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= Original Article =

Tyrosine phosphorylation of the flagellar aldose reductase is involved in the boar sperm capacitation

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ABSTRACT

In mammals, only the sperm that have undergone stepwise activation, including maturation in the epididymis and capacitation in the tubal isthmus, are able to initiate both hyperactivated motility and acrosome reaction to fertilize an ovum. Although the mechanisms in capacitation remain unclear, the tyrosine phosphorylation of functional proteins and the production of reactive oxygen species (ROS), are thought to be particularly important. Comprehensive analyses of the proteins that become tyrosine-phosphorylated during the capacitation process have identified a number of proteins, including aldose reductase (AR). Subsequent functional analysis showed that AR in the particulate fraction of the porcine sperm plays a central role in capacitation. In the present study, after fractionating sperm into cytosolic, membrane, flagellar, and head fractions, we scrutinized the localization of AR to be tyrosine-phosphorylated during capacitation and whether its activity was increased. It was found that the AR localized to the flagellum was tyrosine-phosphorylated, followed by the stimulation of its activity. Furthermore, NADPH levels in the sperm was found to be decreased during capacitation, while ROS levels increased. AR is the rate-limiting enzyme of the polyol pathway and uses NADPH as a coenzyme to convert glucose into sorbitol. These results suggest that the activation of flagellar AR by tyrosine phosphorylation reduces the amount of NADPH in sperm. This impairs the action of the glutathione cycle, which is important for its ROS elimination mechanism, leading to an increase in ROS levels. The present study suggests that sperm have a physiologically relevant ROS production system that is regulated by flagellar AR activities.

Key words: polyol pathway, NADPH, reactive oxygen species, sperm maturation

INTRODUCTION

When mammalian sperm are produced in the testes, they are immature and lack both forward motility and fertilizing capacity. While sperm are transported through ductus epididymidis, factors derived from its epithelium are provided to the immature sperm, either directly or via vesicles called epididymosomes [24], which confer the capabilities of flagellar motility and fertilizing ability on sperm. However, two more activation processes must be completed for the sperm to exert forward motility and fertility. The first activation process occurs at ejaculation to induce the forward motility. When the sperm leave the

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low bicarbonate concentration (2 mM) of the secretions in the cauda epididymis and are exposed to high bicarbonate concentration (20 mM) of the seminal vesicle secretions, a bicarbonate sensitive adenylate cyclase is activated. This promotes a cAMP-dependent phosphorylation cascade in the sperm, which is thought to act as a trigger for the initiation of forward movement [19,20,25]. The second activation is capacitation, which occurs in the isthmus of the uterine tube and sperm motility is further stimulated, so called hyperactivation [1,31]. Only sperm that have undergone the process of capacitation can perform the acrosome reaction at the ovum and may potentially fertilize it. The induction of capacitation has been shown to involve complicated reactions, including the detachment of cholesterol from the sperm cell membrane, influx of intracellular HCO_3^- and Ca^{2+} [2,29], an increase in pH inside the sperm [33], an increase in cAMP levels [6,21], reactive oxygen species (ROS) production [9,22,23], and phosphorylation of protein tyrosine residues [5,12,14,28]. A large number of studies have implicated both the ROS production and the tyrosine phosphorylation of functional proteins, in particular, as key factors in capacitation [11,12,13,16,28,32].

We have identified several kinds of proteins that are specifically tyrosine-phosphorylated during capacitation, including AR, and a functional analysis of these proteins has been performed. Although the protein kinase C (PKC)-dependent phosphorylation of AR has been reported [27], we were the first to discover its tyrosine phosphorylation during capacitation in porcine sperm. In addition, it was determined that AR is not a protein expressed by the sperm themselves but taken up into sperm from Sertoli cells during sperm formation and from the epididymal epithelial cells during the maturation process. Moreover, it was found that AR in the particulate fraction are activated by the tyrosine phosphorylation that accompanies capacitation and regulates both ROS levels and capacitation [8]. However, the location of the AR performing these reactions within the sperm remains unclear.

