Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 125-129

BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF THE ACROSOME REACTION-INDUCING SUBSTANCE (ARIS) OF HFF

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Received December 28, 1993

Summary: Gel filtration experiments with ^{3}H-progesterone labeled hFF demonstrated that the AR-inducing activity of hFF might be mediated by a progesterone-binding protein. Further immunological investigations added evidence in support to ARIS being identical with the SERPIN CBG. Moreover, only the protein-progesterone-complex was able to induce AR. It is suggested that the CBG-progesterone-complex is proteolytically cleaved at the plasma membrane of spermatozoa, releasing a high local concentration of progesterone which leads to induction of the AR.

The acrosome of the mammalian spermatozoon is a membrane-bound organelle which appears during spermatogenesis as a product of the Golgi's complex. The acrosome reaction, which is a prerequisite for fertilization in mammals, is an exocytotic process involving fusion of the sperm plasma membrane and outer acrosomal membrane (1,2). Only acrosome-reacted spermatozoa can penetrate the zona pellucida (3). The physiological site and the initiation of the AR are still controversial. In hFF, the physiological environment of human gametes, two stimulators of the AR are discussed: (i) a 50 kD protein (4,5) and (ii) progesterone (6,7). To further clarify the molecular basis of the induction of the AR by hFF, we started to characterize the 50 kD protein of hFF.

Material and Methods

Sperm collection and processing
Semen was obtained from normozoospermic patients attending the andrological outpatient clinic at the Department of Dermatology and Andrology, Justus Liebig University, Giessen. The ejaculates were collected by masturbation after 4 to 7 days of sexual abstinence, then allowed to liquefy at room temperature, and analyzed according

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Abbreviations: AR, acrosome reaction; ARIS, acrosome reaction-inducing substance; CBG, corticosteroid-binding globulin; hFF, human follicular fluid; HSA, human serum albumin; HTF, human tubular fluid medium; SERPIN, serine proteinase inhibitor.

0006-291X/94 $5.00

to WHO criteria. Sperm preparation and capacitation were performed as described earlier (8). Samples of hFF were collected by follicle aspiration from women undergoing in vitro fertilization treatment at the Department of Gynecology and Obstetrics, Justus Liebig University, Giessen.

Quantification of AR
In all experiments, the triple stain technique (9) was used to quantify the AR. The treated slides were examined at 1000x magnification under oil immersion with a brightfield microscope. For estimation of the acrosome reaction, 400 morphologically normal spermatozoa were evaluated in randomly selected fields, and the proportion of live acrosome-reacted spermatozoa was determined in percent of all vital spermatozoa.

Statistical analysis was performed by one-factorial variance analysis according to Scheffé. Data were analyzed by calculating mean values ± SEM.

Results and Discussion

Apparent molecular masses
The molecular mass of ARIS was determined by gel filtration on Sephacryl S-200 SF. Detection of AR-inducing activity was followed by the triple stain technique. One peak of AR-inducing activity at an apparent molecular mass of 45 ± 8 kD was found (Fig.1). This was in good agreement with previously reported molecular masses for ARIS (4,5). After labeling of hFF with ^{3}H-progesterone and subsequent elution on the same gel filtration column, two peaks of radioactivity were obtained. The peak in the high molecular range (protein-bound ^{3}H-progesterone) was eluted exactly in the same

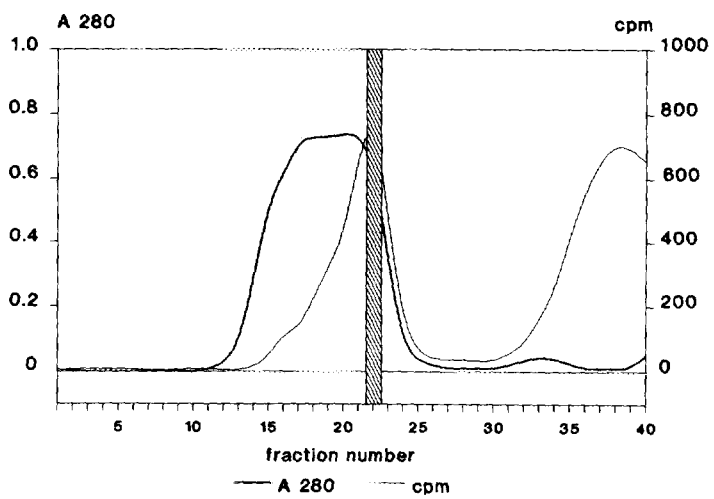

Figure 1: Gel filtration

One ml of hFF was treated for 1 h at 4°C with the pellet of 5 ml of dextrane-coated charcoal suspension (11). After centrifugation, 1 ml of the supernatant was incubated with 10 µl of ^{3}H-progesterone (200 ng progesterone, 0.185 MBq; NEN, Dreieich, Germany) at RT for 1 h. This solution was eluted with HSA-free medium, pH 7.5, on a Sephacryl S-200 SF column (1 x 52 cm) at 4°C and a flow rate of 2 ml·h^{-1}·cm^{-2}. Elution was monitored at 280 nm (——— A 280) and the fractions were tested for their AR-inducing activity (hatched area) and radioactivity (— cpm). Molecular mass was determined after calibration of the column with proteins of different molecular masses and by optical inspection of the plotted K_{av} value.

protein fraction that was found for the AR-inducing activity (Fig.1). The second peak corresponds to free radioactive progesterone. According to these data, and as recently suggested by other authors (10,11), ARIS is likely to be a progesterone-binding protein. However, induction of the AR in human spermatozoa was also observed after stimulation with progesterone alone (6), although these concentrations were much higher than those of bound progesterone used in our experiments. From the data of radioactively labeled progesterone we calculated a concentration of 50 $ng \cdot ml^{-1}$ progesterone, which was sufficient to elicit AR in spermatozoa (Fig.1).

