Expression and Signaling of G-Protein-Coupled Estrogen Receptor 1 (GPER) in Rat Sertoli Cells

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ABSTRACT

The aim of the present study was to investigate the expression and signaling of the G protein-coupled estrogen receptor 1 (GPER) in cultured immature rat Sertoli cells—in which we have previously described the classical estrogen receptors (ESR1 and ESR2). Expression of GPER in cultured Sertoli cells from 15-day-old rats was detected by RT-PCR and immunoassays. Gper transcripts also were present in testes from 5-, 15-, and 120-day-old rats. Short-term treatment of Sertoli cells with 17β-estradiol (E2), the GPER agonist G-1, or the ESR antagonist ICI 182,780 (ICI) rapidly activated MAPK3/1 (ERK1/2), even after down-regulation of ESR1 and ESR2, suggesting a role for GPER in the rapid E2 action in these cells. MAPK3/1 phosphorylation induced by ICI or G-1 was blocked by pertussis toxin, selective inhibitor of the SRC family of protein tyrosine kinases, metalloprotease inhibitor, MAP2K1/2 inhibitor, and epidermal growth factor receptor (EGFR) kinase inhibitor. Furthermore, E2, but not G-1, induced up-regulation of cyclin D1 in the Sertoli cells. This effect was blocked by ICI. E2 and G-1 decreased BAX and increased BCL2 expression and these effects were blocked by MAP2K1/2 inhibitor and EGFR kinase inhibitor. The pretreatment with ICI did not block the effect of E2. Taken together, these results indicate that in Sertoli cells 1) GPER-mediated MAPK3/1 activation occurs via EGFR transactivation through G protein beta gamma subunits that promote SRC-mediated metalloprotease-dependent release of EGFR ligands, which bind to EGFR and lead to MAPK3/1 phosphorylation; 2) E2-ESRs play a role in Sertoli cell proliferation; and 3) E2-GPER may regulate gene expression involved with apoptosis. ESR and GPER may mediate actions important for Sertoli cell function and maintenance of normal testis development and homeostasis.

INTRODUCTION

It is well established that physiological effects of estrogen are mediated by the classical nuclear estrogen receptors (ESRs) ESR1 and ESR2 (also known as ERα and ERβ; reviewed in [1, 2]). Nevertheless, recent studies have revealed the contribution of a novel ESR, namely G protein-coupled estrogen receptor 1 (GPER, or G protein-coupled receptor 30), which belongs to the family of seven-transmembrane G protein-coupled receptors (GPCRs). Other compounds besides estrogen may also activate GPER: ESR antagonists, such as fulvestrant (ICI 182,780), and G-1, which is a GPER agonist (reviewed in [3–10]). ESRs and GPER can mediate rapid signaling events via the activation of different downstream signaling pathways, for example, the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K) pathways, which, in turn, can modulate nuclear transcriptional events (reviewed in [1, 2, 7–14]).

Estrogen plays an important role in male reproductive function and fertility (reviewed in [15–19]). The expression of ESR1 and ESR2 in the fetal testis occurs very early during development, and their distribution in the different testicular cells has been extensively studied in mammals [18, 20–21]. In fact, our laboratory detected by immunofluorescence the presence of ESR1 and ESR2 in the nuclei of cultured Sertoli cells obtained from 15-day-old rats [22]. In these cells, 17β-estradiol (E2) at physiological concentration activates a translocation of ESRs to the plasma membrane mediated by SRC (SRC family of tyrosine kinases), which results in the activation of epidermal growth factor receptor (EGFR) and MAPK3/1 (ERK1/2). Moreover, activation of ESR1 and/or ESR2 by E2 is also involved in the proliferation of immature Sertoli cells [22, 23] and may suppress differentiation [23]. In addition, the 5α-dihydrotestosterone metabolite 5α-androstan-3β, 17β-diol, is a potent modulator of ESR2-mediated gene transcription in mouse Sertoli cell line [24]. A recent study using estrogen nonresponsive ESR1 knock-in mice, which have a point mutation in the ligand-binding domain of ESR1 that significantly reduces interaction with endogenous estrogens, without affecting ligand-independent ESR1 pathways mediated by growth factors, confirmed that estrogen-dependent ESR1 signaling is required for germ cell viability, most likely through support of Sertoli cell function [25].

