Testosterone induces the expression of the uteroglobin gene in rabbit epididymis

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Uteroglobin was characterized in the rabbit epididymis by radioimmunoassay and electrophoretic determinations, as well as by analysis of its mRNA by means of 'Northern blot' and nuclease-S1 mapping. Treatment of sexually immature rabbits with testosterone during 5 days increased up to 8-fold the concentrations of both uteroglobin and its mRNA in the epididymis. The amounts of β -tubulin mRNA, measured as reference, remained unchanged after the hormonal treatment. The synthesis of uteroglobin occurred mainly in the middle region of the epididymis, progressively decreasing toward the distal part of the organ. Uteroglobin was not detected in the testis by radioimmunoassay. The results are discussed in relation to a possible role of uteroglobin in the reproductive functions.

INTRODUCTION

Uteroglobin, a small secretory protein, is mainly synthesized in the endometrium and the lung of the rabbit and related species (for reviews see Beato *et al.*, 1983; Wesphal, 1985). The synthesis of uteroglobin is subject to differential hormonal control in the two organs mentioned, being regulated by ovarian steroid hormones in the uterus (Suske *et al.*, 1983; Bailly *et al.*, 1983) and by glucocorticoids in the lung (Torkkeli *et al.*, 1978; Fernández-Renau *et al.*, 1984; López de Haro & Nieto, 1985a). In addition to those organs, uteroglobin-like antigens have been reported to occur in other tissues of both the female and the male genital tract, such as the oviduct (Kirchner, 1976; Goswami & Feigelson, 1974) and the epididymis (Noske & Feigelson, 1976; El Etreby *et al.*, 1983). In the latter organ, however, the nature of the antigen has been poorly studied, and both its synthesis and its possible hormonal control have not been determined. Such studies are of interest, as they could throw more light on the physiological role of uteroglobin as well as on the mechanisms of the hormonal control of this gene.

Moreover, the study of epididymal secretory proteins is important, as some of them appear to have an important role in the acquisition of motility, fertilizing ability and storage of spermatozoa (Rifkin & Olson, 1985; Orgebin-Crist *et al.*, 1975).

In the present work we characterized epididymal uteroglobin and its mRNA, also showing that both are correlatively increased after administration of testosterone.

MATERIALS AND METHODS

Materials

Enzymes were obtained from Boehringer-Mannheim. L-[³⁵S]Methionine, [α -³²P]- and [γ -³²P]-dATP were from Amersham International. Testosterone propionate (Testoviron) was from Schering-España. Plasmid pUG119 contained a rabbit uteroglobin cDNA of

390 bp (López de Haro & Nieto, 1985b). Plasmid pUG5, which contained a 5 kbp fragment of the 5' region of the uteroglobin gene, was a gift from Dr. M. Beato (Marburg University, Germany). Plasmid pT2, containing a β -tubulin cDNA, was kindly provided by Dr. F. Wandosell (C. B. M., Madrid, Spain).

Animals and hormonal treatment

Sexually immature New Zealand rabbits (about 2 months old) were normally used. In each experiment two or three animals were pooled to serve as control or experimental groups respectively. Animals from the experimental group received daily intramuscular injections of 5 mg of testosterone during 5 days. The animals were killed 12 h after the last injection. For studies on the regional distribution of uteroglobin synthesis, adult animals (10–15 months old) were used.

Preparation of RNA

Epididymides were quickly trimmed of fat and powdered with liquid N₂ in a pre-cooled mortar. A small portion was taken for RIA measurements as described below. Total RNA was prepared from the remaining powder by extraction with a phenol mixture and precipitation with 3 M-sodium acetate (Fernández-Renau *et al.*, 1984). RNA from either endometrium of pseudopregnant female rabbits or rat lung was obtained similarly.

Immunological techniques

Portions of the powdered epididymides were homogenized in 1–2 ml of phosphate-buffered saline containing 1% Triton X-100. After centrifugation at 10000 *g* for 10 min, samples of the supernatants were taken for measurement of the tissue protein concentration (Lowry *et al.*, 1951) and for RIA determination of uteroglobin (Nieto & Beato, 1980). Samples of the homogenates were also analysed by SDS/polyacrylamide-gel electrophoresis (Nieto & Beato, 1980). At the end of the run, gels were sliced and the fractions extracted as described by Nieto &

Abbreviations used: RIA, radioimmunoassay; bp, base pair.

