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Anti-estrogen ICI 182.780 and anti-androgen flutamide induce tyrosine phosphorylation of cortactin in the ectoplasmic specialization between the Sertoli cell and spermatids in the mouse testis ☆

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Abstract

Our previous study revealed that the ectoplasmic specialization (ES) was deleted by the treatment of anti-estrogen, ICI 182.780 (ICI), and anti-androgen, flutamide (FLUT) in mouse testis. Also, expression of cortactin, an F-actin-binding protein, was decreased by the treatment of FLUT in mouse testis. Cortactin has been suggested to promote actin polymerizer at the ES in the testis, and also actin depolymerization is induced by tyrosine phosphorylation of cortactin. The present study revealed that exogenous treatment of ICI and FLUT caused the deletion of the cortactin in the apical ES and the increase of tyrosine phosphorylated cortactin in mouse testis. These results suggest that the sex hormone antagonists', ICI and FLUT, induced actin depolymerization and tyrosine phosphorylation of cortactin in the mouse testis. Also, the present study may be a key to elucidate the adverse affect exogenous compounds that affect spermiation.

Keywords: Cortactin; Ectoplasmic specialization; Tyrosine phosphorylation; Depolymerization; ICI 182.780; Flutamide

The ectoplasmic specialization (ES) is an actin-based (cell–cell) junctional structure in the Sertoli cell of the testis, found at the apical and basal sites of the seminiferous epithelium. The apical ES develops in the Sertoli cells which face the plasma membrane covering the acrosomal cap in the elongated spermatids. The structure of the ES is composed of the plasma membrane of the Sertoli cell, a subsurface cistern of endoplasmic reticulum that runs parallel to the plasma membrane, and a layer of actin filaments that is sandwiched between the plasma membrane and the subsurface cistern [1–8]. The shaping of the sperm head [4], holding developing spermatids until spermiation [1], orientating the elongating spermatids with their heads towards the base of the seminiferous epithelium [8], and releasing mature spermatids at the time of spermiation [6] have been suggested as four main roles of the apical ES. In the apical ES, several proteins that associate with cell–cell adhesion exist. Cortactin (p80/p85), an F-actin-binding protein, is widely expressed in multiple tissues

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and organs, and is also found in the actin layer in the ES [9,10]. Cortactin has been reported as a major of phosphotyrosine-containing protein [11], and tyrosine phosphorylation of cortactin has been suggested to reduce its actin polymerization [12]. Our previous studies revealed depression of the expression of cortactin protein and the decrease of the immunoreactivity in flutamide (FLUT) treated mouse testis [10]. FLUT also induces the deletion of the apical ES and the deformation of nucleus and acrosomes of spermatids [13]. FLUT is a potent anti-androgenic compound and used for the treatment and prevention of prostatic cancer [14,15].

In the present study, we investigated the relationship between the depression of cortactin protein and the tyrosine phosphorylation of cortactin in the mouse testis after the treatment of ICI 182.780 (ICI), an anti-estrogenic compound [16], and FLUT in the present study. Also, ICI and FLUT induce the similar histological changes, such as the deletion of ES to mouse. The present results may be a key to elucidate the adverse effect of exogenous chemicals on spermiation because this study suggests that both ICI and FLUT induce the actin depolymerization in ES, following the tyrosine phosphorylation of cortactin in mouse testis.

Materials and methods

Animals and experimental design. ICR mice were purchased from CLEA Japan Co. (Hamamatsu, Japan) and housed under a 12:12 h light:dark cycle, in a controlled temperature (24–26 °C) room. Water and food were available ad libitum. All animals were treated according to the Chiba University Guide for the Care and Use of Laboratory Animals. Either ICI (TOCRIS, Ellisville, MO, USA), or FLUT (Sigma, St. Louis, MO, USA), dissolved in corn oil (Sigma) respectively, was administered subcutaneously to 12-week-old ICR mice ($n = 7$). The dose was 0.012 μg/g body weight/day. Control animals received the vehicle (corn oil). The volume for each dose was 0.1 ml. Animals were injected for five sequential days, and then were sacrificed on the 6th day. The injection protocol was based on that of Anahara et al. [13].

Western-blot analysis. The decapsulated testes from ICI ($n = 7$) and control ($n = 7$) mice after five sequential days treatment were lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA 2Na, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The lysates were then homogenized on ice and centrifuged at 15,000*g* for 20 min at 4 °C.

