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·*Original Article*·

Protease activated receptor 2 and epidermal growth factor receptor are involved in the regulation of human sperm motility

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Abstract

Aim: To investigate mechanisms of tryptase-induced reduction of sperm motility and explore whether epidermal growth factor receptor (EGF-R) and protease activated receptor 2 (PAR-2)- associated pathways are involved. **Methods:** Fresh semen was collected from healthy donors (n = 15). Semen parameters and quality were assessed in accordance with the World Health Organization (WHO) criteria. Swim-up sperm were fixed and subjected to immunocytochemistry and immunoelectronmicroscopy with specific antibodies directed against PAR-2 and EGF-R. Protein extractions from swim-up spermatozoa were analyzed by Western blotting with antibodies for both receptors. Motility of spermatozoa was evaluated by computer-assisted semen analysis. **Results:** Immunocytochemistry found PAR-2 and EGF-R in approximately 30% of examined human ejaculated spermatozoa. Both receptors were localized in the plasma membrane. Like tryptase, the PAR-2 synthetic agonist SLIGKV reduced sperm motility, and this effect was inhibited by application of two specific EGF-R pathway blockers (AG1478 and PD168393). **Conclusion:** The observed reduction of sperm motility by tryptase through the PAR-2 receptor involves EGF-R pathways. *(Asian J Androl 2007 Sep; 9: 690–696)*

Keywords: spermatozoa; motility; epidermal growth factor receptor; protease activated receptor

1 Introduction

Mast cells (MC) are located throughout the male and female genital tract and secrete a plethora of potent mediators [1–3]. Although their function in reproduction is largely unknown, the importance of MC secretory products is indicated by clinical studies showing improved semen parameters after treating subfertile and infertile men with MC blockers [4–6].

The main MC product, the serine protease tryptase, is of special interest and has been described as elevated in the seminal plasma of infertile subjects [3]. Tryptase has been shown to exert action through activation of the protease-activated receptor 2 (PAR-2) [7–9], but other models of action suggest an activation of phosphatidylinositol

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3-kinases proteolytically independently from PAR-2 [10].

PAR-2 belongs to the family of G protein-coupled receptors with seven transmembrane-spanning domains, comprising four members, known as PAR-1, PAR-2, PAR-3 and PAR-4 [11]. PAR-2 can be activated by multiple trypsin-like enzymes, including trypsin, and MC tryptase. The mechanism of activation of this receptor is the irreversible proteolytic cleavage of the N-terminal external domain of the protein. The newly generated N-terminus serves as a tethered ligand capable to activate the receptor itself. Synthetic activating peptides, like SLIGKV, which mimic the tethered ligand of PAR-2, specifically activate PAR-2 independently of receptor proteolysis [11].

In our recent studies, we localized PAR-2 in the plasma membrane of human spermatozoa and found that activation of PAR-2 receptors by human recombinant tryptase reduced human sperm motility in a dose-dependent and time-dependent fashion involving MAP-kinases [3, 12].

Epidermal growth factor (EGF) is also present in seminal fluid [13] and some authors have observed an effect of the molecule on several sperm motility parameters [14]. Interestingly, Darmoul *et al.* [15] described a novel pathway in which MAP kinase activation is linked to EGF-R transactivation, raising the question of whether such interactions might also occur in human spermatozoa.

Here, we show that PAR-2 and EGF-R are both localized on human spermatozoa. Specific activation of PAR-2 using a selective agonist peptide *in vitro* leads to a decrease in sperm motility, which can be blocked by application of inhibitors of EGF-R pathways, pointing towards the involvement of EGF-R in PAR-2 mediated inhibition of human sperm motility.

2 Materials and methods

2.1 Chemicals

Human tubal fluid medium (HTFM) based on Earle's balanced solution containing 26 mmol/L sodium bicarbonate, 0.8 mmol/L sodium pyruvate, 2.8 mmol/L glucose, 1 mL/L synthetic serum replacement SSR, 1% human serum albumine, 15 mmol/L HEPES, 50 000 IU/L penicillin-G, 50 mg/L streptomycin sulphate and 0.01 g/L (pH 7.3) phenol red were all obtained from Stephan Gück GmbH (Berlin, Germany). The following were used: SLIGKV (peptide PAR-2 agonist; NeoMPS, Strasbourg, France), AG1478 (EGF-R tyrosine kinase-specific inhibitor; Calbiochem, Bad Soden, Germany), PD168393 (EGF-R tyrosine kinase-specific inhibitor; Calbiochem, Bad Soden, Germany) and recombinant EGF (Sigma-Aldrich, Schnelldorf, Germany). Antibodies were purchased from the following vendors: polyclonal anti-human EGF-R and the respective blocking peptide from Dunn Labortechnik GmbH (Asbach, Germany); polyclonal anti-human PAR-2 from Lifespan Biosciences (Seattle, WA, USA); and monoclonal anti-human PAR-2 from Invitrogen GmbH (Karlsruhe, Germany). The secondary antibodies for immunofluorescence were coupled to fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC). For Western blots, the secondary antibodies were coupled to peroxidase.