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Lombardero (1982) for determination of immuno-reactivity by RIA.

Measurements of the relative synthesis of uteroglobin were carried out by immunoprecipitation of the [35 S]-methionine-labelled proteins synthesized by epididymal explants cultured *in vitro* for 2 h, as described previously for other tissues (Fernández-Renau *et al.*, 1984; Nieto & Beato, 1980).

Nucleic acid hybridizations

For Northern-blot analysis, the RNA was electrophoresed in 1.5%-agarose gels containing formaldehyde (Maniatis, 1982), blotted on nitrocellulose paper and hybridized as described by López de Haro *et al.* (1984). After washing, blots were autoradiographed at -70°C with intensifying screens. The probes used were pUG119 or pT2 labelled by nick translation up to a specific radioactivity of 5×10^7 c.p.m./ μg .

Nuclease-S1 digestion of RNA-DNA hybrids was done essentially as described by Maniatis (1982), with an excess of the *Bst*EII fragment of plasmid pUG5, which contains the main initiation site of the uteroglobin mRNA (Suske *et al.*, 1983). Usually, hybridizations were carried out at 52°C in 80% (v/v) formamide and the products were digested for 2 h with 3000 units of nuclease S1/ml. In some experiments the temperature was raised to 57°C and the time of digestion increased to 4 h. DNA was 5'-end-labelled with polynucleotide kinase (Maniatis, 1982). The digested products were analysed on 6%-polyacrylamide sequencing gels (Maxam & Gilbert, 1980). After autoradiography, the band generated by the

mature uteroglobin mRNA was cut out of the gel and its radioactivity measured in a scintillation counter.

Dot-blot hybridizations were performed as described previously (Fernández-Renau *et al.*, 1984).

RESULTS

Fig. 1(a) shows the competition curves generated in the RIA by increasing doses of either homogeneous uterine uteroglobin or an extract of the epididymal tissue. The identity of both curves suggests that the epididymal antigen is similar to authentic uteroglobin. On the other hand, extracts from rabbit testis do not show immunoreactivity, which is in agreement with previous immunohistochemical observations (El Etreby *et al.*, 1983). To obtain more information on the nature of the antigen, the epididymal extract was analysed by SDS/polyacrylamide-gel electrophoresis. The results, shown in Fig. 1(b), indicate that the epididymal antigen behaves like authentic uteroglobin in the electrophoresis.

Next, we analysed the uteroglobin mRNA sequences in the epididymis by the Northern-blot technique. RNA from pseudopregnant-rabbit endometrium, a very rich source of uteroglobin mRNA (Arnemann *et al.*, 1977; Atger & Milgrom, 1977), was analysed in parallel as marker. Endometrial RNA (Fig. 2, lane a) displays a strong hybridization signal corresponding to the mature form of uteroglobin mRNA, which is known to contain about 600 nucleotides (Snead *et al.*, 1981). The RNA from the epididymis of testosterone-treated rabbits (Fig. 2, lane b) also shows a weak band at the position of the

Fig. 1. Characterization of epididymal uteroglobin

(a) RIA curves generated by increasing amounts of either the standard of uterine uteroglobin (○) or extracts from epididymides (●) and testis (■) from adult rabbits. (b) and (c) The epididymal extract was subjected to SDS/polyacrylamide-gel electrophoresis either in the absence (b) or in the presence (c) of 2-mercaptoethanol, and the gel fractions were assayed by RIA. Arrows point to the position of the dimeric (UG_2) and monomeric (UG_1) forms of the uteroglobin marker run in parallel.

Fig. 2. Northern-blot analysis of epididymal RNA

Total RNA extracted from the tissues listed below was electrophoresed on a 1.5%-agarose gel, blotted on nitrocellulose and hybridized either to the pUG119 uteroglobin cDNA (a) or to the pT2 tubulin cDNA probe (b). RNA was from: (a) rabbit endometrium, used as marker (5 μ g); (b) and (c) epididymis from testosterone-treated and control rabbits respectively (20 μ g in each case); (d) rat lung, used as negative control (20 μ g); (e) and (f) epididymis from testosterone-treated and control animals respectively (20 μ g). Autoradiography was for 5 h in (a), for 10 days in (b)–(d) and for 2 days in (e) and (f). The approximate numbers of nucleotides ($\times 10^{-3}$) of the markers (28 S and 18 S rRNA and the mature uteroglobin mRNA) are given on the left.

mature uteroglobin mRNA. This band is present neither in the epididymal RNA from the untreated rabbits nor in the RNA from rat lung used as control (Fig. 2, lanes c and d). Owing to the long exposure time, some signals were also obtained at the zones corresponding to rRNA. Northern-blot analysis of the tubulin mRNA sequences in the epididymis shows that its content is little affected by the hormonal treatment (Fig. 2, lanes e and f).