The resulting supernatants were dissolved in SDS sample buffer and used for polyacrylamide gel electrophoresis. Equal amounts of the proteins (2 μg/lane) from the ICI-treated or control mice were applied on 10% SDS–polyacrylamide gels. The gels were then transferred onto polyvinylidene fluoride membranes (Immobilon, Millipore, Bedford, MA) for semi dry electrophoresis (ATTO Corporation, Osaka, Japan). Then, the membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 10% skim milk and then incubated at 4 °C for 24 h in a 1:4000 dilution of mouse anti-cortactin monoclonal antibody (Upstate Biotechnology, Lake Placid, NY). Following incubation, the membranes were washed with TBS containing 0.5% Tween 20 (TBS-T) and incubated for 1 h at room temperature in a 1:20,000 dilution of peroxidase-labeled goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA). The ECL Western-blotting detection system (Amersham, Piscataway, NJ) was used to visualize the results. Analysis of the protein bands was densitometric-performed, using a software package from Scion Image (Frederick, MD, USA).

Immunoprecipitation and phosphorylation detection. The decapsulated testes from ICI- ($n = 7$) and FLUT-treated ($n = 7$), and control ($n = 7$) mice were lysed in a lysis buffer containing 50 mM Tris–HCl (pH 8.0), 1% NP40, 0.5% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 1 mM Na_3VO_4, 1 mM NaF, 10 mM/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The lysates were then homogenized on ice and centrifuged at 14,000*g* for 5 min at 4 °C. Lysates were then precleared with protein G-Sepharose (Amersham) and proteins were immunoprecipitated with 1 μg/μl of incubated cortactin antibody for 1 h at 4 °C and then were collected by incubating with protein G-Sepharose (Amersham) for 1 h at 4 °C. Immunoprecipitates were washed three times with ice-cold lysis buffer. Bound proteins were separated by SDS–PAGE, transferred to polyvinylidene fluoride membranes (Immobilon; Millipore, Bedford, MA). The membrane was blocking in blocked buffer with 0.2 g of block reagent (ECL phosphorylation detection system RPN 2220, Amersham) to 20 ml of TBS-T (10 mM Tris–HCl, pH 7.5, 100 mM sodium chloride, and 0.1% Tween 20) for one hour at room temperature. Following blocking, the membrane was washed in TBS-T twice, and incubated with anti-phosphotyrosine-HRP conjugate (ECL phosphorylation detection system RPN 2220, Amersham) 1:1000 in TBS-T for 1 h at room temperature. Following incubation, the membrane was washed with TBS-T and detected the ECL Western-blotting detection system (Amersham) as aforesaid.

Electron microscopy. The control- and treated-mice were perfusion fixed with 3% glutaraldehyde, and the testes were removed and cut into small pieces. The testicular material was then post fixed with 1% OsO_4 and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate for electron microscopy (JEM 1200EX; JEOL, Tokyo, Japan).

Statistics. The data of Western blots and immunoprecipitation from the ICI- and FLUT-treated groups and the control were expressed as means ± standard error (SD), and compared by an independent *t* test. The significance level for all the statistical tests was set at $P < 0.03$–0.05.

Results

ICI treatment decreases the cortactin in the testis

Immunoblot analysis using anti-cortactin antibody in the ICI-treated and control testes was shown in Fig. 1. The immunoreactive band in the ICI-treated testis appeared to be weaker than that in the control. In the quantitative analysis of immunoreactivity between the control and ICI treatments, the ratio of the ICI treatments to the control was 0.57 ± 0.11 ($P = 0.03$). The similar immunoreactivity depression of cortactin in FLUT treatment is shown in our previous paper [10].

Tyrosine phosphorylation of cortactin is increased in testis after ICI or FLUT treatment

To reveal the effect of ICI or FLUT on cortactin tyrosine phosphorylation in the testis, phosphotyrosine was detected in immunoprecipitated cortactin in ICI-, FLUT-treated and control testes (Fig. 2). Cortactin was immunoprecipitated with a monoclonal anti-cortactin antibody from lysates of ICI-, FLUT-treated, and control testis. Immunoprecipitated proteins were then analyzed by immunoblotting using anti-phosphotyrosine antibody. As shown in Fig. 2, tyrosine phosphorylated cortactin was significantly accumulated in ICI- and FLUT-treated testes compared to the control. The quantitative analysis of the immunoreactivities was also shown in Fig. 2. The ratio of the immunoreactivity of the ICI treatments to the control was

