Estrogen receptor ESR1 mediates activation of ERK1/2, CREB, and ELK1 in the corpus of the epididymis

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Abstract

Expression of the estrogen receptor ESR1 is higher in the corpus than it is in the initial segment/caput and cauda of the epididymis. ESR1 immunostaining in the corpus has been localized not only in the nuclei but also in the cytoplasm and apical membrane, which indicates that ESR1 plays a role in membrane-initiated signaling. The present study investigated whether ESR1 mediates the activation of rapid signaling pathways by estradiol (E2) in the epididymis. We investigated the effect of E2 and the ESR1-selective agonist (4,4',4''-(4-propyl-(1H)-pyrazole-1,3,5-triy)-trisphenol (PPT) on the activation of extracellular signal-regulated protein kinases (ERK1/2), CREB protein, and ETS oncogene-related protein (ELK1). Treatment with PPT did not affect ERK1/2 phosphorylation in the cauda, but it rapidly increased ERK1/2 phosphorylation in the initial segment/caput and corpus of the epididymis. PPT also activated CREB and ELK1 in the corpus of the epididymis. The PPT-induced phosphorylation of ERK1/2, CREB, and ELK1 was blocked by the ESR1-selective antagonist MPP and by pretreatment with a non-receptor tyrosine kinase SRC inhibitor, an EGFR kinase inhibitor, an MEK1/2 inhibitor, and a phosphatidylinositol-3-kinase inhibitor. In conclusion, these results indicate that the corpus, which is a region with high expression of the estrogen receptor ESR1, is a major target in the epididymis for the activation of rapid signaling by E2. The sequence of events that follow E2 interaction with ESR1 includes the SRC-mediated transactivation of EGFR and the phosphorylation of ERK1/2, CREB, and ELK1. This rapid estrogen signaling may modulate gene expression in the corpus of the epididymis, and it may play a role in the dynamic microenvironment of the epididymal lumen.

Key Words

- ESR1
- ERK1/2
- CREB
- ELK1
- epididymis

Introduction

The epididymis plays an important role in sperm transport, maturation, protection, and storage. In rodents, this organ can be functionally divided into four regions: the initial segment, the caput, the corpus, and the cauda (Turner 1995, Turner et al. 2003). Each of these segments possesses a distinct pattern of gene expression related to the physiological functions of the epididymis (Johnston et al. 2005, Henderson et al. 2006, Guyonnet et al. 2009; reviewed by Belleannée et al. 2012).
Epididymal development and physiology are regulated by a complex interplay of hormones and testicular factors (lumicrine factors) (Robaire & Viger 1995, Hinton et al. 1998, Hess et al. 2011). Androgens and lumicrine factors have been extensively studied, and their contribution to the regulation of epididymal function is indubitable (Robaire & Viger 1995, Hinton et al. 1998). Androgens and lumicrine factors mainly influence the expression of genes specific to the initial segment and caput, and they are less efficient in the more distal corpus and cauda regions of the epididymis (Lan et al. 1998, Sipilä et al. 2006). Therefore, other factors (i.e., estrogens, temperature, pressure, and small RNAs) may contribute to the regulatory mechanisms found in the epididymis (reviewed by Belleannée et al. 2012).

Estrogen biosynthesis may occur not only in the luminal sperm (Hess et al. 2011) but also in the epididymal epithelium (Pereyra-Martinez et al. 2001, Carpino et al. 2004, Shayu & Rao 2006, Joseph et al. 2011). Cytochrome P450 aromatase has been shown to be present in the caput epithelium and interstitium of the epididymis of the mouse (Joseph et al. 2011), rat, human, and monkey (Pereyra-Martinez et al. 2001, Carpino et al. 2004). Estrogen sulfotransferase has also been shown to be present in the epithelium and intraluminal fluid of the epididymis (Tong & Song 2002, Frenette et al. 2009, Hoffmann et al. 2010), and it may control the pool of bioactive luminal estrogens in this organ (Frenette et al. 2009).

