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Ubiquitin-activating enzyme E1 inhibitor PYR-41 retards sperm enlargement after fusion to the egg

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ABSTRACT

The ubiquitin-proteasome system, which is initiated by a single ubiquitin-activating enzyme E1 (UBE1), is involved in male reproduction via spermatogenesis and function in mammals. Here we explored the influence of UBE1-specific inhibitor, 4[4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester (pyrazone-41 or PYR-41) in female reproduction. UBE-1 was detected by immunoblotting and immunocytochemistry in mouse eggs and was localized mainly under the egg plasma membrane. PYR-41 pretreatment suppresses the development of eggs into two-cell embryos. Specifically, pretreatment retarded sperm enlargement and meiotic chromosomal division after sperm-egg fusion. PYR-41 pretreatment disturbed β-catenin, a well-known target protein for ubiquitination, localization under the egg plasma membrane and on spindle microtubules in wild-type eggs. Otherwise, PYR-41 treatment had no effect on the two-cell development of eggs lacking β-catenin. Our results raise the possibility that inhibition of the ubiquitin-proteasome system suppresses sperm enlargement through impaired β-catenin-mediated mechanism.

1. Introduction

The ubiquitin-proteasome system is a eukaryotic mechanism of intracellular protein degradation, which regulates basic cellular processes such as cell cycle, division, differentiation, and death [1–3]. Therefore, aberrations of this system are closely related to the pathogenesis of human diseases. Ubiquitination is catalysed by the sequential action of a single ubiquitin-activating enzyme E1 (UBE1) and multiple ubiquitin-conjugating E2 (UBE2) and ubiquitin-protein ligase E3 (UBE3) enzymes, and ubiquitinated proteins are finally degraded by proteasomes [3]. Since UBE1 is the common gatekeeping enzyme for ubiquitination, inhibition of UBE1 enzymatic activity blocks the ubiquitin-proteasome system. Target proteins are degraded by the 26S proteasome after ligation with lysine 48 (K48)-linked ubiquitin chains [3]. Otherwise, K63-linked ubiquitin chains contribute to the cytokine-induced activation of nuclear factor-κB (NF-κB) [4].

The 4[4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester (hereafter PYR-41) and related pyrazones are cell-permeable UBE1 inhibitors identified in high-throughput screening of the UBE1–UBE2–UBE3 cascade, which selectively and irreversibly inhibits UBE1 activity [3]. PYR-41 and other related pyrazones enter cells and decrease UBE1-ubiquitin thiolester formation via covalent modification, resulting in irreversible inhibition of the enzymatic activity of UBE1 [5,6]. Since PYR-41 exposure inhibits NF-κB activation and increases the expression level and activity of p53, an anti-tumour protein, it is considered a potent drug for selectively killing transformed cells [5].

Dysfunction of the ubiquitin-proteasome system causes abnormal organelle morphology and impairs sperm formation and maturation steps resulting in male infertility [7–9]; this indicates that ubiquitination is essential for spermatogenesis and sperm

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function during fertilization. β-catenin is a well-known target protein for ubiquitination, and its cellular level is tightly controlled by ubiquitination-mediated degradation [10]. In fertilization, β-catenin degradation is triggered by membrane adhesion between sperm and egg, and treatment of wild-type eggs, but not β-catenin-deficient eggs, with PYR-41 affects sperm-egg fusion [11]. To further investigate the effect of PYR-41 on egg function, we here examined wild-type and β-catenin-deficient eggs pretreated or treated continuously with PYR-41.

2. Materials and methods

2.1. Antibodies and chemicals

For immunostaining and immunoblotting, a rabbit anti-UBE1 monoclonal antibody [clone No. EPR14204(B)] was purchased from Abcam plc (Cambridge, UK). For immunostaining, a rabbit anti-K48-linked polyubiquitin chain mAb (clone No. Apu2) was purchased from Merck Millipore (Billerica, MA). For immunostaining, a mouse anti-β-catenin mAb (clone No. 15B8) conjugated with phycoerythrin was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO). A secondary antibody for immunostaining was Alexa Fluor 488-conjugated IgG purchased from Molecular Probes (Eugene, OR). A horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich Co. LLC.) was used for immunoblotting. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (WAKO Pure Chemical Industries, Tokyo, Japan). Mouse eggs (7–32 eggs per experiment) were incubated in a 100 μl TYH medium containing various PYR-41doses (0, 1, 5, and 10 μM) (Biogenova Corp., MD), which is a cell-permeable, irreversible inhibitor of UBE1 activity (half maximal inhibitory concentration [IC50] less than 10 μM in cell-free UBE1 ubiquitination reactions) [5]. The experiments using PYR-41 treated eggs or sperm were repeated at least 3 times.