Although ESR1 and/or ESR2 present in/near the plasma membrane can mediate the rapid E2 actions in the Sertoli cells [22], the involvement of GPER cannot be excluded. Recently, GPER has been identified in a variety of human and rodent estrogen target tissues (reviewed in [9–10, 14, 26]). There are conflicting data regarding the presence of mRNA for GPER in human and rodent testis (reviewed in [26]). Using RNase protection analysis, mRNA for Gper was not detectable in the mouse testis [27]. However, Gper was located in blood vessels of the testis, using a mutant mouse model (Gpr30-ΔlacZ) [28]. In addition, mRNA for Gper was found in the mouse spermato-
gonadal GC-1 cell line [29]. In these cells, E2 rapidly activates the EGFR/MAPK3/1/FOS pathway through a cross talk between GPER and ESR1, leading to cell proliferation [29]. Recently, the expression of GPER (mRNA and protein) has also been detected in rat pachyteme spermatocytes [30]. E2 through both GPER and ESR1 is able to activate the EGFR/MAPK3/1/JUN pathway, which in turn triggers an increase in BAX expression and a reduction of CCNA1 and CCNB1 [30]. In situ hybridization studies also demonstrated the presence of mRNA for gper in early germ cells and Sertoli cells in zebrafish [31]. In ovarian cancer cells, it has been demonstrated that both GPER and ESR1 must be present to mediate the E2-induced activation of EGFR signaling, MAPK3/1 activation, and cell proliferation [32]. On the other hand, in MCF-7 breast cancer cells, E2 stimulates cell proliferation via a sequential activation of insulin-like growth factor 1 receptor (IGFIR) and EGFR. Additionally, knockdown or blockade of ESR1, but not of GPER, blocks E2-induced MAPK activation [33]. Overall, the apparent discrepancies found in the literature seem to result from differences in cellular context and/or experimental approaches. Although the disruption of Gper does not seem to affect fertility [27, 28], the expression and function of GPER in the male reproductive tract have not been analyzed in detail up to now.

In Sertoli cells, different mechanisms may regulate the balance between cell proliferation and apoptosis, which is essential for normal testis homeostasis and development. However, there are few reports concerning this interesting topic. Studies have demonstrated that proliferating Sertoli cells display increased apoptotic frequency after injury when compared to quiescent Sertoli cells (reviewed in [34]). Nonetheless, Sertoli cell apoptosis may also occur in adult mouse testis, as reported in Bcl-2 (Bclw)-deficient mice [35]. BCL2, a member of the antiapoptotic BCL2 family of proteins, mediates the survival of postmitotic Sertoli cells via inhibition of the proapoptotic BCL2 family member BAX [35]. Considering that estrogen supports cell survival and/or induces cell death/apoptosis depending on the cell context (reviewed in [36–37]) and that GPER may be involved in these processes [29, 38], the aim of the present study was to investigate the signaling and function of GPER in rat Sertoli cells.