2.2 Sperm preparation

Fresh semen was collected from healthy donors (n = 15) with no history of diseases related to infertility and who had given informed consent. Semen samples were allowed to liquefy at room temperature for 30 min, then they were washed twice (500 × g, 10 min) and the pellet was resuspended in 2 mL of HTFM medium. After 1 h at 37ºC, the swim-up sperm [16], in concentrations of 1–2 × 10^7 cells/mL, were analyzed. Semen parameters and quality were assessed in accordance with World Health Organization (WHO) criteria [17]. Motile spermatozoa were evaluated by computer-assisted semen analysis (CASA) using the Stroemberg–Mika cell motion analyzer (version 4.4; Mika Medical GmbH, Rosenheim, Germany). Briefly, 5 µL aliquots of sperm suspensions were transferred into disposable counting chambers (10 µm depth). Measurements of motility parameters were performed and observed by a high resolution CCD-video camera and an Optiphot-2 microscope (Nikon, Tokyo, Japan). A minimum of 100 spermatozoa from at least 10 different fields were analyzed. The motility was graded as follows: classes a and b, fast and weak forward motility; class c, no progressive motility; and class d, immobile spermatozoa. Sperm velocity and kinematics characteristics were evaluated only for motile sperm and expressed as mean values of straight progressive velocity (a + b). Sperm vitality was assessed using Eosin staining. For motility experiments, sperm were incubated with: SLIGKV (100 µmol/L), AG1478 (10 µmol/L) or PD168393 (2 µmol/L) and a combination of the blockers with SLIGKV peptide. The samples were collected and measured after 60 min. Motility results reported in the present study represent percentage of motility (WHO a + b) normalized to the control group considered as 100%.

2.3 Statistical analysis

All results were normalized to their respective controls, considered as 100%, and expressed as the average percentage of motility ± SEM of 3–12 experiments. Results were analyzed statistically by using the computer program PRISM (GraphPad, San Diego, CA, USA). To assume normal distribution, all data were subjected to arcsine square root transformation. Statistical analysis was performed using parametric tests. Comparison between columns was performed with analysis of variance and the Tukey post test. Values with $P < 0.05$ compared with control were considered statistically significant.

2.4 Immunoelectronmicroscopy

Gold-immunoelectronmicroscopy for EGF-R was performed on ejaculated spermatozoa (fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.05 mol/L phosphate-buffered saline [PBS], pH 7.4) using Lowicryl (K4M; Polysciences, Eppenheim, Germany) with goat anti-EGF-R polyclonal antibodies and a secondary gold-labelled (10 nm) antibody (1:20; Aurion, Wageningen, The Netherlands). Electron microscopy was performed with a Zeiss Electron microscope EM10 using a previously described method [3, 18]. For control purposes, the antiserum was omitted or replaced with normal rabbit serum.

2.5 Immunocytochemistry

Indirect immunofluorescence was performed on swim-up sperm. Briefly, 500 µL of swim-up sperm were fixed with 500µL of 4% paraformaldehyde for 5 min, washed twice with PBS (500 × *g*, 5 min) and resuspended to yield 1 000 000 cells/mL. Drops of this solution were placed on cover slips previously coated with 20 µg/mL of polylysine and air-dried. Specimens were re-hydrated and unspecific binding was blocked using 2% goat or donkey serum. Slides were incubated with the first antibody at 4ºC overnight (EGF-R 1:100 and PAR-2 1:250). Later, they were rinsed in saline solution (three times at room temperature) and re-incubated for 1 h with second antibody coupled with FITC or TRITC. Slides were rinsed in saline solution and mounted with glycerin for fluorescence microscope examination (Zeiss Axiomat, Jena, Germany).

Fixed and immobilized spermatozoa (described above) were incubated with fluoresceinated Pisum Sativum lectin, to check whether the fixation procedure and manipulation induced membrane rupture.