The present study was performed with the aim of determining the localization of AR which was tyrosine-phosphorylated during capacitation in porcine sperm. The mechanisms how tyrosine phosphorylation of AR regulates ROS levels and capacitation were also discussed.

MATERIALS AND METHODS

Animals

All animal experiments performed in the present study were approved by the Animal Experiment Committee of the University of Tsukuba and the Animal Research Committee of the Ibaraki Prefectural University of Health Sciences. Fresh porcine testes and epididymes were purchased from the local slaughter house.

Preparation of cauda epididymal sperm and induction of capacitation

Spermatozoa were collected from the porcine cauda epididymis by perfusing through the ductus epididymidis with air and washed at 20 ºC with Non-cap medium (4.8 mM KCl, 1.2 mM KH_2PO_4, 95 mM NaCl, 2 mM sodium pyruvate, 5.56 mM glucose, pH 7.4) by centrifugation at 400 × *g* for 5 min. The sperm pellets were washed twice with Non-cap medium by the centrifugation as above. When capacitation was induced, Cap medium (2 mM $CaCl_2$, 0.4% BSA, 25 mM $NaHCO_3$ in Non-cap medium) was used for washing the sperm pellets. Only the samples with over 95% of the sperm showing progressive motility were used for the experiments. Sperm capacitation was induced according to the method described in the previous paper [8]. The washed sperm were incubated either in 15 ml of Non-cap medium or in Cap medium (5×10^6 cells/ml) at 37 ºC for 3 h in a 5% CO_2 atmosphere. Capacitated sperm were assessed on the basis of the changes in the chlortetracycline fluorescence staining [34] and the percentage of sperm with hyperactivated motion in all of the motile sperm were calculated by using a computer-assisted sperm analysis (CASA) system (HTM-CEROS; Hamilton Thorne Research, Beverly, MA, USA).

Isolation of sperm fractions and extraction of sperm protein

Fractionation of sperm was performed according to the methods of Okamura & Sugita. [18] with slight modifications. Sperm were separated from incubation buffer by layering 10 ml of semen on 20 ml of 1.3 M sucrose in 0.15 M NaCl and centrifuged at 12,100 × *g* for 70 min at 4 ºC. The sperm pellet was suspended in PBS and washed twice by centrifugation at 12,100 × *g* for 5 min. After washed sperm were resuspended in 10 ml of 5 mM HEPES, pH 7.0, and homogenized by sonication with ULTRA S homogenizer (TAITEC, Saitama, Japan) at the

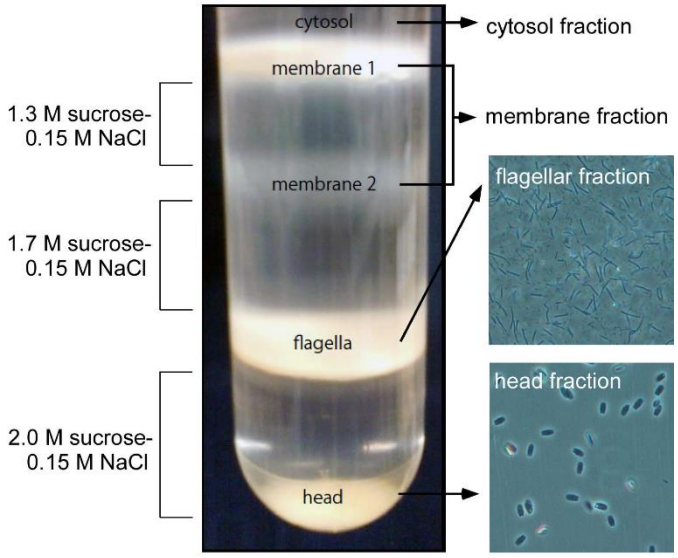

Figure 1. Sperm fractionation by sucrose density-gradient centrifugation. The preparation of cytosol, membrane, flagellar and head fraction from the porcine sperm were determined with described in 'Materials and Methods' section.

