northern blots of intact or gonadectomized female rats, but could be induced by the administration of androgens both in intact (results not shown) and gonadectomized female rats (Fig. 1). In fact, the same 3 size variants of CRP mRNA are observed as in male rat prostate and lacrimal gland. As has been shown elsewhere [8], the largest two bands differ by the length of their polyA-tail, whereas the smallest form is the result of alternative splicing. It should be noted, however, that the induction in female rats was relatively slow, as compared to that observed in 2 or 7 day castrated male rats suggesting that preexposure to androgens might have promoted the secondary response. Moreover, in male rats the duration of the interval between castration and androgen treatment was of major importance for the time course of induction (Fig. 1). Glucocorticoids, estrogens or progesterone were unable to induce CRP mRNA in the exorbital lacrimal gland of female rats (results not shown).

Lack of expression of CRP mRNA in the exorbital lacrimal glands of male Tfm rats

The expression of CRP mRNA was also studied in genetically male rats presenting the syndrome of testic-

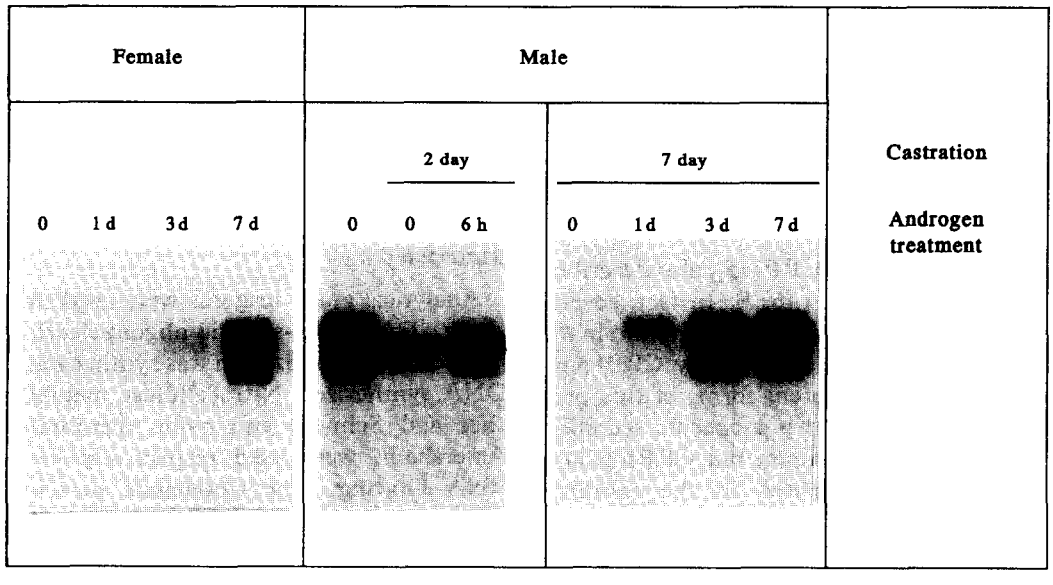

Fig. 1. Effect of androgen treatment (1 mg daily) on the levels of CRP1 mRNA in the lacrimal gland as shown by Northern blotting. Total RNA was prepared from the lacrimal glands (5 rats per sample) of intact female rats and of female rats treated for 1, 3 or 7 days with androgens, of intact male rats and 2-day castrates before and after 6 h of androgen treatment and of 7-day castrates before and after further androgen treatment for 1, 3 or 7 days. RNA (10 μg) was separated by electrophoresis on a 1.7% agarose gel, blotted onto a nylon membrane and hybridized with a CRP1 mRNA-specific oligo.