2.6 Protein extraction and Western blot

Swim-up sperm were washed twice with PBS and the pellet was resuspended in sample buffer to yield 2 000 000 total cells/10 µL. We performed immunoblots, as described by Frungieri *et al.* [19], using a mouse monoclonal antibody against human PAR-2 (1:250) and a polyclonal antibody anti-human EGF-R (1:100). For EGF-R blockage we incubated the polyclonal anti-human EGF-R antibody (2 µg/mL, dilution 1:100) together with blocking peptide (20 µg/mL). The secondary antibodies were coupled to peroxidase and developed with chemiluminescent substrate (PierceSierce, Rockford, IL, USA), as described previously [19].

3 Results

3.1 PAR-2 and EGF-R-like determinants are present on human spermatozoa

As a prerequisite for a possible crosstalk between PAR-2 and EGF-R, we investigated whether these two receptors are present on swim-up sperm. In Western blots, specific antibodies indicated the presence of both receptors. The PAR-2 protein had an expected molecular weight of 50 kDa [20]. The EGF-R protein displayed a size of 85 kDa, which was lower than the expected molecular weight of 170 kDa. To test the specificity of the antibody signal, we blocked the EGF-R antibody with a specific peptide. The absence of a band under these conditions suggests that the antibody recognizes a possibly truncated EGF-R protein on human sperm extracts (Figure 1).

The presence of PAR-2 and EGF-R was also indicated by immunocytochemistry on swim-up spermatozoa (Figure 2A). Immunocolocalization for both receptors showed the presence of PAR-2 and EGF-R (Figure 2B) in approximately 30% of the sperm cells.

Localization of PAR-2 in the plasma membrane has been shown by our group previously [3]. By using immunoelectronmicroscopy, we now also localize the EGF-R in the plasma membrane of ejaculated human spermatozoa (Figure 3).

3.2 EGF-R blockage inhibits the PAR-2 mediated effect on human sperm motility

Having established the presence of both receptors on human sperm, we investigated whether PAR-2 mediated effects on sperm motility could be altered by inhibiting EGF-R intracellular pathways. The stimulation of

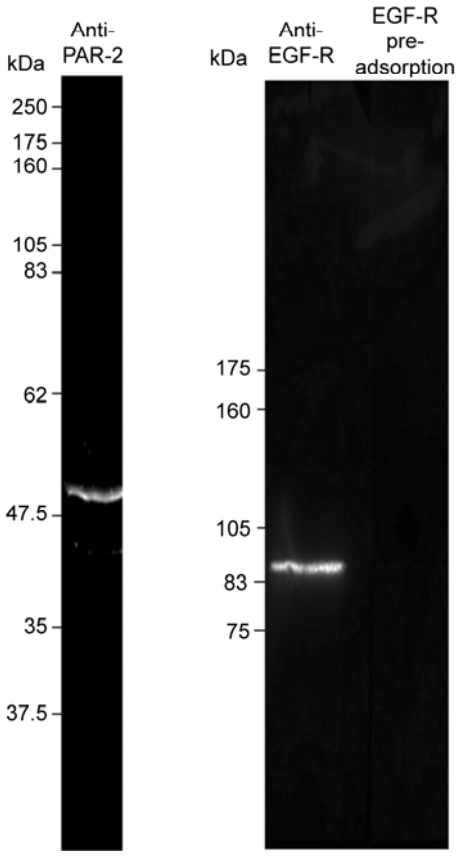

Figure 1. Detection of protease activated receptor 2 (PAR-2) and epidermal growth factor receptor (EGF-R) proteins by Western blot. Western blots were performed with proteins extracted from swim-up sperm, and showed immunoreactive bands corresponding to PAR-2 and EGF-R proteins. For EGF-R, the specificity of the signal was furthermore tested by blocking the antibody with the appropriate blocking peptide. Western blots were performed for three times and one representative experiment is shown.