The classical estrogen receptors ESR1 and ESR2 (also known as ERα and ERβ respectively) and the G-protein-coupled estrogen receptor (GPER, GPR30) are present in the epididymis (Hess et al. 2011). Knockout of ESR1 (Esr1−/−) in the male mouse leads to the misregulation of acid/base transporters, failure of epididymal acidification, and infertility (Joseph et al. 2010a). Spermatozoa recovered from the epididymides of these animals exhibit abnormal flagellar coiling and increased incidence of spontaneous acrosome reactions (Joseph et al. 2010b). Whereas ESR2 expression does not change among the epididymal regions in adult rats, the expression of ESR1 is higher in the corpus as compared with the initial segment/caput and cauda, which indicates that the effects of estrogen may vary among epididymal regions (Hess et al. 2011) and may contribute to regional differences in gene expression. Furthermore, cytoplasmic and apical membrane immunostaining for ESR1 have been observed in the corpus region (Hess et al. 2011), which indicates that ESR1 plays a role in membrane-initiated signaling.

In addition to the well-established transcriptional effects of E2 (reviewed by Heldring et al. 2007, and Nilsson & Gustafsson 2011), this steroid also mediates rapid effects that occur within seconds or minutes. These rapid effects may be mediated by: i) ESR1 and ESR2 localized at or near the plasma membrane after exposure to ligand (reviewed by Song & Santen 2006, Acconcia & Marino 2011 and Levin 2014); ii) a variant of ESR1 called ERα-36 (Wang et al. 2005, 2006); and/or iii) GPER (reviewed by Lucas et al. 2011 and Prossnitz & Barton 2014). These rapid responses include the activation of different downstream signaling pathways, for example, the MAPK (ERK1/2) and phosphatidylinositol-3-kinase (PI3K) pathways, which in turn modulate nuclear transcriptional events in a variety of cells (reviewed by Lucas et al. 2011, Levin 2014 and Prossnitz & Barton 2014). These protein kinases play a key role in cell growth, differentiation, and function at both the transcriptional and the post-transcriptional levels by phosphorylating a range of proteins, including nuclear transcription factors, such as CREB protein and ETS-oncogene-related protein (ELK1), cytoskeletal proteins, other protein kinases, and receptors for hormones and growth factors. However, it is not known whether the ERK1/2 (MAPK3/1) and PI3K pathways are activated by the rapid action of E2 in the epididymis and whether they play a role in the activation of transcription factors, such as CREB and ELK1.

In the present study we therefore investigated whether ESR1 mediates the E2-induced activation of rapid signaling pathways in the epididymis.

Materials and methods

Animals and tissue preparation

Ninety-day-old male Wistar rats were born and housed in the Animal Facility at the Instituto Nacional de Farmacologia e Biologia Molecular (INFAR), Escola Paulista de Medicina, Universidade Federal de São Paulo (EPM-UNIFESP), and they were maintained on a 12 h light:12 h darkness schedule at 23 °C and were allowed to feed and drink water ad libitum. The experimental procedures were approved by the Research Ethical Committee at EPM-UNIFESP (no. 2043/11). Epididymides were dissected and sectioned into three regions: initial segment/caput, corpus, and cauda (Turner et al. 1990, Turner 1995). For the removal of sperm and luminal fluid, each region was cut into pieces (approximately 1 mm³), which were placed in six-well dishes and washed three times with nutrient solution (Vreeburg et al. 1992, Siu et al. 2006, Gomes et al. 2011) with the following composition: 136.89 mM NaCl, 5.63 mM KCl, 1.80 mM CaCl2, 0.36 mM NaH2PO4, 14.88 mM NaHCO3, and 5.55 mM glucose (pH 5.1530/JME-15-0086 DOI: 10.1530/JME-15-0086 © 2015 Society for Endocrinology Published by Bioscientifica Ltd.
centrifuged at 1610 × g for 30 min at 4 °C. The supernatant was separated, and the protein concentration was determined with the Bio-Rad protein assay using BSA as the standard (Bio-Rad Laboratories, Inc.). Total proteins (5 µg/lane) were incubated with sample buffer containing β-mercaptoethanol and subjected to 10% SDS–PAGE.

