Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Decrease in the cytosolic NADP$^+$-dependent isocitrate dehydrogenase activity through porcine sperm capacitation

Yuki Katoh [a, b, *], Michiko Tamba [b], Manabu Matsuda [c], Kazuhiro Kikuchi [d], Naomichi Okamura [b]

[a] Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan
[b] Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, 305-8575, Japan
[c] Division of Basic Medical Sciences, Kindai University Faculty of Medicine, Osaka, Sayama, 589-8511, Japan
[d] Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, 305-8602, Japan

ARTICLE INFO

*Article history:*
Received 7 February 2018
Accepted 8 February 2018
Available online xxx

*Keywords:*
Sperm
Capacitation
Tyrosine phosphorylation
IDPc

ABSTRACT

In order to understand the molecular mechanisms involved in the sperm capacitation, we have identified the proteins tyrosine-phosphorylated during the capacitation especially in conjunction with the regulation of the levels of reactive oxygen species (ROS) in sperm. In the present study, the effects of the tyrosine phosphorylation of cytosolic NADP$^+$-dependent isocitrate dehydrogenase (IDPc) on its catalytic activity and on the levels of ROS in sperm have been studied. The tyrosine phosphorylated IDPc showed a significantly lowered enzymatic activity. The immunocytochemical analyses using the highly specific antisera against IDPc revealed that IDPc was mainly localized to the principal piece of the porcine sperm flagellum. As IDPc is one of the major NADPH regenerating enzymes in porcine sperm, it is strongly suggested that the decrease in IDPc activity is involved in the increased levels of ROS, which results in the induction of hyperactivated flagellar movement and capacitation.



## 1. Introduction

It has been strongly suggested that the increases in protein phosphorylation at tyrosine residue(s) and in ROS levels are the key phenomena during the process of sperm capacitation [1–7]. Extensive studies have been carried out to identify the proteins that are tyrosine phosphorylated at capacitation and to clarify the effects of the various reagents that regulate intracellular levels of ROS on capacitation [6,8,9].

We have recently reported that aldose reductase is phosphorylated at tyrosine residue(s) during porcine sperm capacitation *in vitro* [10]. The activity of aldose reductase in the flagellar particulate fraction was highly enhanced by tyrosine phosphorylation, resulting in decreased glutathione (GSH) contents and an increase in ROS. Furthermore, alrestatin, a membrane-permeable and specific inhibitor of aldose reductase, inhibited capacitation as well as hyperactivation. These results suggest that tyrosine phosphorylated aldose reductase controls porcine sperm capacitation through the enhanced consumption of NADPH and ROS generation by the increased activity.

In contrast, sufficient NADPH must be provided to glutathione reductase to maintain intracellular levels of GSH, which is an effective antioxidant for decreasing the levels of ROS and controlling the cellular redox state. The pentose phosphate pathway is a major source of NADPH, and the appropriate regulation of its activity is suggested to be important for the fertilizing capacity of human and mouse sperm [11–15]. Although the generation of NADPH in the sperm head and midpiece via the pentose phosphate pathway has been proven, the occurrence of the pentose phosphate pathway in the principal piece of flagellum has not been demonstrated [16,17]. Furthermore, it has been shown that this pathway is

*Abbreviations:* NADPH, nicotinamide adenine dinucleotide phosphate; GST, glutathione S-transferase; $H_2$DCFDA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, diacetoxymethyl ester; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; MOPS, 3-(N-morpholino) propanesulfonic acid; DEPC, diethylpyrocarbonate; DW, distilled water; DAPI, 4′,6-diamidino-2-phenylindole.

* Corresponding author. Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan.
*E-mail address:* yukikatoh@keio.jp (Y. Katoh).

https://doi.org/10.1016/j.bbrc.2018.02.087
0006-291X/

not operating in the sperm of other species such as bull and boar [18,19]. These results indicate that alternate pathways such as malic enzyme and IDPc are also responsible for producing NADPH in sperm.

In addition to aldose reductase, we found that IDPc was also phosphorylated at tyrosine residue(s) during porcine sperm capacitation [10]. Although mitochondrial $NAD^+$-dependent isocitrate dehydrogenase plays a major role in the tricarboxylic acid cycle, the physiological roles of mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm) and IDPc have been poorly elucidated. In the present study, we examined the expression and localization of IDPc in the porcine testis and epididymal matured sperm, looking at changes in its enzymatic activities during sperm capacitation in relation to tyrosine phosphorylation, and its roles in the regulation of NADPH and ROS levels in sperm.

## 2. Materials and methods

### 2.1. Preparation of cauda epididymal sperm and induction of capacitation

Spermatozoa were collected from the porcine cauda epididymis. Sperm preparation and induction of capacitation were carried out according to the method of Katoh et al. [10]. Sperm capacitation was assessed by the CTC-fluorescence staining pattern of the sperm head [21] and by the flagellar movement analyses both on the flagellar beat angle and the flagellar curvature ratio [22] as described previously [10]. Over 70% of the sperm represented the characteristic patterns for capacitation at 180 min of the incubation under the capacitation-inducing conditions of the present experiments.