MATERIALS AND METHODS

Sertoli Cell Culture

Primary cultures of Sertoli cell were obtained from 15-day-old male Wistar rats housed in the Animal Facility at Instituto de Farmacologia e Biologia Molecular, Universidade Federal de São Paulo-Escola Paulista de Medicina (UNIFESP-EPM), and maintained on a 12L:12D lighting schedule at 23°C and water ad libitum. The experimental procedures were conducted according to guidelines for the care and use of laboratory animals as approved by the Research Ethical Committee from UNIFESP-EPM. The testes were removed and capsulated, and Sertoli cells were prepared as previously described [22, 39–40]. The cells were plated at a density of approximately 4 × 10⁵ cells/ml in phenol red-free Ham F12/Dulbecco modified Eagle medium (F12/DMEM 1:1; Gibco, Invitrogen) containing 0.02 g/L gentamicin (Sigma), 10 ng/ml sodium selenite, and 10 ng/ml epidermal growth factor (EGF; Sigma). (F12/DMEM 1:1; Gibco, Invitrogen) containing 0.02 g/L gentamicin (Sigma), 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate, 0.5 μM of each primer, and 2.5 U of Taq DNA polymerase (Invitrogen) in the appropriate PCR buffer. Samples were amplified in an Applied Biosystem 9600 Fast Thermal Cycler (Applied Biosystem). The thermal cycles were usually one denaturation cycle at 94°C, 1 min; 35 cycles at 94°C, 1 min, 65°C, 1 min, 72°C, 2 min; and an extension period of 72°C, 7 min. The PCR products were resolved onto agarose gels (1%) containing ethidium bromide (0.5 μg/ml) and visualized under ultraviolet transillumination. After extraction from the gel (gel extraction kit; Qiagen), the identity of the product was confirmed by direct nucleotide sequencing in an ABI PRISM 377 automated sequencer (Applied Biosystems) using the DYEEnamic ET Terminator Sequencing kit (Amersham Biosciences).

Immunofluorescence

Sertoli cells were grown as described above on 0.1% gelatin-coated coverslips and placed into six-well plates. The cells were washed with PBS, fixed with 2% paraformaldehyde for 20 min at room temperature and washed with PBS containing 0.1 M glycine. Cells were then permeabilized with 0.01% saponin and blocked with PBS containing 1% bovine serum albumin (BSA) for 10 min at room temperature. After blocking, the cells were incubated with rabbit antibody generated against a synthetic peptide derived from the C-termesus of the deduced amino acid sequence of human GPER polypeptide (a kind gift from Edward J. Filardo, Rhode Island Hospital) [42, 43], diluted 1:50 in PBS containing 0.01% saponin and 1% BSA (Sigma), for 1 h at room temperature. Afterwards, the cells were washed with PBS containing 0.01% saponin and 1% BSA, and incubated with Alexa Fluor 594-labeled secondary antibody (anti-rabbit antibody 1:300; Molecular Probes) for 30 min at room temperature. Coverslips were washed and mounted on slides with Fluoromount G (Electron Microscopy Sciences) [22, 44]. Human embryonic kidney (HEK-293) cells and MCF-7 breast cancer cells were used as negative and positive controls, respectively [5, 7, 42]. Negative control was also performed using normal rabbit serum at the same dilution of antibody or in the absence of primary antibody.

In another series of experiments, the cells were incubated in the absence (control) and presence of a high concentration of E2 (100 μM; Sigma) for 12 h at 35°C [45]. The immunooassays for detection of ESRs were performed as described by Lucas et al. [22].

Immunolocalization of the receptors was visualized under a Nikon E800 fluorescence microscope equipped with a short arc mercury lamp, exciter filters of 330–380 nm and 528–553 nm and respective barrier filters of 435–485 nm and 600–660 nm (Nikon). Images were processed with a CoolSNAP-Pro CCD digital camera and the Image-Pro Express software program (Media Cybernetics).

Western Blot for Detection of MAPK3/1, Phospho-MAPK3/1, and Cyclin D1

Primary Sertoli cell cultures were incubated in the absence (vehicle; control) and presence of 1 nM of ICI 182,780 (AstraZeneca) or G-1 (Calbiochem) for 5–30 min at 35°C [22]. ICI 182,780 was prepared diluting commercially available Faslodex (0.1 M solution of fulvestrant; AstraZeneca) in PBS. G-1 was prepared in dimethyl sulfoxide (0.01 M) and diluted in PBS. The vehicle was also treated or pretreated with pertussis toxin PTX (100 nM; Calbiochem) for 30 min, or the EGFR kinase inhibitor AG 1478 ([4-(3-chloroanilino)-6,7-dimethoxyquinazoline]; 50 μM; Calbiochem) for 15 min at 35°C [22, 43–44]. Afterwards, the cells were stimulated with ICI 182,780 (1 nM; AstraZeneca) or G-1 (1 nM; Calbiochem) for 10 min at 35°C. The concentrations used of the inhibitors are highly selective, as previously reported.
GPER EXPRESSION AND SIGNALING IN SERTOLI CELLS