Proteins were electrotransferred onto PVDF membranes (pore size 0.45 µm, ImmobilonP, Millipore, Bedford, MA, USA) using 20 V overnight at 4 °C. Membranes were blocked in Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl) containing 0.1% Tween 20 (TBST) and 5% nonfat dry milk (pH 7.6) for 2 h at room temperature. After washes in TBST, membranes were incubated overnight at 4 °C with a rabbit polyclonal antibody raised against a synthetic peptide (KLH-coupled) derived from the sequence of the

Figure 1

Effects of E2 and EGF on the expression and phosphorylation of ERK1 and ERK2 in the corpus of the epididymis. Tissues were incubated at 30 °C in the absence (control, C) and presence of epidermal growth factor (EGF, Sigma, 10 ng/ml, 5 min) (A), E2 (10 and 0.1 nM, 5 min) (B), or E2 (10 nM, different periods of time) (C). Total tissue lysates (5 µg of protein/lane) were resolved in 10% SDS–PAGE, transferred to PVDF membrane, and probed with antibody specific to phosphorylated ERK1/2 (p-ERK1/2) or with antibody that recognizes total (phosphorylation state-independent) ERK1/2. The relative positions of p-ERK1/2 and total ERK1/2 proteins are shown on the right. The data shown are representative of three (A and B) and two (C) independent experiments. Bars represent the densitometric analysis of the western blot. White bars = ERK1; black bars = ERK2. Results were normalized to total ERK1/2 expression in each sample and plotted (mean ± S.E.M.) in relation to the control (C = 1). *ERK1/2 activation was significantly greater than that of the control (P < 0.05, ANOVA and Newman–Keuls). #Significantly different from E2 10 nM (P < 0.05, ANOVA and Newman–Keuls).

Western blot to detect total and phospho-ERK1/2

Western blotting was performed as previously described by Lucas et al. (2008). Briefly, 25 mg of pulverized tissue were lysed in ice-cold lysis buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 µg/ml aprotinin, 10 µM leupeptin, 1 mM phenylmethysulphonyl fluoride, 2 mM Na3VO4, 50 mM NaF, and 10 mM Na2P2O7) and homogenized in Ultra Turrax once at 8200 × g for 30 s at 4 °C. The homogenate was incubated for 30 min at 4 °C and then

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ESR1 activates ERK1/2, CREB, and ELK1

C-terminus of rat p44 MAPK (no. 9102, Cell Signaling Technology, 1:2000 dilution) or a polyclonal antibody raised against a synthetic phosphopeptide that corresponded to the residues that surround Thr202/Tyr204 of human p44 MAPK (no. 9101, Cell Signaling Technology, 1:1000 dilution), which cross-reacts with rat p44 MAPK. Proteins were visualized using ECL reagent (GE Healthcare UK Ltd, Buckingham, UK) after being incubated with donkey anti-rabbit HRP-conjugated secondary antibody (GE Healthcare UK Ltd) at 1:3000 dilution for 1 h at room temperature. Actin levels were monitored to ensure equal protein loading by using a rabbit antibody raised against a synthetic peptide derived from actin residues 20–33 with N-terminus-added lysine (1:4000; A5060, Sigma) overnight at 4°C. Apparent molecular masses were determined from molecular mass standards (no. 26634, Thermo Fisher Scientific, Inc., Suwanee, GA, USA).