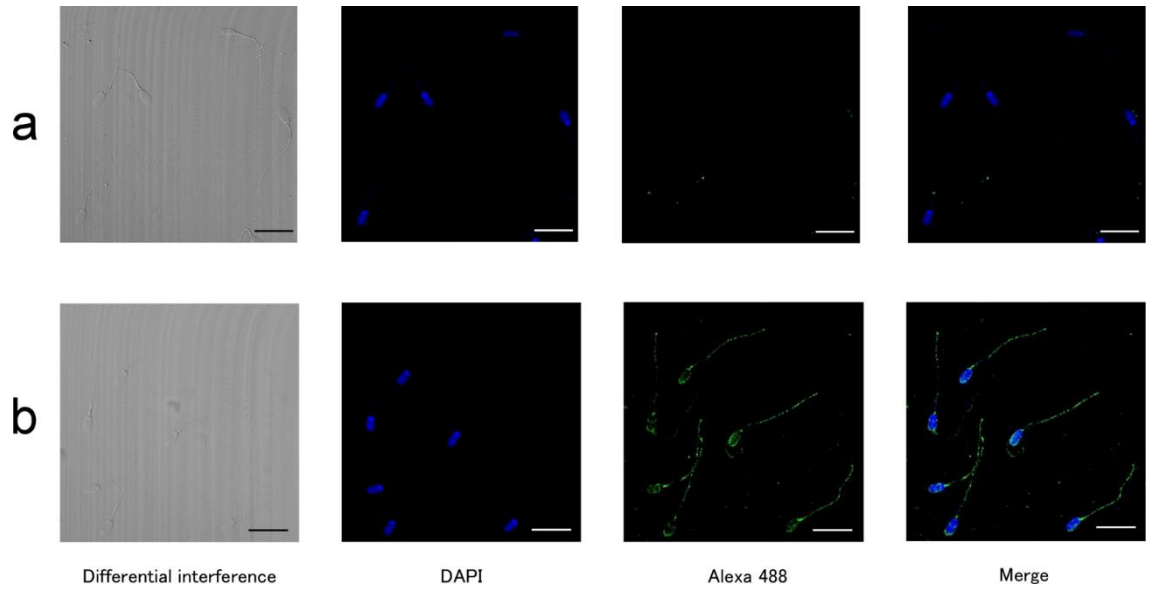

Figure 2. Immunofluorescent staining of AR in porcine cauda epididymal sperm. Localization of AR in the porcine cauda epididymal sperm was analyzed with anti-AR antisera (b). Anti-AR antisera neutralized with antigen were used as a negative control (a). The nuclei were counterstained with DAPI. Bars = 20 μm.

maximum output. Ten milliliter of sperm homogenate were layered on discontinuous gradients consisting of 8 ml of 1.3 M sucrose–0.15 M NaCl, 10 ml of 1.7 M sucrose–0.15 M NaCl, 10 ml of 2.0 M sucrose–0.15 M NaCl. The solutions were centrifuged at 100,000 × *g* for 3 h at 4 ºC in Beckman SW 28 rotor. The cytosol fractions were collected from the top layer of each gradient and membrane fractions (plasma membranes and outer acrosomal membranes) were collected from the sample, 1.3 M sucrose and the 1.3 M–1.7M sucrose interface. Flagellar fractions were collected from 1.7 M–2.0 M sucrose interface and head fractions were collected from the precipitates (Fig. 1). Subsequently, membrane, flagellar and head fractions were respectively washed with PBS by centrifugation, resuspended in the extraction buffer (1% Trition-X 100, 20 mM Tris-HCl, 2.5% protease inhibitor cocktail, 0.25% phosphatase inhibitor cocktail, pH 7.4), and homogenized by sonication with ULTRA S homogenizer at the maximum output. After centrifuged at 105,000 × *g* for 30 min at 4 ºC, each supernatant was collected and stored at –20 ºC until use.