Uteroglobin-mRNA sequences in the epididymis were also characterized by nuclease-S1 mapping of the 5' end of the mRNA as described in the Materials and methods section. The results obtained with the marker of endometrial RNA (Fig. 3, lane a) indicated the presence of a major and broad band corresponding to the expected protected fragments of about 78 nucleotides, extending from the 'cap' site to the 5' end of the *Bst*II cut site. Some minor bands corresponding to larger fragments were also detected, which may represent minor alternative points of initiation upstream of the 'cap', as previously observed (Bailly *et al.*, 1983; Suske *et al.*, 1983). The pattern obtained with epididymal RNA from testosterone-treated rabbits was similar (Fig. 3, lane b), although it was necessary to use about 200-fold more RNA to get a signal similar to that generated by endometrial RNA. Besides the major signal corresponding to the main initiation point, epididymal RNA from testosterone-treated rabbits also produced minor

Fig. 3. S1-nuclease mapping of the 5'-end of epididymal uteroglobin mRNA

Total RNA was hybridized to the 5'-labelled probe, digested with S1 nuclease, and the products were analysed in denaturing polyacrylamide gels as described in the Materials and methods section. RNA was from: (a) rabbit endometrium (1 μ g); (b) and (c) epididymis from testosterone-treated or control rabbits respectively (200 μ g each); and (d) rat lung (100 μ g). The diagram shows the complementary strand of the *Bst*EII fragment of genomic DNA used as probe. The bracket indicates the expected 78-nucleotide fragment protected from S1-nuclease digestion. To the right are given the numbers of nucleotides (bases, b) of the following markers: *Hind*III restriction fragments of phage ϕ 29 (271, 170 and 72 b respectively) and the *Bst*EII/*Bam*HI fragment of the probe (66 b).

bands of 270–240 nucleotides, whose significance will be commented on in the Discussion section. Epididymal RNA from untreated rabbits (Fig. 3, lane c) produced a very weak signal, indicating a much lower concentration of uteroglobin mRNA. No bands were seen with RNA from rat lung (Fig 3, lane d).

The above results indicated that testosterone increased the amounts of uteroglobin mRNA in the epididymis. Therefore we determined in several separate experiments the correlation between the concentrations of uteroglobin and its mRNA in the epididymis of control or testosterone-treated rabbits. The results in Table 1 indicate that the hormone concomitantly increases both parameters. On the other hand, dot-blot analysis showed that the amounts of β -tubulin mRNA were not affected by the hormonal treatment (Table 1), which substantiated the specificity of the effect on uteroglobin mRNA.

Finally, it was decided to see whether the synthesis of uteroglobin occurs uniformly through the epididymis or is restricted to some regions. The results are shown in Fig. 4. Maximal synthesis occurs in the corpus segment, with a progressive decrease toward the distal region. No detectable synthesis was found in the first half portion of the caput, and little, if any, in the proximal portion of the

Table 1. Effect of testosterone on uteroglobin content and uteroglobin and tubulin mRNA concentrations in epididymis from immature rabbits

Paired groups of animals of the same age were either used as controls or treated for 5 days with testosterone. Epididymides from each group were pooled, frozen and powdered. Portions of the powder were used to measure uteroglobin by RIA, uteroglobin mRNA by nuclease-S1-resistance experiments and tubulin mRNA by dot-blot hybridization as described in the Materials and methods section.