Immunological characterization

A few steroid-binding transport proteins in the plasma are well characterized (12). According to our findings, the molecular mass of ARIS ranges between 37 and 53 kD. Only one of the steroid-binding proteins that are already known fits into this molecular mass range, the corticoid-binding globulin with a molecular mass of 52 kD (13,14). For further characterization of ARIS, we incubated hFF with a polyclonal antibody against human CBG. Thereafter, the antibody-CBG-complex was removed by binding to sepharose-bound protein A and subsequent centrifugation. The CBG-free supernatant was used for induction of AR in human spermatozoa. After removal of CBG by a specific antibody from hFF, hFF failed to induce the AR (Table 1).

The dAR value obtained after antibody incubation was not significantly different from that of the negative control (incubation with HTF medium). The presented findings suggest that CBG is a causative agent in hFF, inducing the exocytosis in human spermatozoa.

Direct stimulation of AR by CBG

To clarify inasmuch free or bound progesterone is responsible for induction of the AR, we incubated capacitated human spermatozoa with the protein CBG, the CBG-

Table 1: Treatment of hFF with anti-CBG

n = 12	$\bar{x}$ [%]	dAR [%]	SD	p
HTF/HSA	9.0	-	4.1	-
hFF	16.1	7.1	4.0	< 0.0025
hFF after anti-CBG treatment	10.3	1.3	4.3	< 0.1

100 µl hFF was incubated with 500 µl anti-CBG (1:85; DAKO GmbH, Hamburg, Germany) for 1 h at 37°C. Thereafter, the solution was added to a pellet of 500 µl washed protein A-Sepharose suspension, incubated for 1 h at 37°C and centrifuged. The supernatant was tested for AR-inducing activity. The positive control was treated in the same way without addition of antibody. $\bar{x}$ [%]: mean value of 12 different determinations. dAR [%]: Difference between spontaneous and total AR. SD: standard deviation. p: Significance between value and negative control value (HTF/HSA).

Table 2: AR-inducing activity of progesterone, CBG and CBG-progesterone-complex

n = 5	$\bar{x}$ [%]	dAR [%]	SD	p
HTF/HSA	7.0	-	0.8	-
progesterone	7.5	0.5	2.0	< 0.35
corticosteroid-binding globulin (CBG)	6.3	-0.7	1.0	< 0.2
CBG/progesterone complex	11.45	4.45	1.5	< 0.0005

Capacitated human spermatozoa were incubated for 30 min at 37°C with 225 ng·ml^{-1} progesterone, 40 µg·ml^{-1} CBG (Biogenesis, Bournemouth, UK) and CBG-progesterone-complex (40 µg·ml^{-1} CBG, 225 ng·ml^{-1} progesterone, pre-incubated for 1 h at 37°C). The negative control was incubated with medium alone (HTF/HSA). $\bar{x}$ [%]: mean value of 5 different determinations. dAR [%]: Difference between spontaneous and total AR. SD: standard deviation. p: Significance between value and control value.

progesterone-complex and free progesterone. Only CBG preincubated with progesterone induced AR (significantly compared to negative control), while induction was not achieved by incubation with the protein or free progesterone alone (Table 2). The concentration of CBG used in this experiment was within the physiological range (6,15). Progesterone was added in equimolar concentrations corresponding to 225 ng·ml^{-1}. Incubation with free progesterone was performed at the same level. Other authors who described induction of AR by free progesterone used concentrations ranging between 1 and 400 µg·ml^{-1}, which were at least 5 times higher than those in our experiments.

Concluding Comments

According to earlier investigations, AR was induced by a protein with an apparent molecular mass of 50 kD (4,5). These findings are consistent with our data suggesting a 45 kD fraction to be responsible for induction of exocytosis. Moreover, other authors demonstrated that progesterone was able to induce the AR in human spermatozoa (6,7,11). After exchange of progesterone by ^{3}H-progesterone, radioactivity and AR-inducing activity were eluted in identical fractions, indicating that ARIS might be a progesterone-binding protein. By removal of AR-inducing activity by anti-CBG and subsequent adsorption to protein A we showed that ARIS of hFF is identical with CBG. In further experiments, AR-inducing activity could only be demonstrated for the CBG-progesterone-complex, while CBG and progesterone alone failed to induce AR. The progesterone concentrations in our studies were much lower than those used by other authors (7,15). At these low concentrations, free progesterone did not show significant induction of the AR in human spermatozoa. At physiological conditions, the CBG-progesterone-complex might therefore play an important role in hFF-mediated AR induction.

CBG is a member of the SERPIN superfamily (16). CBG tightly binds progesterone and releases it after proteolysis by serine proteinases (17). This mechanism is thought to be essential for activation of neutrophils by delivering high local concentrations of corticoids in inflammatory processes (16,18).

The serine protease acrosin was localized at the plasma membrane of human spermatozoa (19). Inactive proacrosin is usually located within the acrosome. During capacitation, proacrosin and acrosin are exposed at the plasma membrane of the spermatozoon. Hence, the CBG-progesterone-complex might be bound at and proteolytically cleaved by exposed acrosin, which leads to high local concentrations of progesterone and induction of AR. This theory is supported by findings from other authors (20). Serine proteinase inhibitors are able to inhibit progesterone-induced Ca^{2+} influx, which is required for acrosomal exocytosis. The suggested mechanism for the processes occurring during CBG-progesterone-induced AR might explain most of the controversial data.

Acknowledgment

This work was supported by Bundesminister für Forschung und Technologie, project no. 01KY9105/6.

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