2.2. Animals

To produce eggs with a single gene deleted, floxed mutant mice for β-catenin were cross-mated with transgenic (Tg) mice expressing cre-recombinase under the control of the egg-specific zona pellucida protein 3 (ZP3) promoter (TgZp3-cre/+), kindly provided by Dr. Barbara B. Knowles [12]. β-cateninfloxed/floxedTgZp3-cre/+ was propagated through brother-sister mating. The presence of the cre-recombinase gene was detected by PCR analysis using the following set of primers: Cre-S (5'-TGATGAGGTTCGCAAGAACC-3′; nucleotide no. 170–189 [GenBank Accession no. AB449974.1]) and Cre-A (5'-CCATGAGTGAACGAACCTGG-3'; nucleotide no. 539–558 [GenBank Accession no. AB449974.1]); this primer set yielded a band of 389 base pairs (bp). Eight- to 12-week-old C57BL/6 female and male mice were purchased from Japan SLC Inc (Shizuoka, Japan).

All mice were housed under specific pathogen-free controlled conditions. Food and water were available ad libitum. The procedures for performing animal experiments were in accordance with the principles and guidelines of the Care and Use of Laboratory Animals at the National Research Institute for Child Health and Development. The animal committee of the National Research Institute for Child Health and Development approved the experiments, including the use of live animals (#2004–004).

2.3. Immunostaining

Mouse eggs were collected from oviducts of 8–12-week-old C57BL/6 superovulated female mice. The eggs (10–15 eggs per experiment) were fixed for 20 min at room temperature in a solution (termed PFA-GLA-PVP) containing 2% paraformaldehyde (PFA), 0.1% glutaraldehyde (GLA) and 0.1% polyvinylpyrolidone (PVP). After washing in phosphate-buffered saline (PBS), they were permeabilized with 1% Triton X-100 in PBS and washed 3 times in PBS. The eggs were then incubated with the primary antibodies (Abs) (2.5 μg/ml) in HEPES-buffered saline (HBS) containing 10 mM HEPES (pH 8.0), 0.15 M NaCl, and 3% fetal bovine serum (FBS) for 2 h at 4 °C. The eggs were then treated with the secondary Abs (1.25 μg/ml) and Alexa488-conjugated IgG (Molecular Probes), and washed 3 times in HBS.

Sectioned fluorescent images were captured using a confocal microscope (LSM 510 model; Carl Zeiss).

2.4. Immunoblotting

A total of 40 eggs was collected from oviducts of superovulated female mice. As UBE1 control, the testicular extracts were prepared as described previously [11]. The eggs and testicular extracts were lysed in Laemmli's SDS sample buffer, boiled, resolved by SDS-PAGE on a 10% acrylamide gel, and immunoblotted as described previously [11].

2.5. In vitro fertilization (IVF)

Eggs were collected from the oviductal ampullas of superovulated 8-–12-week-old C57BL/6 female mice 14–16 h after human chorionic gonadotropin (hCG) injection, placed in a 30 μl drop of TYH medium covered with paraffin oil (Nacalai), and equilibrated with 5% CO2 in air at 37 °C. Eggs were collected from the oviductal ampullas of superovulated β-catenin floxed/floxedTgZp3cre/+ female mice on the C57BL/6 genetic background (8–12 weeks old). The eggs collected from floxed/floxed mice were also inseminated with C57BL/6 sperm as a control. Sperm collected from the epididymides of 8–12-week-old C57BL/6 male mice were induced to capacitate by incubating in TYH medium for 2 h in an atmosphere of 5% CO2 in air at 37 °C before insemination. The ovulated eggs (cumulus-intact eggs) were treated with hyaluronidase (Sigma-Aldrich Co. LLC.) at a final concentration of 300 μg/ml for 15 min at 37 °C in TYH medium.

To estimate the fertilizability of the eggs, capacitated sperm (1.5 × 10[superscript 5] sperm/ml final concentration) were added to the cumulus-intact or cumulus-free eggs. The number of eggs which developed into two-cell embryos was then counted after 24 h incubation under a stereoscopic microscope without fixation.

To count the number of eggs fused to the sperm, zona-free eggs were preincubated with DAPI at a final concentration of 10 μg/ml in TYH medium for 20 min at 37 °C, and washed 3 times by transfer to separate drops of TYH medium. DAPI is a fluorescent dye that can slowly permeate the living cell membrane (semi-permeable), with minimal leakage out of cells after washing relative to Hoechst33342 (permeable). This preincubation procedure with DAPI enables the staining of only fused sperm nuclei, probably through a mechanism in which the dye present within an egg is transferred to the sperm upon membranous fusion. C57BL/6 sperm (1.5 × 10[superscript 5] sperm/ml) were added to a 30-μl drop of TYH medium containing DAPI-treated zona-free eggs and the dish was incubated for 1 h at 37 °C. After incubation, the eggs were fixed with PFA-GLA-PVP solution for 20 min at room temperature. The proportion of eggs fused with sperm was determined by counting DAPI-transferred sperm on each egg under a fluorescence microscope. In this case, eggs fused with sperm were defined as those with at least one DAPI-positive sperm. The influence of UBE1 inhibitor on fertilization was evaluated on the proportion of eggs developed to two-cell stage or the proportion of egg in which "the enlarged sperm" after fertilization. Before and immediately after sperm fusion, the length of the sperm DNA is limited to below 8 μm. We defined "enlarged sperm" as 10 μm and above in the length of the sperm DNA.