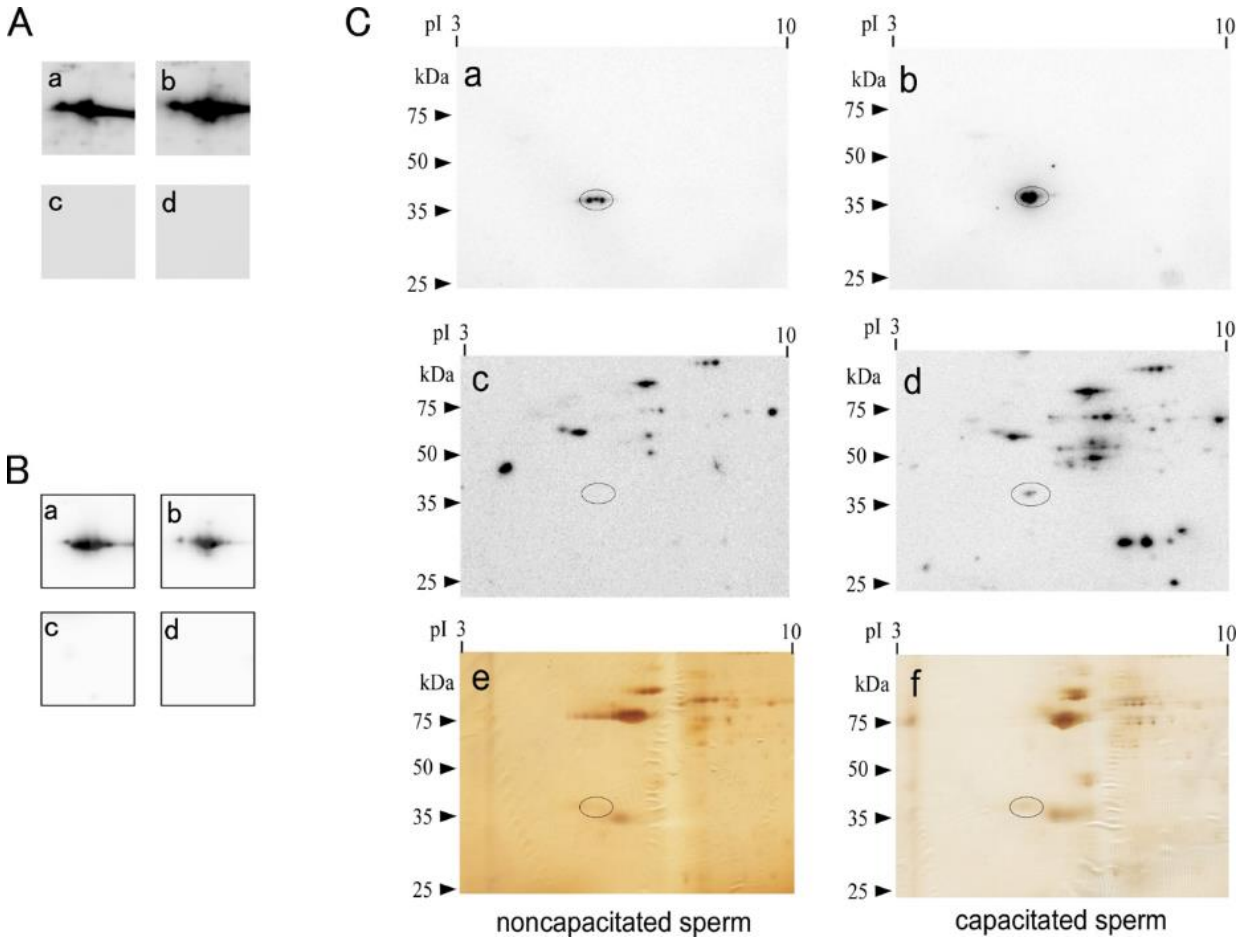

Figure 3. Tyrosine phosphorylation of AR in cytosol, membrane and flagellar fraction of sperm during capacitation. Protein extract of cytosol (A), membrane (B) and flagellar (C) fraction was separated by 2-DE. Western blotting analyses either on AR in the noncapacitated sperm (a) or capacitated sperm (b) or on tyrosine phosphorylated proteins in the noncapacitated sperm (c) or capacitated sperm (d) were done as described in Materials and Methods. Silver staining in the noncapacitated sperm (e) or capacitated sperm (f).