Band intensities of total ERK1/2 and phosphorylated ERK1/2 from individual experiments were quantified by densitometric analysis of linear-range autoradiograms using an Epson Expression 1680 scanner (Epson America, Long Beach, CA, USA) and Quick Scan 2000 WIN Software (Helena Laboratories, Beaumont, TX, USA). Results were normalized based on total ERK1/2 expression in each sample and plotted (mean ±S.E.M.) in relation to control (C = 1) (Lucas et al. 2008).

Western blot to detect phospho-CREB (Ser133) and phospho-ELK1 (Ser 383)

Nuclear fractions were obtained as previously described by Royer et al. (2012). Nuclear extracts (40 µg/lane) were incubated with sample buffer containing β-mercaptoethanol and subjected to 10% SDS–PAGE. Proteins were electrotransferred onto PVDF membranes using 20 V overnight at 4°C. Membranes were blocked in TBST containing 10% nonfat dry milk (pH 7.6) for 2 h at room temperature. After washes in TBST, membranes were incubated overnight at 4°C with a rabbit MAB against phospho-CREB Ser133 (phospho-CREB (Ser 133), no. 9198, Cell Signaling Technology) diluted in blocking solution (1:1000) or a rabbit polyclonal antibody against the synthetic phosphopeptide derived from the amino acids that contain the Ser383 from human ELK1 (p-ELK1, Ser383, no. 9181S, Cell Signaling Technology) diluted in blocking solution (1:250). Proteins were visualized using ECL reagent after being incubated with donkey anti-rabbit HRP-conjugated secondary antibody (GE Healthcare UK Ltd) at 1:3000 dilution for 1 h at room temperature.

Band intensities of phospho-CREB, phospho-ELK1, and lamin A from individual experiments were quantified as described in the previous section for ERK1/2. Results were normalized to the respective lamin A expression and plotted (mean ±S.E.M.) in relation to control (C = 1).

Statistical analysis

Data were expressed as mean ±S.E.M. Statistical analysis was carried out by ANOVA followed by the Newman–Keuls test. P values of <0.05 were accepted as significant.

Figure 2

Effects of ESR1-selective agonist PPT on the expression and phosphorylation of ERK1 and ERK2 in the corpus of the epididymis. Tissues were incubated in the absence (control, C) and presence of PPT (100 nM) for 5–30 min at 30°C. (A) Tissues were pretreated or not pretreated with ESR1-selective antagonist MPP (100 nM, 60 min). Afterward, tissues were stimulated with PPT (100 nM) for 5 min at 30°C. (B) Total tissue lysates (5 µg of protein/lane) were resolved using 10% SDS-PAGE, transferred to PVDF membrane, and probed with antibody specific to phosphorylated ERK1/2 (p-ERK1/2, top panels) or with antibody that recognizes total (phosphorylation state-independent) ERK1/2 proteins (bottom panels). The relative positions of p-ERK1/2 and total ERK1/2 proteins are shown on the right. The data shown are representative of three or four independent experiments. Bars represent the densitometric analysis of the western blot. White bars = ERK1; black bars = ERK2. Results were normalized to total ERK1/2 expression in each sample and plotted (mean ±S.E.M.) in relation to the control. *ERK1/2 activation was significantly greater than that of the control (P < 0.05, ANOVA and Newman–Keuls). †Significantly different from PPT (P < 0.05, ANOVA and Newman–Keuls).
Results

Effects of the E2 and ESR1-selective agonist PPT on the expression and phosphorylation of ERK1 and ERK2 in the corpus of the epididymis

EGF receptor is present in the epididymis (Hamzeh & Robaire 2011). Therefore, EGF (10 ng/ml, 5 min) was used as a positive control for the phosphorylation of ERK1/2 in the corpus of the epididymis (Fig. 1A).

Activation of ERK1/2 induced by a 5 min treatment with E2 was concentration-dependent, and it was greater with 10 nM of E2 (2.3- and 2.5-fold respectively for ERK1 and ERK2) than it was with 0.1 nM (Fig. 1B). A whole representative gel is shown to demonstrate that no other protein bands were detected in control or E2-treated tissues. E2 (10 nM) induced a rapid and transient increase in the phosphorylation state of ERK1/2, with a peak at 5 min (Fig. 1C). No differences were observed in total ERK1/2 protein expression under any of these conditions (Fig. 1B and C). The expression of total ERK1 protein was eightfold higher than that of ERK2 in the corpus of the epididymis.