### 2.2. Production of anti-IDPc antisera

The *Idpc* cDNA fragment (nucleotides 317–1288 of *Idpc* cDNA, accession no. XM_005672137. 2 [updated to XM_005672137.3, May 13, 2017]; Fig. S1) synthesized by RT-PCR using porcine testis DNase I-treated total RNA as a template was ligated into the multicloning site (EcoR I - Sal I) of pGEX-6P-2 and transformed into *E. coli* DH5α. Oligonucleotides, 5′- CACCATCCGAATTCTCTTGGGTGGCAC -3′ and 5′- AGGCCAATTCGTCGACAAAGCCATCTG -3′, were used as primers. The recombinant IDPc-GST fusion protein was bound to Glutathione-Sepharose 4B beads and IDPc was cut out from GST using PreScission Protease, according to the manufacturer's instructions. The beads were centrifuged at 1200×*g* for 10 min. The supernatant was then purified by PD-10 Columns and DEAE-Sepharose. The purified recombinant IDPc (2 μg) was separated on SDS-PAGE gel and detected as a single band by Coomassie Brilliant Blue staining, which was used as the antigen. The amino acid sequences of antigen has low homology with those of other types of isocitrate dehydrogenase so as to produce antisera specifically reacting only with IDPc. Rabbit antisera against IDPc were prepared by subcutaneous injection of 200 μg of antigen with Freund's complete adjuvant, followed by five additional booster injections of 300 μg of antigen in Freund's incomplete adjuvant given at 1-week intervals.

### 2.3. Western blot analysis of tyrosine phosphorylated proteins and IDPc

Testes and sperm proteins were extracted with homogenizing buffer (2.5% Protease Inhibitor Cocktail, 0.25% Phosphatase Inhibitor Cocktail, 20 mM Tris-HCl, pH 7.4) and separated by SDS-PAGE following to a previously published method [10]. The proteins were electroblotted to PVDF membranes under semidry conditions. After blocked in TBST (100 mM NaCl, 0.1% Tween 20, 10 mM Tris-HCl, pH 7.4) containing 3% BSA at 4 °C overnight, the membranes were treated with primary antibodies at 37 °C for 60 min: anti-phosphotyrosine (pY), or anti-IDPc. After three washed with TBST, the membranes were incubated with corresponding secondary antibodies at 37 °C for 60 min. Signals were detected with Chemi-Lumi One L.

### 2.4. Immunoprecipitation

Five hundred microliters of total sperm protein extracts (1 mg protein) were incubated with 10 μl of Protein A resin equilibrated with 20 mM Tris-HCl (pH 7.4) at 4 °C for 90 min and then centrifuged at 2500 × *g* for 10 min at 4 °C. Following this, 450 μl of the supernatant was incubated with 50 μl of preimmune sera or anti-IDPc antisera at 4 °C for 120 min. Twenty microliters of Protein A resin was added to the mixture, and this was then incubated at 4 °C for 90 min. Protein A resin was precipitated by centrifugation at 2500 × *g* for 10 min at 4 °C and washed three times with 0.1% Triton-X 100 in 20 mM Tris-HCl (pH 7.4) by centrifugation. The precipitated immune complexes were then denatured and resolved by SDS-PAGE for further Western blot analysis.

### 2.5. Determination of IDPc activity

IDPc activity was determined according to the methods of Alp et al. [23] and Córdoba et al. [24] with slight modifications. The washed sperm ($1.0 \times 10^9$ cells) were suspended in 5 ml of DW and then frozen overnight at −20 °C. The sperm suspensions were thawed at room temperature and were homogenized by sonication with ULTRA S homogenizer (TAITEC, Saitama, Japan) at the maximum output and centrifuged at 105,000 × *g* for 30 min at 4 °C. The supernatant was used to determine IDPc activity. One hundred microliters of supernatants were incubated with 70 mM Tris-HCl, 8 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM citric acid, 1.2 mM DL-isocitric acid, 0.5 mM NADP, pH 7.5 at 37 °C for 5 min, and the absorbance at 340 nm was monitored.

### 2.6. Determination of ROS and NADPH concentrations

The concentrations of ROS in the sperm were determined following the method of Katoh et al. [10]. $H_2$DCFDA (200 μM) was added to the incubation mixture for the induction of capacitation ($2.5 \times 10^8$ cells in 5 ml of Cap medium) at 0, 60 and 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 fluorescence of sperm at 530 nm in response to 488 nm excitation [26] was evaluated using a microplate reader (Varioskan; Thermo Fisher Scientific, Yokohama, Japan).

The concentration of NADPH in sperm ($1.25 \times 10^8$ cells), which were incubated for 0, 30, 90, 180 min, was determined by use of an EnzyChrom™ NADP/NADPH Assay Kit according to the manufacturer's instrument.

### 2.7. RT-PCR analysis and synthesis of DIG-labeled cRNA probe

Total RNA was extracted from porcine testis and caput, corpus, and cauda epididymes with Isogen II. RNA concentration was estimated by optical density and electrophoresis. First-strand cDNA reverse transcribed using an oligo(dT) primer was further amplified by PCR. Oligonucleotide primers used for PCR were 5′-TGGAACTGGATCTGCACAGCTACG -3′ and 5′- CTCCTGTCCTTTCTG GGACATTTG -3'.

In the preparation of the DIG-labeled cRNA probe, the RT-PCR

Please cite this article in press as: Y. Katoh, et al., Decrease in the cytosolic $NADP^+$-dependent isocitrate dehydrogenase activity through porcine sperm capacitation, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.02.087