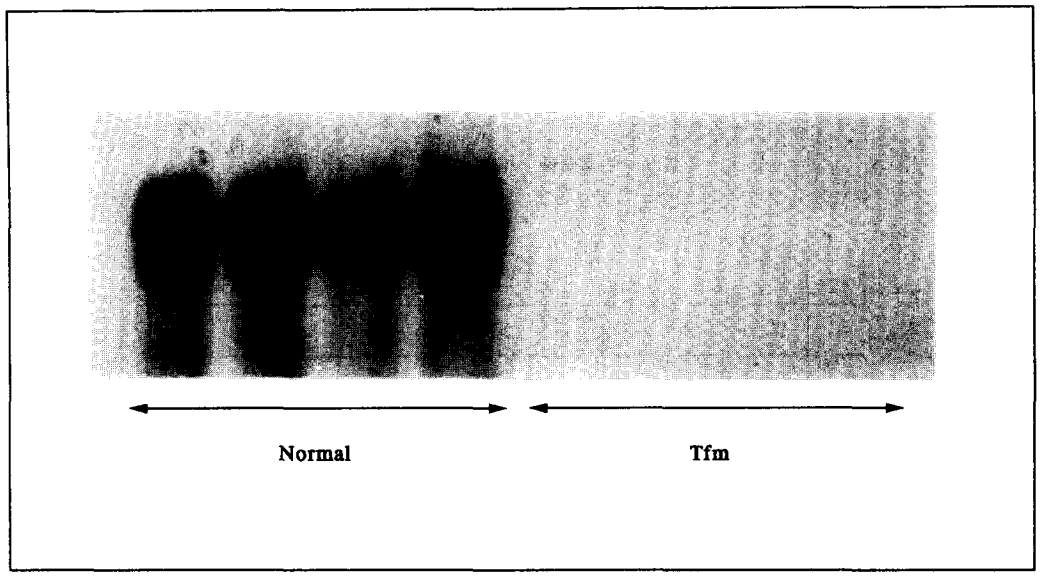

Fig. 2. Northern blot analysis of CRP mRNA in the lacrimal gland of intact adult normal male rats and testicular feminized rats. RNA was extracted from lacrimal glands of 4 intact adult male rats and of 4 adult testicular feminized rats and analyzed by Northern blot as in Fig. 1.

ular feminization. Affected males have a functionally deficient androgen receptor and do not respond to androgens [14]. In these rats the ventral prostate is not developed, making an evaluation of CRP mRNA impossible in this organ. Such evaluation, however, is possible in the exorbital lacrimal gland, which is of similar size as in non-affected animals. In spite of normal male serum levels of androgens, CRP mRNA was non-detectable (Fig. 2) indicating the crucial importance of the androgen receptor for the induction.

CRP in tears of male rats and androgen-treated female rats

In view of the presence of a signal peptide [5] it was most likely that CRP is secreted by the epithelial cells of the exorbital lacrimal gland and therefore should be recovered in the tears of the animals. For that reason tears of adult male or female rats were analyzed by SDS–PAGE followed by Western blotting. As expected, the corresponding 22-kDa band was observed in tears of male rats, but, not of untreated female rats (Figs 3 and 4). Moreover, this band was somewhat microheterogeneous, possibly reflecting partial proteolytic breakdown or differences in glycosylation. Because tears can be collected by a relatively simple, non-invasive procedure, longitudinal studies became possible, wherein the effect of androgens on CRP secretion could be followed in individual animals.

This technique was used to study the induction of CRP by androgens in adult female rats. As shown in Fig. 3(A), CRP becomes detectable after 2 days of androgen treatment (500 μg daily) and is induced gradually thereafter. The extent and rate of induction are dependent on the dose of androgen [Fig. 3(B)]. At a daily dose of 50 μg minimal induction is observed after 3 days and even after 7 days of hormone treatment the degree of induction is limited. At a dose of 250 μg induction is also observed after 3 days, but the degree of induction is more marked. Finally, when 1.25 mg was given daily there is already slight induction after 1 day of treatment and the degree of induction after 3 or 7 days is much more prominent. Similar experiments were performed to follow the disappearance of CRP from tears after castration of adult male rats or after interruption of androgen treatment in female rats. In castrated male rats CRP disappears more rapidly than after the interruption of androgen treatment of female rats, but this may be due to the slower elimination of injected androgens. When androgens are administered again after an interval of 7 days, the rate of reinduction of CRP by androgens, however, is much slower in female rats, in spite of the fact that these animals had already been stimulated by androgens to levels exceeding those observed in adult male rats.

DISCUSSION

The present studies indicate that cystatin-related protein is synthesized and secreted under androgen control in the exorbital lacrimal gland of the rat. The function of this protein is still unknown, but it shows marked structural similarity to cystatin type 2 proteins [5]. The structure of the genes encoding CRP [15] are also clearly related to these cystatin genes, although the CRP genes contain an additional exon, which is probably the result of partial duplication of the second exon from a cystatin gene. Whether there is also a functional relation to cystatin has not yet been demonstrated; in fact, CRP did not inhibit the activity of papain, that is inhibited by most cystatins [16].