The ESR1-selective agonist PPT (100 nM) also induced a rapid increase of ERK1/2 phosphorylation that was already detected 5 min after treatment with PPT. The activity of ERK1/2 almost returned to control levels by 30 min (Fig. 2A top panels). After 5 min of stimulation with PPT, ERK1 and ERK2 activation were 2.2- and 2.4-fold higher respectively as compared with the control. No differences were observed in total ERK1/2 protein expression under any of these conditions (Fig. 2A, lower panel). No significant differences among different controls were observed in the expression or phosphorylation state of ERK1/2 (P>0.05) (Fig. 2A). Thus, the results were normalized based on the total ERK1/2 expression in each sample and plotted in relation to the average value for controls (C=1) (Fig. 2A).

Activation of ERK1/2 induced by a 5 min treatment with PPT (100 nM) was blocked by pretreatment with an ESR1-selective antagonist (MPP, 100 nM, 60 min) (Fig. 2B), which indicates that ESR1 is the upstream component that regulates ERK1/2 activity in this rapid action. Pretreatment with MPP in the absence of PPT did not have any effect on basal ERK1/2 phosphorylation (Fig. 2B).

The effects of PPT were also examined using the three epididymal regions: the initial segment/caput, the corpus, and the cauda (Supplementary Figure S1, see section on supplementary data given at the end of this article). The total ERK1/2 protein expression, which was normalized to the endogenous control actin, was similar among the distinct epididymal regions (Supplementary Figure S1). The ESR1-selective agonist PPT (100 nM, 5 min) increased the phosphorylation state of ERK1/2 in the initial segment/caput (1.8-fold for both ERK1 and ERK2) and in the corpus of the epididymis (2.5- and 2.8-fold respectively for ERK1 and ERK2), but it did not change the ERK1/2 activity in the cauda of the epididymis, which indicates that the effects of PPT are stronger in the corpus of the epididymis and could be related to the higher expression of ESR1 in this region.
Figure 4

Effects of PPT and signaling pathways involved in the phosphorylation of CREB (Ser133) and ELK1 (Ser383) in the corpus of the epididymis. Tissues were incubated in the absence (control, C) and presence of PPT (100 nM) for 5 min at 30°C. Tissues were pretreated or not pretreated with the ESR1-selective antagonist MPP (100 nM, 60 min) (A), a non-receptor tyrosine kinase SRC inhibitor (PP2, 2.5 μM) (B), an EGFR kinase inhibitor (AG1478, 25 μM) (C), a MEK1/2 inhibitor (U0126, 1 μM) (D), or a PI3K inhibitor (Wortmannin, 1 μM) (E) for 30 min. Afterward, tissues were stimulated with PPT (100 nM) for 5 min at 30°C. Nuclear extracts (40 μg of protein/lane) were resolved in 10% SDS–PAGE, transferred to PVDF membrane, and probed with antibody specific to phosphorylated CREB Ser133 (p-CREB, top panels). The membrane was stripped and reused with antibody specific to phosphorylated ELK1 Ser383 (p-ELK1, middle panels) or with antibody that recognizes the nuclear protein lamin A (bottom panels). The relative positions of p-CREB, p-ELK1, and lamin A proteins are shown on the right. The data shown are representative of two (A) or four (B, C, D, and E) independent experiments. Bars represent the densitometric analysis of the western blot. Black bars = p-CREB; hatched bars = p-ELK1. Results were normalized to lamin A expression in each sample and plotted (mean ± S.E.M.) in relation to the control (C = 1). *CREB or ELK1 activation was significantly greater than that of the control (P < 0.05, ANOVA and Newman–Keuls). #Significantly different from PPT (P < 0.05, ANOVA and Newman–Keuls).
Signaling pathways involved in the phosphorylation of ERK1/2 induced by PPT in the corpus of the epididymis