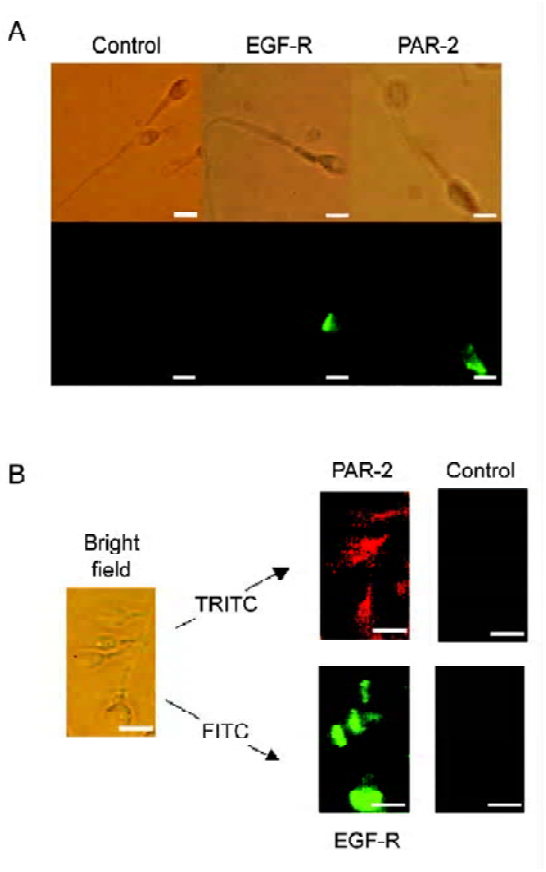

Figure 2. Protease activated receptor 2 (PAR-2) and epidermal growth factor receptor (EGF-R) immunocytochemistry. Immunocytochemistry confirmed the presence of PAR-2 and EGF-R in ejaculated spermatozoa (A) and also showed the presence of both receptors on some of the sperm (B). Controls were performed by omitting the respective first antibody. Bar = 5 μm. FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate.

spermatozoa with the PAR-2 agonist peptide SLIGKV resulted in a significant decrease of motility (range: 74%–86% compared to control; see Figure 4 for two sets of experiments) after 60 min, as measured by CASA. This confirmed our previously published results on the effect of human recombinant tryptase on human sperm motility [3]. Compared to tryptase, which as serine protease might exert various and so far undetermined effects on human sperm, SLIGKV is a selective PAR-2 agonist. Therefore, our results show, for the first time, that the reduction of human sperm motility is indeed a result of activation of PAR-2.

Incubation of spermatozoa with SLIGKV in the presence of two different and specific EGF-R inhibitors (AG1478 and PD168393) reverted the SLIGKV effect on sperm motility to control values (motility [% of control]: SLIGKV = 86.50% ± 4.05%; SLIGKV + AG1478 = 107.29% ± 3.83% [Figure 4A]; SLIGKV = 74.15% ± 4.48%; SLIGKV + PD168393 = 92.02% ± 7.27% [Figure 4B]). Inhibitors alone had no effect on motility (data not shown).

4 Discussion

The present study extends previous work by showing that PAR-2 on human sperm can be activated by SLIGKV peptide [12]. In addition, we found that PAR-2 and EGF-

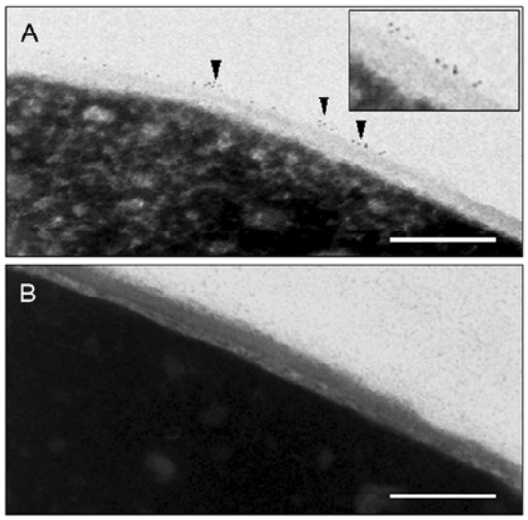

Figure 3. Subcellular localization of epidermal growth factor receptor (EGF-R). Gold immunoelectronmicroscopy displayed the localization of EGF-R in the plasma membrane of human ejaculated spermatozoa (A). Negative controls were performed by omitting the primary antibody (B). Scale bar = 300 nm.