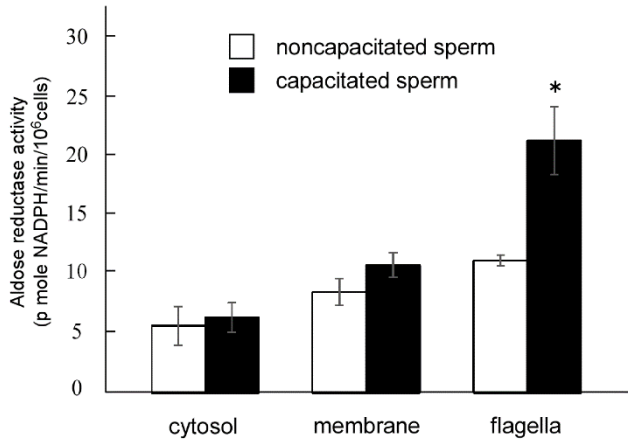

Figure 4. Changes in AR activities of the sperm fractions through capacitation. The AR activities of cytosol fraction, membrane fraction and flagellar fraction from the noncapacitated sperm (open bar) or capacitated sperm (closed bar) were determined as described in 'Materials and Methods' section. Data are expressed as mean ± S.E.M. from three determinations. *P < 0.05.

Immunocytochemical analysis of porcine sperm

The capacitated and noncapacitated sperm (5.0×10^7 cells) were washed twice with PBS by centrifugation at 400 × g for 5 min. Sperm were fixed on MAS coat slide glass with 4% paraformaldehyde for 60 min at room temperature. After washing with PBS, sperm were incubated with 3% H_2O_2 in PBS at room temperature for