Activation of ERK1/2 induced by a 5 min treatment with PPT (100 nM) was tested in the presence of the following inhibitors: a non-receptor tyrosine kinase src inhibitor (PP2, 2.5 μM), an EGFR kinase inhibitor AG 1478 (25 μM), a MEK1/2 inhibitor (U0126, 1 μM), and a PI3K inhibitor (Wortmannin, 1 μM) for 30 min (Fig. 3). The effect of each inhibitor (PP2, AG1478, U0126, and Wortmannin) on the basal phosphorylation of ERK1 and ERK2 was tested and compared with the control (absence of an inhibitor). Any change produced by the inhibitor on the basal phosphorylation of ERK1/2 was subtracted from the effect of the inhibitor on phosphorylation induced by PPT. Only U0126 produced a statistically significant inhibition of the basal phosphorylation of ERK1/2 (78 ± 5% of ERK1 and 79 ± 7% of ERK2 basal phosphorylation; n = 4 independent experiments). The results shown in the graphs (Fig. 3) show this result. All of the inhibitors completely blocked the phosphorylation of ERK1/2 that was induced by PPT (Fig. 3).

Activation of ESR1 induces phosphorylation of CREB (Ser 133) in the corpus of the epididymis

The ESR1-selective agonist PPT (100 nM, 5 min) increased phosphorylation of CREB about 3.5-fold. Pretreatment with MPP, a selective antagonist of ESR1, blocked this effect, which indicates that ESR1 is the upstream component that regulates CREB activity (Fig. 4A). No differences were observed in lamin A expression under any of these conditions (Fig. 4, lower panels).

The involvement of SRC, EGFR, ERK1/2, and PI3K in the phosphorylation of CREB induced by PPT (100 nM, 5 min) was tested in the presence of the non-receptor tyrosine kinase src inhibitor (PP2), the EGFR kinase inhibitor (AG 1478), the MEK1/2 inhibitor (U0126), and the PI3K inhibitor (Wortmannin). The activation of CREB induced by PPT was blocked by pretreatment with PP2 (Fig. 4B), AG 1478 (Fig. 4C), U0126 (Fig. 4D), and Wortmannin (Fig. 4E).

Pretreatment with MPP, PP2, AG 1478, U0126, or Wortmannin in the absence of PPT did not affect the phosphorylation of CREB (Fig. 4A, B, C, and E).

Activation of ESR1 induces phosphorylation of ELK1 (Ser383) in the corpus of the epididymis

The ESR1-selective agonist PPT (100 nM, 5 min) increased the phosphorylation of ELK1 about 2.5-fold. Pretreatment with the selective antagonist of ESR1 (MPP) blocked this effect, which indicates that ESR1 is the upstream component that regulates ELK1 activity (Fig. 4A).

The activation of ELK1 induced by PPT was blocked by pretreatment with a non-receptor tyrosine kinase src inhibitor (PP2, Fig. 4B), the EGFR kinase inhibitor (AG 1478, Fig. 4C), the MEK1/2 inhibitor (U0126, Fig. 4D), and the PI3K inhibitor (Wortmannin, Fig. 4E).

Pretreatment with MPP, PP2, AG 1478, U0126, or Wortmannin in the absence of PPT did not affect the phosphorylation of ELK1 (Fig. 4A, B, C, and E).