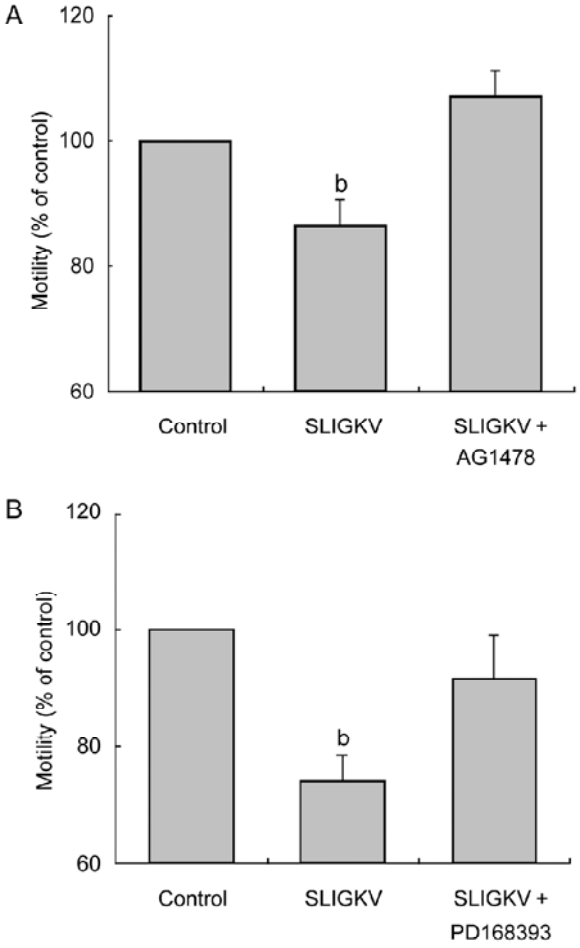

Figure 4. Determination of sperm motility. Spermatozoa were incubated with SLIGKV, SLIGKV plus epidermal growth factor receptor (EGF-R) inhibitors: SLIGKV + AG1478 or SLIGKV + PD169393 (A and B, respectively). Motility was measured by computer-assisted semen analysis (CASA) after 60 min. Results were normalized to non stimulated control values and expressed as the average percentage of motility ± SEM (n = 3–12). $^{b}P < 0.05$ compared with non stimulated controls

R are both present on human sperm and report that EGF-R pathway blockers inhibit the inhibitory effect of PAR-2 action on human sperm motility.

Western blot studies revealed that PAR-2 in human sperm had the expected size of 50 kDa, whereas EGF-R showed a signal at 85 kDa. The most common form of the EGF-R is a 170-kDa transmembrane glycoprotein [21], but a variety of EGF-R forms with wide range of molecular weights have also been described. This might be a result of differences in the glycosylation pattern of the receptor in different cell types or possible truncation of the protein.

EGF-R has previously been described in human sperm by other authors [14, 21–23]. Our dual-labeling studies using specific antibodies showed that at least 30% of the cells coexpressed EGF-R and PAR-2; in addition, immunoelectronmicroscopy indicates that PAR-2 [3] and EGF-R (the present study) are located in the human sperm membrane.

The functions of EGF in the male reproductive tract are still not very well established. Because EGF is present in seminal fluid [13, 24] it could alter sperm function. This is suggested by Naz and Kaplan [14], who showed that incubation of sperm with various amounts of EGF for 7–8 h enhances sperm motility. Moreover, animal *in vivo* experiments showed that the administration of EGF can improve epididymal sperm content and motility [25]. Our studies with recombinant EGF at a concentration of 60 nmol/L did not reveal any alteration of sperm motility (data not shown), which might be explained by the fact that in contrast to the experimental settings used by Naz and Kaplan [14], we incubated the sperm for a shorter period.

In addition to our previous studies, where tryptase reduced sperm motility, our results obtained with the

specific PAR-2 agonist peptide SLIGKV confirm the specificity of PAR-2 activation. This is of importance because tryptase, besides activating PAR-2, might also exert other currently unknown proteolytic effects, whereas SLIGKV only acts on PAR-2 and specifically inhibited human sperm motility. This is the first report showing clearly that the tryptase effect on human sperm motility is a result of PAR-2 activation and not to any other proteolytic products that might be generated by tryptase.

To investigate whether EGF-R associated signal transduction events might contribute to sperm motility we used SLIGKV to activate PAR-2, and two different specific EGF-R tyrosine kinase inhibitors (AG1478 and PD168393) to block EGF-R mediated signal transduction.

Importantly, the blockage of EGF-R upon PAR-2 stimulation by SLIGKV reverted the impairment of sperm motility induced by SLIGKV alone. This clearly implies the contribution of both receptors to this effect and also suggests an interaction of their signaling pathways. Therefore, we conclude that the novel mechanism of receptor crosstalk, first described for other cell types, also occurs in human sperm [15].

The mechanisms by which the two receptors can share signaling pathways are still far from being understood. In particular, the fact that human spermatozoa can display a high compartmentalization leads to the question of how this barrier is bypassed by signal transduction events.

The localization of PAR-2 and EGF-R in ejaculated sperm might hint to other events as well. Whether, for example, it might also participate in the acrosomal reaction, needs to be evaluated in future studies.

In summary, our data demonstrate that PAR-2 activation in human spermatozoa leads to signaling events that result in a reduction of sperm motility by utilizing EGF-R-associated signal transduction pathways.

Aknowledgment

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