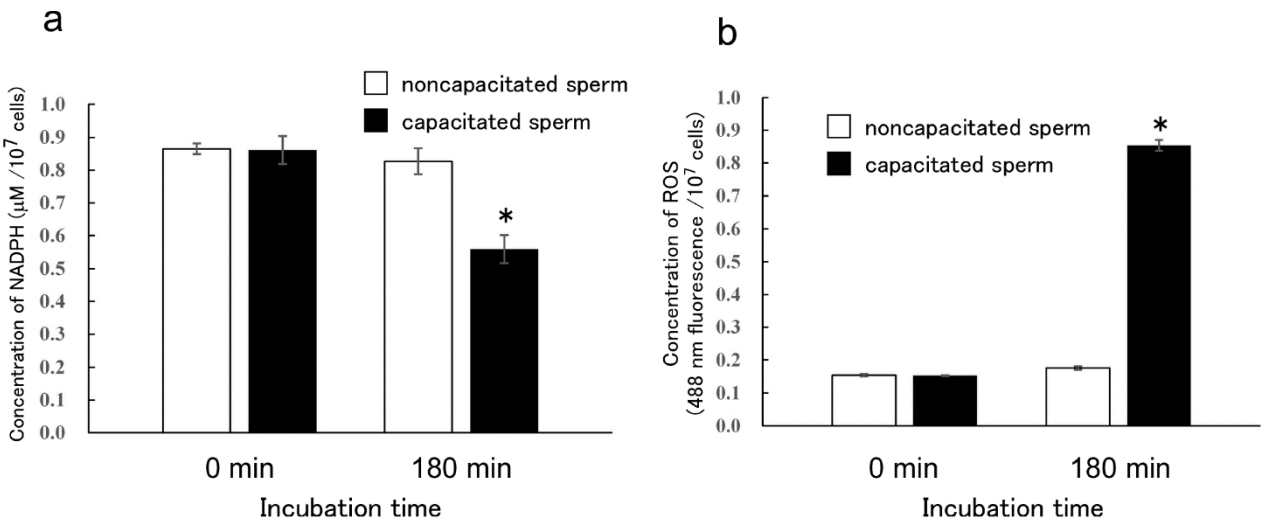

Figure 5. The time-dependent changes in NADPH and ROS levels of porcine sperm during capacitation. The concentration of NADPH (a) and ROS (b) either in noncapacitated sperm (open bar) or in capacitated sperm (closed bar) were determined as described in 'Materials and Methods' section. Data are expressed as mean ± S.E.M. from three determinations. *P < 0.05.

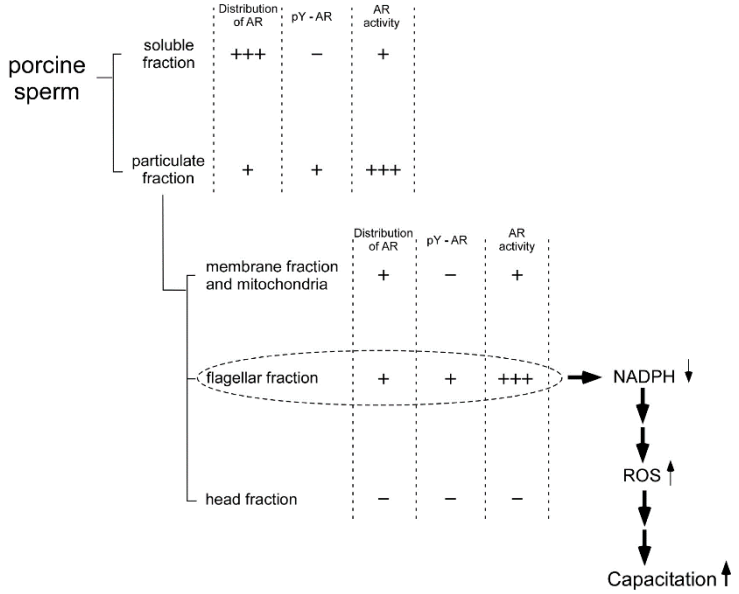

Figure 6. Tyrosine phosphorylation and enzyme activity of the flagellar aldose reductase is involved in the boar sperm capacitation. pY : tyrosine phosphorylation

30 min. The samples were washed with PBS and then blocked with PBS containing 3% BSA for 60 min at 37 ºC. The samples were incubated with anti-AR antisera in PBS at 4 ºC overnight. After washing three times with PBS, the samples were incubated with Goat Anti-Rabbit IgG H&L (Alexa Fluor 488, Abcam plc, Cambridge, UK) for 60 min at 37 ºC. After washing with PBS, nucleus were counterstained with DAPI (NucBlue Fixed Cell Stain ReadyProbesTM reagent, Life technologies). After washing with PBS, the sections were covered with cover glass and observed with a confocal laser scanning microscope (LSM-780, ZEISS, Oberchoken, Germany).

Determination of AR Activity

AR activity was determined according to the method of Katoh et al. [8]. Two hundred microliters of each samples were incubated with 0.15 mM NADPH and 10 mM DL-glyceraldehyde in 120 mM phosphate buffer (pH 6.2) at 20 ºC for 10 min, and the absorbance at 340 nm was monitored.

Determination of ROS and NADPH concentrations

The concentrations of ROS in the sperm were determined according to the method of Katoh et al. [8]. H_2DCFDA (200 μM) was added to the incubation mixture