Discussion

Several pieces of evidence are indicative of a role for estrogen in the control of epidydimal fluid absorption and secretion (Hess et al. 2011). Whereas fewer genes are regulated by estrogens as compared with androgens in the mouse epididymis (Hamzeh & Robaire 2010), estrogen regulates the expression of genes involved in solute and water transport (Hess et al. 1997, 2001, Snyder et al. 2009), which is a function that is essential for normal reproductive performance (Hess et al. 1997). Among these genes, aquaporin 9 contains both AREs and EREs, and its expression is regulated by both estrogen and androgen (Pastor-Soler et al. 2010, Joseph et al. 2011). Furthermore, we have also previously reported that treatment with the anti-estrogen fulvestrant affected the expression of proteins that are important for epithelial organization and absorption/secretion in the rat epididymis (Pereira et al. 2014). This indicates that the balance of estrogen and androgen actions may be important to ensure proper epidydimal function.

The higher expression of the estrogen receptor ESR1 in the corpus of the rat epididymis and its localization not only in the nucleus but also in the cytoplasm and membrane regions of the epithelial cells led us to investigate the role of this receptor in E2-mediated rapid signaling pathways.

Regulation of the ERK and AKT pathways is important for cell survival, proliferation, metabolism, and motility (reviewed by Mendoza et al. 2011). The high level of activity of the ERK pathway components seems to be essential for differentiation of the epithelium from the initial segment of the mouse epididymis (Xu et al. 2010, 2011). Similar to the results of previous studies using mice (Xu et al. 2010), the relatively high basal activity was detected in the present study of ERK1/2 in all of the epidydimal regions in adult rats. The role of the ERK1/2 pathway in the adult epididymis is still not clear, but it
may be important for cell survival and secretion. The high activity of the ERK pathway in the epididymis of mice was abolished by efferent duct ligation (Xu et al. 2010, 2011), which indicates that the testicular luminal fluid may contain factors that activate the ERK pathway. In fact, the results of the present study indicated that E2 is a possible component of the testicular luminal fluid that is involved in the regulation of the ERK1/2 pathway in the epididymis.

ERK1/2 activation by the ESR1-selective agonist PPT was stronger in the corpus of the epididymis, which correlates with the higher concentration of ESR1 in this region. This rapid action of PPT was blocked by the ESR1-selective antagonist MPP, which indicates that ESR1 is the upstream estrogen receptor that regulates ERK1/2 activity.

The intracellular mechanism by which the E2-ESR1 complex initiates rapid signaling in epididymal cells is not known. The non-receptor tyrosine kinase SRC has been implicated in the activation of ERK1/2 by E2 and androgen. Both steroids induce the interaction of ESR1 and androgen receptor (AR) with SRC as well as a conformational change that activates this kinase in breast cancer MCF-7 and prostate cancer LNCaP cells (Migliaccio et al. 2000; reviewed by Migliaccio et al. 2005). An SRC-dependent ERK activation by 5α-dihydrotestosterone (DHT) has also been described in the mouse proximal caput epididymis PC-1 cell line (Hamzeh & Robaire 2011). The phosphorylation of ERK1/2 induced by the activation of ESR1 was blocked by pretreatment with a non-receptor tyrosine kinase Src inhibitor (PP2) in the corpus of the epididymis, which indicates that SRC is involved in this signal transduction.

The complex E2-ESRs (Razandi et al. 2003, Lucas et al. 2008) and E2-GPER (Filardo et al. 2000, Lucas et al. 2010), via the activation of SRC, induce transactivation of EGFR with a consequent phosphorylation of ERK1/2 in breast cancer cells and rat Sertoli cells. Another model of this cross-talk has been proposed in which the ligand-activated EGFR phosphorylates ESR1 on tyrosine 537, thereby inducing ESR1-SRC association and kinase activation. SRC phosphorylates EGFR, which amplifies receptor activity and induces signal transduction activation (reviewed by Migliaccio et al. 2010). PPT-induced ERK1/2 phosphorylation in the corpus of the epididymis was decreased by the EGFR kinase inhibitor, which indicates the involvement of ESR1-SRC-EGFR in the activation of ERK1/2.