Expt. no.	Treatment	Uteroglobin (ng/mg of tissue protein)	Uteroglobin mRNA (c.p.m./mg of total RNA)	Tubulin mRNA (c.p.m./μg of total RNA)
1	Control	5.3	1460	75
	Testosterone	9.2	3650	62
2	Control	1.5	340	52
	Testosterone	11.9	1950	56
3	Control	1.8	245	52
	Testosterone	14.5	2000	62
4	Control	8.7	1835	65
	Testosterone	37.5	8255	57

Fig. 4. Uteroglobin synthesis in the different regions of the rabbit epididymis

The relative synthesis of uteroglobin (expressed as a percentage of the total protein synthesis) was determined in explants prepared from the indicated regions. Values are means of two different experiments whose range is indicated by the vertical bars. Abbreviation: Def., proximal portion of vas deferens.

vas deferens. The synthesis of total proteins (expressed per mg of tissue protein) was not significantly different in all epididymal regions (results not shown).

DISCUSSION

The present study unambiguously demonstrates by several criteria that the antigen detected in the rabbit

epididymis by immunohistochemical techniques (El Etreby *et al.*, 1983) is authentic uteroglobin. This demonstration appears important, since the presence of other antigens cross-reacting with the uteroglobin antibodies cannot be ruled out. Thus the C₁ and C₂ subunits of prostatic steroid-binding protein of the rat show amino acid sequence similarity with uteroglobin (Baker, 1983) and cross-react with anti-uteroglobin antibodies (Aumüller *et al.*, 1985).

We also show here that the concentrations of both uteroglobin and its mRNA in the epididymis increase after administration of testosterone to sexually immature rabbits. The mechanism of this effect remains to be established, but the data presented here are compatible with a transcriptional effect, according to the typical mechanism of action of steroid hormones. Other mechanisms, including testosterone-induced cell differentiation or proliferation, cannot be ruled out. Nevertheless, the latter mechanism seems unlikely, since our experiments indicated a slight increase in epididymal size after 5 days of testosterone treatment. The tissue-specific multi-hormonal regulation of the uteroglobin gene can offer an interesting model in which to study the molecular mechanisms of steroid-hormone action.

The extent of uteroglobin synthesis in the epididymis appears to be low compared with those reported in other uteroglobin-producing organs. Thus the relative synthesis (0.1–0.2%) observed in the adult male (animals with maximal synthesis) is far below the values of 1.5% and 9% observed in the lung (Lombardero & Nieto, 1981) and the stimulated uterus (Nieto & Beato, 1980) respectively. The low synthesis of uteroglobin in the epididymis was also confirmed by the determinations of the relative amounts of its mRNA (Figs. 2 and 3). It should be noted, however, that in spite of this low synthesis the total amount of uteroglobin in the luminal fluid of the epididymal cauda is rather high, reaching up to more than 30 μg in some adult animals (M. S. López de Haro, unpublished work).

Nuclease-S1 protection experiments (Fig. 3) showed that the main point of transcription initiation in the uteroglobin gene is the same in the uterus and the epididymis. However, epididymal RNA also generated minor bands corresponding to transcripts apparently initiated upstream of the 'cap' site. These bands persisted when both the hybridization stringency and the nuclease digestion time were increased, as described in the Materials and methods section (results not shown), which indicated a high degree of sequence homology with the probe. Furthermore, after testosterone administration these bands appeared concomitantly with the signal corresponding to authentic uteroglobin mRNA. It seems likely that these minor transcripts represent alternative points of initiation of the uteroglobin gene in the epididymis.

The remarkable regional differences in uteroglobin synthesis along the rabbit epididymis resemble those found for other proteins of the rat epididymis (Jones *et al.*, 1980; Brooks *et al.*, 1986). This fact might reflect a requirement for localized high concentrations of these proteins for specific functions of the spermatozoa.

The function of uteroglobin remains still unknown, but the fact that it is found in the genital tracts of both the male and the female rabbit suggests a role of the protein in some reproductive process. In the case of the epididymis, the protein might be implicated in the

maturation of the spermatozoa, like other epididymal proteins whose presence appears to be important for the acquisition of motility and fertilizing abilities (Rifkin & Olson, 1985; Orgebin-Crist *et al.*, 1975). Alternatively, it has been suggested that the binding of uteroglobin to the spermatozoa surface could mask the antigenicity of the male gamete (Mukherjee *et al.*, 1983).

Uteroglobin might be an interesting marker of the androgen action in the epididymis, since it is so far the only epididymal secretory protein whose gene has been extensively characterized.

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