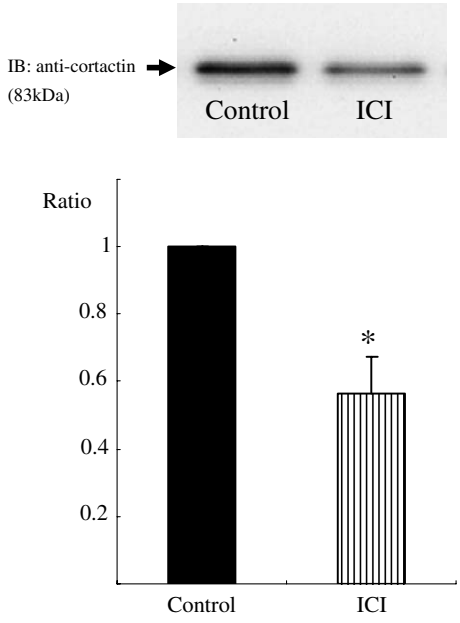

Fig. 1. Western-blot analysis of cortactin in control and ICI-treated mouse testis. The immunoreactive bands of anti-cortactin protein (2 μg/lane) in control and ICI-treated mouse testes and the relative level of cortactin in the control and ICI-treated testes. Compared with the control, the immunoreactive band from ICI-treated testis is faint, and the relative level of cortacitn from ICI-treated testis is significantly decreased. Values are expressed as ratio ± SD. (*) significantly different ($P < 0.05$) from the control.

3.27 ± 0.44 ($P = 0.02$) (Fig. 2A), and, that of the immunoreactivity of the FLUT treatments to the control was 5.55 ± 0.81 ($P = 0.04$) (Fig. 2B). These results clearly indicate that ICI and FLUT treatments induce increase of the cortactin tyrosine phosphorylation in the testis.

Discussion

The study by Fan et al. has shown that actin depolymerization-induced cortactin phosphorylation was mediated by the Fyn/Fer kinase pathway, independent of Src and c-Abl [12]. Also, Daly [17] has reported that tyrosine phosphorylation of cortactin reduces its actin-binding capacity. Our previous study has revealed that FLUT depressed the expression of cortactin in the apical ES in the specific stages of the seminiferous epithelium [10]. The present study demonstrated that ICI also depressed the expression of cortactin in the testis. The results revealed that ICI and FLUT induced the actin depolymerization by the tyrosine phosphorylation of cortactin in the ES. Therefore, the relationship between the deletion of the apical ES by ICI and FLUT treatment and tyrosine phosphorylated cortactin was strongly suggested. The effect of ICI and FLUT on the apical ES was illustrated in Fig. 3. In the normal apical ES, the layer of F-actin is folded, and locates in the Sertoli cell (Fig. 3A). To the knowledge of the previous study, we speculated that the cortactin binds the polymerized F-actin, and the F-actin combined the several focal adherence associated proteins (Fig. 3C). ICI and FLUT induced the tyrosine phosphorylation of cortactin following the actin depolymerization in the apical ES (Fig. 3F). It is interesting that the hormonal actions of ICI and FLUT were different, but the adverse effect of ICI and of FLUT compounds on the apical ES is very similar. Recent reports have shown

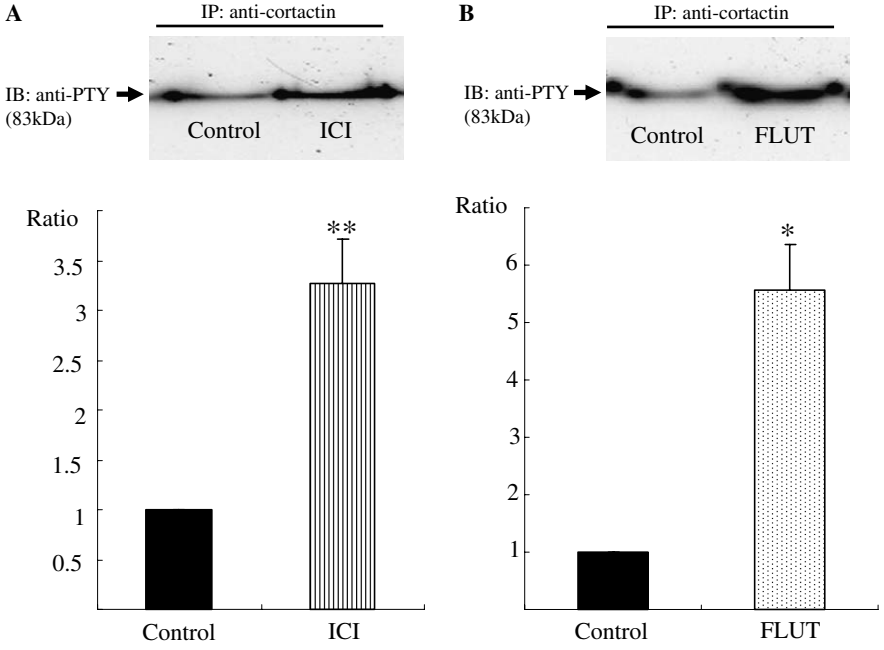

Fig. 2. Analysis of tyrosine phophorylation in control and ICI- and FLUT-treated mouse testis. Lysates of the control, ICI- (A), and FLUT- (B) treated testes were then immunoprecipitated with anti-cortactin antibodies. Immunoprecipitated proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and incubated with anti-phosphotyrosine (PTY) antibody after confirmation of equal total protein. The relative levels of PTY in the control and ICI- (A) and FLUT- (B) treated testes are shown. Values are expressed as ratio ± SD. (*) significantly different ($P < 0.05$) from the control. (**) significantly different ($P < 0.03$) from the control.