PPT-induced activation of ERK1/2 in the corpus of the epididymis was inhibited by the PI3K inhibitor Wortmannin. In fact, there is substantial evidence of cross-talk between the ERK1/2 and the PI3K/AKT pathways, either positively by cross-activation or negatively by cross-inhibition, depending on the cellular environment (reviewed by Mendoza et al. 2011). PI3K-mediated activation of the ERK1/2 pathway induced by relaxin in rat Sertoli cells (Nascimento et al. 2012) and by DHT in the mouse proximal caput epididymis PC-1 cell line (Hamzeh & Robaire 2011) has been shown.

Overlapping and interconnected actions of E2 (in the present study), androgens (Hamzeh & Robaire 2011), and growth factors (reviewed by Cotton et al. 2008) on ERK1/2 activation may occur in the epididymis. An imbalance of these actions may result in infertility, and much remains to be learned about the complex interplay of hormonal actions in the epididymis. Further experimental approaches using a combination of these hormones and growth factors are necessary to address these issues of epididymis biology.

It is now well known that rapid, membrane-initiated signaling mechanisms stimulated by E2 may lead to the activation of gene transcription (reviewed by Lucas et al. 2011, Levin 2014 and Prossnitz & Barton 2014). The phosphorylation of CREB protein at Ser133 induced by E2 through the MAPK and PI3K pathways plays a role in gene transcription in several cells (Aronica et al. 1994, Genua et al. 2009, Royer et al. 2012). E2-mediated CREB phosphorylation has not, to our knowledge, been analyzed in the epididymis so far. The phosphorylation of CREB induced by E2-ESR1 in the corpus of the epididymis was blocked by PP2, AG 1478, U0126, and Wortmannin, which indicates that the SRC-EGFR-MEK-ERK1/2 and PI3K pathways are involved in the phosphorylation of CREB.

The phosphorylation of CREB may be involved in the regulation of gene expression related to epidydimal secretion. For instance, CREB plays a role in the transcriptional regulation of pannexin1, a channel-forming protein that has been implicated in cellular communication through the secretion of biomolecules, such as ATP and glutamate, in the epididymis (Dufresne & Cyt 2014).

Another transcription factor that may be activated by the E2-ERK1/2 pathway in different cells is ELK1 (Song et al. 2002, Kim et al. 2011). The presence of ELK1 in the epididymis has not, to our knowledge, been analyzed so far. The pathway responsible for the activation of ELK1 is triggered by the relocation of the GRB2/SOS complex from the cytoplasm to the cell membrane, where GRB2 is recruited to phosphorylated residues of EGFR. Translocation of the GRB2/SOS complex facilitates the interaction of membrane-associated RAS with SOS and RAS-RAF-1-
MEK1/2-ERK1/2 activation. Activation of the ERK1/2 pathway causes de-SUMOylation and phosphorylation of ELK1, which permits ELK1 to transition from a transcriptionally repressive to a transcriptionally active form (reviewed by Jorissen et al. 2003 and Yang & Sharrocks 2006). The activation of ELK1 induced by PPT was seen in the corpus of the epididymis.

ELK1 modulates the expression of genes that encode proteins that control proteolytic activity, such as the inhibitors of plasminogen activator 1 and metalloproteinase 2 and 9 (MMP2 and MMP9) (reviewed by Kasza 2013). MMP2 and MMP9 are present in the epididymis (Warinrak et al. 2015). Whether the phosphorylation of ELK1 induced by E2 plays a role in the regulation of the metalloproteinases remains to be explored.

In conclusion, the present results indicate that estrogen, when it interacts with the classical estrogen receptor ESR1, induces transactivation of EGFR via the activation of SRC, with the consequent phosphorylation of ELK1, which permits ELK1 to transition from a transcriptionally repressive to a transcriptionally active form of CREB and ELK1 phosphorylation in the corpus of the epididymis. This rapid action of estrogen may ultimately modulate the transcription of genes, and it may play a role in the dynamic microenvironment of the epididymal lumen.

Supplementary data
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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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