for the induction of capacitation (2.5×10^8 cells in 5 ml of Cap medium) at 150 min. The mixture was further incubated for 30 min and sperm were collected by centrifugation at 400 × *g* for 5 min. The basal levels of ROS in sperm were determined with Non-Cap medium instead of Cap medium. The sperm pellet was suspended in PBS and washed twice by centrifugation at 400 × *g* for 5 min. The fluorescence of sperm at 530 nm in response to 488 nm excitation was observed under the fluorescence microscope and evaluated using a microplate reader (Varioskan; Thermo Fisher, Yokohama, Japan). The concentration of NADPH in sperm were determined by use of EnzyChrom™ NADP/NADPH Assay Kit. Sperm (1.25×10^8 cells) were collected from the incubation mixture for induction of capacitation at 0, 180 min and washed twice with PBS by centrifugation at 400 × *g* for 5 min. The sperm pellets were incubated with 100 µl of NADPH Extraction Buffer at 60 ºC for 10 min and then 20 µl of Assay Buffer and 100 µl of NADP Extraction Buffer were added. The mixture was briefly vortexed and centrifuged at 12,100 × *g* for 15 min at 4 ºC. Forty microliters of supernatants (2.3×10^7 cells) or 10 µM NADP Standard were incubated with 80 µl aliquots of Working reagent (60 µl of Assay Buffer, 10 µl of 1 M Glucose, 14 µl of formazan [MMT] solution, 1 µl of Enzyme Mix) at room temperature for 30 min and the absorbance at 565 nm was determined.

Statistical Analysis

Data were expressed as the means ± SEMs of at least 3 experiments. Statistical significance was calculated by two-way ANOVA. The difference between two means was determined by t-test, and differences among more than two means were determined by Tukey's test. The general linear models of Statistical Analysis System (SAS Inc., Cary, NC, USA) were used for these analyses. Differences were considered significant at $P < 0.05$.

RESULTS

It is known that AR is principally a soluble and cytosolic protein. In sperm, it has been reported that soluble AR mainly exists in the acrosomes. We have previously discovered that soluble AR is less active than that of the particulate fractions which is activated tyrosine phosphorylation during capacitation. On the other hand, the soluble AR is not phosphorylated at tyrosine residue [8].

Thus, in the present study, the particulate fraction was further fractionated into membrane, flagellar, and head fractions, and both the distribution of AR among those fractions and the levels of the tyrosine phosphorylation were determined by the immunohistochemical analyses, the two-dimensional gel electrophoresis and western blotting. However most AR was found in the cytosol, small amount was also found to localize in the flagellum (Fig. 2). Upon fractionating sperm by sucrose gradient centrifugation AR was found to be present at levels of 70% in the cytosol fraction, 20% in the membrane fraction, and 10% in the flagellar fraction, but was not present in the head fraction (data not shown). In contrast to the AR in the cytosol and membrane fractions, which was not tyrosine-phosphorylated (Fig. 3A, B), AR in the flagella was tyrosine-phosphorylated during capacitation (Fig. 3C). Next, enzyme activity was measured for the cytosol, membrane, and flagellar fractions where AR was localized. In the flagellar fraction, the activity of capacitated sperm was markedly higher than that of noncapacitated sperm. The activity of AR in the cytosol and membrane fractions was the same before and after capacitation and was lower than that in both the noncapacitated and capacitated sperm flagellar fractions (Fig. 4). With the results shown in Fig. 3, these findings strongly imply that increases in AR activity of the particulate fraction during capacitation are due to the tyrosine phosphorylation and activation of AR in the flagella.

AR is the rate-limiting enzyme of the polyol pathway, which produces fructose, a primary energy source for sperm, from glucose, using NADPH as a coenzyme to convert glucose into sorbitol. Consequently, an increase in AR activity by tyrosine phosphorylation is expected to lead to an increased consumption of NADPH to form $NADP^+$. Accordingly, we measured the NADPH levels in Non-cap and Cap sperm and determined that NADPH levels did not change in Non-cap sperm. However, in Cap sperm, NADPH levels were reduced by approximately 35% during capacitation (Fig. 5a). Moreover, although no changes in ROS levels were found in Non-cap sperm, ROS levels increased approximately 5.5-fold in Cap sperm during capacitation (Fig. 5b).