CRP has originally been described as an androgen-regulated 20-kDa glycoprotein in the rat ventral prostate [9], a well known androgen target tissue. The observation of androgen-dependent CRP expression in the exorbital lacrimal gland adds further evidence to the hypothesis that this organ is a selective target tissue

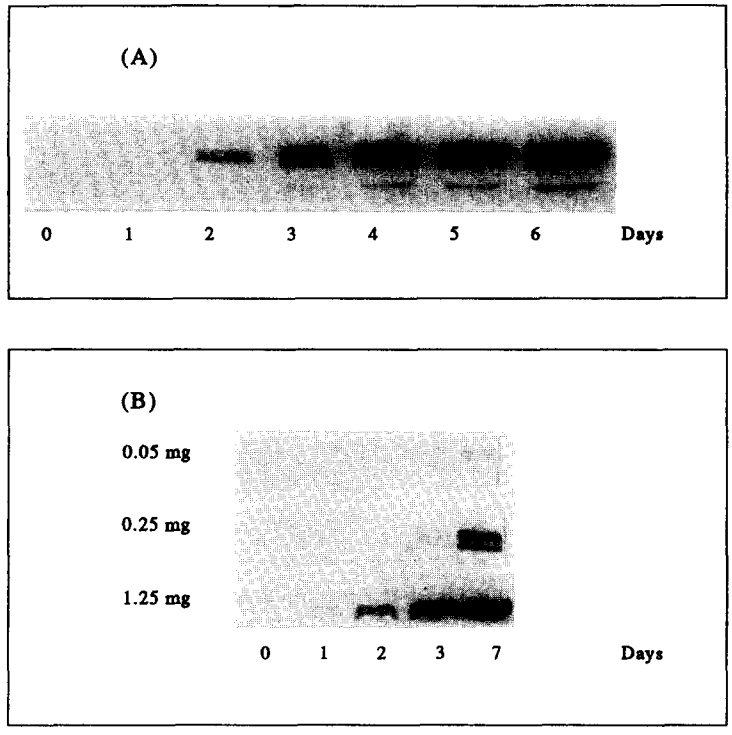

Fig. 3. Induction of CRP excretion in tears of adult female rats by androgen treatment as demonstrated by Western blotting. (A) An adult female rat was treated for 6 days with androgens (0.5 mg daily). Tears were collected daily and their protein concentration was measured. From each sample 4 μg of protein was analyzed by SDS–PAGE followed by immunoblotting using an anti CRP antiserum. (B) Effect of the dose of androgen on the induction of CRP in tears of adult female rats. Groups of 3 rats were treated with 50, 250 or 1250 μg of androgen daily and tears were collected before and after 1, 2, 3 or 7 days of androgen administration. At each time point equal amounts of tear proteins were pooled and analyzed as indicated in A.

for androgens. The lacrimal gland not only contains significant amounts of androgen receptor [17], but androgens also have marked effects on the histomorphometry of this organ [18, 19] and influence the concentration of specific proteins, such as the secretory component (SC) of the immune system [9, 20]. In the latter case, however, the response to androgens is much less pronounced than for CRP: SC is stimulated about 5 times by androgens, whereas CRP is completely non-detectable in female rats and castrated male rats, indicating that androgens are absolutely required for its expression. Moreover, the lack of CRP expression in genetically male rats with testicular feminization in spite of high endogenous levels of androgens underlines the importance of a functional androgen receptor.