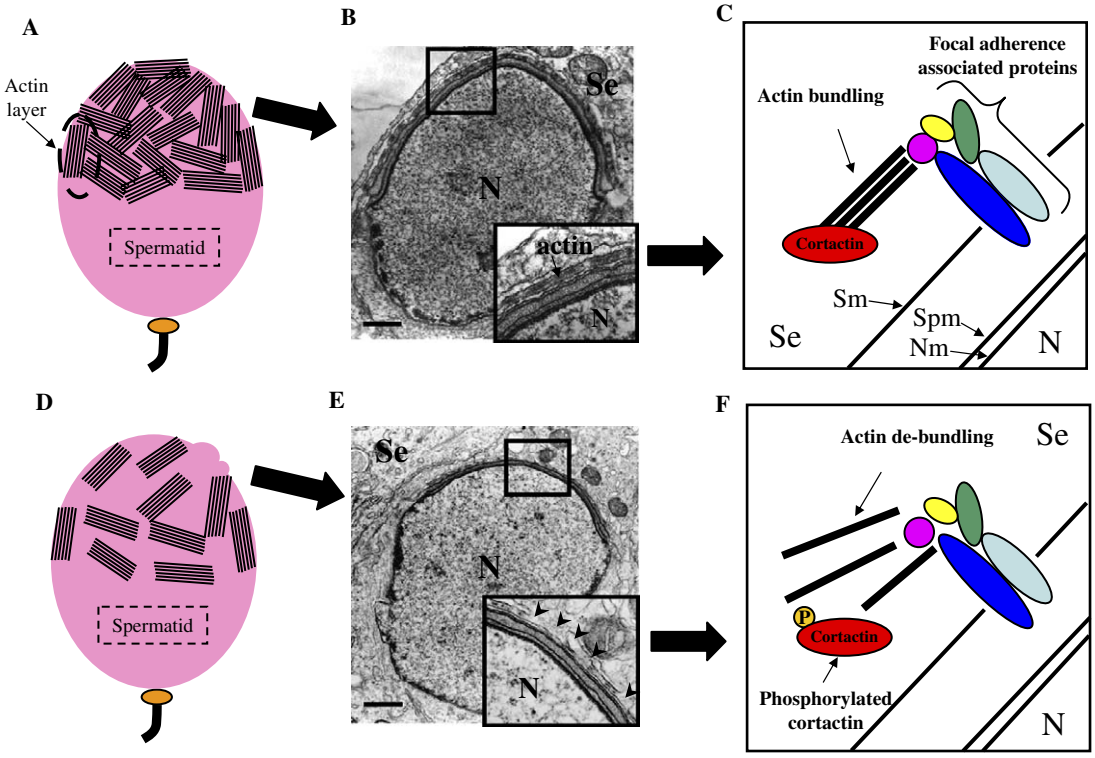

Fig. 3. Hypothetical mechanisms for the apical ES deletion by ICI or FLUT treatment. Normal condition of the apical ES by control treatment (A–C) and abnormal condition of the deleted apical ES by ICI or FLUT treatments (D–F). (A,D) Illustrate the late stage of spermatids. The electron micrographs of the late stage of spermatid from control (B) and ICI-treated testes (E). The regulation of cortactin mediated control of actin polymerization in the apical ES (C,F). The polymerized actin (C) is depolymerized by phosphorylated cortactin (D). Se, the Sertoli cell; N, nucleus; Sm, the Sertoli cell membrane; Spm, spermatid membrane. Bars = 0.5 μm.

that several exogenous chemicals, which act as an agonist or antagonist of sex-hormones, affect the male reproductive systems and spermatogenesis [13,15,18–21]. The previous studies also revealed that β-estradiol 3-benzoate [18], 17β-estradiol [20], bisphenol A [20,21], ICI and FLUT [13] affected the spermiogenesis of the rodents, also, these compounds depressed the cortactin in the apical ES [10,22]. Therefore, we hypothesized that one of the adverse effects of exogenous chemicals, which induces the deletion of apical ES, targets the tyrosine phosphorylation of the cortactin, and these findings may be an index of the elucidation of the chemical to spermiogenesis.

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