DISCUSSION

It is assumed that proteins expressed and taken up by sperm in specific regions of the epididymis are important in the sperm maturation process. Some of the proteins

produced by the epithelial cells of the ductus epididymidis are released into the lumen of the tubes in vesicles called epididymosomes, and sperm are known to take up these vesicles. The inclusion of AR in epididymosomes has been reported by Sullivan et al. [24] and, combined with our previous research [8], it strongly suggests that AR is incorporated into sperm from the epididymosomes and plays important role in the functional maturation of sperm in the epididymis.

In the present study, it was shown for the first time that the activity of AR in the flagellum is increased by tyrosine phosphorylation, which also leads to a reduction in NADPH levels in the sperm and an increase in ROS levels. From these results, we surmise that AR regulates capacitation via the mechanism shown in Fig. 6. First, AR is tyrosine-phosphorylated, and then the enhancement of its activities promote the utilization of glucose via the polyol pathway. This leads to a reduction in NADPH levels in sperm, impairing the action of the glutathione cycle, which is important for its ROS elimination mechanism, and thus leading to an increase in ROS levels. On the other hand, the promotion of the polyol pathway results in fructose accumulation that causes increased production of advanced glycation end products and also leads to ROS production. It has been found that GSH levels throughout the sperm are reduced by approximately 10% owing to capacitation [8]. Because tyrosine-phosphorylated AR is detected only in the flagellum, it seems likely that GSH levels would be even further reduced when only the flagellum is observed. Given a previous suggestion [7] that glutathione (GSH) levels are reduced to around 30% by freezing and thawing in a capacitation-like reaction called cryocapacitation, the increase in ROS levels found in the present study seems highly likely to derive from a reduction in GSH levels in the sperm. As long as ROS is regulated to an appropriate concentration, it may be an important regulatory factor of various sperm functions including capacitation [16,17]. We have reported that a membrane-permeable AR specific inhibitor, Alrestatin, inhibits the increase in the protein tyrosine phosphorylation, the decrease in the GSH levels, the elevation of ROS levels and the induction of both hyperactivated motility and induction of pattern B of chlortetracycline (CTC) fluorescence staining, all of which occur during capacitation [8]. So, AR is strongly suggested to act as the central role of the regulation of capacitation.

There are proteins, some of them tyrosine-phosphorylated proteins, whose localization in the sperm changes upon capacitation [3,30]. AR is known to be dominantly present in the soluble fraction in sperm. However, its specific activity there is lower than that of AR in the insoluble fractions, such as the flagellar fraction. It was found that this was due to that the flagellar AR was tyrosine-phosphorylated whereas soluble one was not. In the present study, no clear changes in the localization of AR were found to result during capacitation. Whether AR localized to the juxtamembrane region is tyrosine-phosphorylated during capacitation or changes in location from the cytoplasm to the membrane occur as a result of tyrosine phosphorylation will be the subject of future research. In this connection, it is interesting that AR is transferred to the mitochondria due to phosphorylation via protein kinase C stimulation [27].

The tyrosine kinase that phosphorylates AR remains to be identified. The involvement of the SRC family in capacitation in human and rats [4,10,15,16] as well as the involvement of a 34–45 kDa tyrosine kinase in capacitation of boar sperm has been reported. These tyrosine kinases are present in the acrosome, equatorial segment, midpiece, and main flagellar component of the sperm, suggesting that they are intimately involved in the acrosome reaction and hyperactivation [26]. Whether they participate in the phosphorylation of AR is a question of profound interest.

The present analysis of AR, a protein that has been identified to be tyrosine-phosphorylated during capacitation, suggests that sperm have a physiologically relevant ROS-production system that is regulated by intracellular NADPH levels. To date, the production of ROS in the sperm has been assumed to originate mostly in the mitochondria, but the results of the present study can be interpreted as revealing the existence of a new ROS-production system. Although it is clear that sperm functions, including capacitation, are regulated by ROS, the species of ROS involved and their roles await identification. A clear picture of the ROS-mediated system regulating sperm function could lead to the effective control of sperm fertility for infertility treatment, contraception, breeding, and preservation of genetic resources.

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