We can only speculate on the significance of CRP secretion by the exorbital lacrimal gland. The fact that CRP is strictly androgen-dependent and recovered both in tears and prostatic secretion strongly suggests that this protein plays an accessory role in reproduction and sexual behavior. In this respect it may be significant that the exorbital lacrimal gland of rodents is

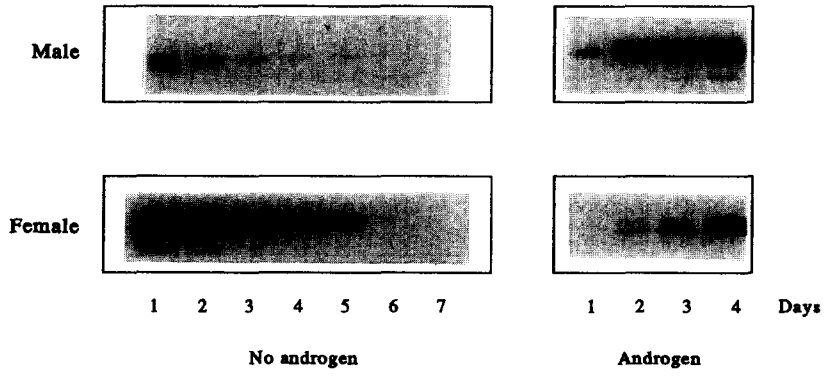

Fig. 4. Effect of androgen withdrawal and androgen readministration on CRP levels in tears of male and female rats. *Top panel*: groups of 4 adult male rats were castrated and treated with androgens (1 mg daily) for 4 days from the 7th day after castration. Tears were collected and analyzed as indicated in Fig. 3. *Bottom panel*: adult female rats were treated with androgens (1 mg daily) for 7 days. After a 7 day interruption androgen treatment restarted at the same dose for 4 days. Tears were collected and analyzed as indicated in Fig. 3.

regarded as a scent gland [21], the animals sniffing each other at the corner of each others' eyes at first encounter, probably to determine age and sex. The presence in tears of alpha-2-urinary globulin, an odorant-binding protein, may also be significant in this respect [22].

When the response to androgen administration is compared in castrated male rats and in female rats, it is obvious that the response to androgens is much more rapid in castrated male rats than in female rats. This is most clearly shown at the mRNA level (Fig. 1), but also apparent, when the secretion of CRP in tears is followed. Previous contact with androgens therefore plays an important role in the time course of induction, suggesting a role of these hormones in the differentiation of the exorbital lacrimal gland. Furthermore, the interval between the previous treatment with androgens and the final androgen treatment is also important for the time course of induction. Consequently, the potential for a rapid response seems to be lost in a rather short time period. Similar observations have been made previously with respect to the expression of CRP in the rat ventral prostate [5], although it was impossible in this case to study the effect of primary induction. The mechanism responsible for the more rapid secondary induction is not clear: it is conceivable that primary induction results in a higher number of epithelial cells able to respond to androgen treatment by the synthesis of CRP mRNA or alternatively the CRP response of these cells to androgens might be more marked. For that reason it might be worthwhile to follow the course of primary and secondary induction by immunohistochemical techniques or *in situ* hybridization.

Because of its rapid and marked response to androgen withdrawal or androgen administration CRP is a promising candidate for the study of the mechanism of action of androgens. The fact that CRP1 is also expressed and androgen regulated in the exorbital lacrimal gland may be a valuable property in this respect. Indeed, the size of this organ is much less dependent on the presence of androgens than that of the ventral prostate, suggesting a less important role of hormone-regulated apoptosis and cell proliferation. Moreover, a defined culture system has been described, which permits the long-term maintenance of viable and differentiated lacrimal gland acinar cells [23]. In view of the importance of tissue specific factors such culture system might be a valuable tool for transfection experiments with constructs linking putative regulatory regions from the CRP genes to reporter genes.

In conclusion, the androgen-regulated expression of CRP by the exorbital lacrimal gland provides a specific and sensitive model system for the study of androgen activity, that can be easily monitored by means of a non-invasive procedure. For that reason it may be worthwhile to elaborate a quantitative immunoassay for CRP, which may be used for *in vivo* studies and for the study of the regulation of CRP by androgens in cultured cells.

Acknowledgements—The authors wish to thank M. Hertogen and H. Geeraerts for excellent technical assistance, A. Devos, B. Peeters and W. Rombauts for fruitful collaboration on other aspects of the project and K. W. Chung and D. Vanderschueren for making available the lacrimal glands from testicular feminized animals. The text presents research results of the Belgian National Incentive Program on Fundamental Research in Life Sciences initiated by the Belgian State Prime Minister's Office-Science Policy Programming. The scientific responsibility is assumed by its authors. The work was further supported by Grant No. 30015.88 from the Nationaal Fonds voor Wetenschappelijk Onderzoek and a grant from the Nationale Loterij.

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