FIG. 1. Identification of Gper mRNA and protein in the Sertoli cells. A) Identification of Gper transcripts in primary culture of Sertoli cells from 15-day-old rats (lane 2), and in testes from 5- (lane 1), 15- (lane 3), and 120- (lane 4) day-old rats by RT-PCR. The PCR products were resolved on 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Arrow indicates the product with the expected size. Molecular size standard (M) is a 1-kb plus DNA ladder. Identity of the transcript was checked by nucleotide sequencing. Results are representative of three different experiments. B) Specific immunostaining for GPER (using rabbit antibody generated against a synthetic peptide derived from the C-terminal of the deduced amino acid sequence of human GPER polypeptide) (red) was observed in Sertoli cells and MCF-7 (positive control). GPER was not detected in the HEK-293 cells (negative control). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Note that GPER is not localized in the cell nuclei. Bar = 30 μm. The data shown are representative of three independent experiments.

AstraZeneca), MAP2K1/2 inhibitor U0126 (20 μM PBS and lysed in ice-cold lysis buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, Na3VO4, 50 mM NaF, 10 mM Na4P2O7; Sigma). Total cell lysates (40 μg of protein/lane) were incubated with sample buffer containing β-mercaptoethanol and subjected to 15% SDS/PAGE. Proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membranes (0.45 μm pore size, Immobilon-P; Millipore) overnight, 20 V at 4°C. Membranes were blocked in Tris-buffered saline (TBS) containing 0.2% Tween 20 and 10% nonfat dry milk, pH 7.6, for 2 h at room temperature. After washes in TBS-T, membranes were incubated with a rabbit polyclonal antibody raised against a synthetic peptide conjugated to keyhole limpet hemocyanin derived from residues near the C-terminus of CCND1 (#2922; Cell Signaling Technology) diluted in blocking solution (1:500) overnight at 4°C. Proteins were visualized after incubation with donkey anti-rabbit HRP-conjugated secondary antibody (Amersham Biosciences) diluted in TBS-T (1:3000) for 1 h at room temperature by enhanced chemiluminescence reagent (ECL, Amersham Biosciences). Beta-actin (ACTB) levels were monitored on the same blot to ensure equal protein loading, by using a rabbit antibody raised against a synthetic peptide derived from residues 20–33 of ACT with N-terminus added lysine (1:4000; Sigma), overnight at 4°C. Apparent molecular masses of these proteins were determined from molecular mass standards (New England Biolabs).

Band intensities of CCND1 and ACTB from individual experiments were quantified as described above for MAPK3/1. Results were normalized to the respective ACTB expression and plotted (mean ± SEM) in relation to control (=100%).

Western Blot for Detection of ESRs

Primary Sertoli cell cultures were incubated in the absence (control) and presence of a high concentration of E2 (100 μM; Sigma) for 12 h at 35°C [45]. Western blot assays for detection of ESRs were performed as described by Lucas et al. [22].

For detection of cyclin D1 (CCND1), the cells were incubated in the absence (control) and presence of E2 (0.1 nM; Sigma) or G-1 (1 nM; Calbiochem) for 24 h at 35°C. Cells were also untreated or pretreated with ICI 182,780 (1 nM, AstraZeneca), MAP2K1/2 inhibitor U0126 (20 μM; Cell Signaling) for 30 min, or the EGFR kinase inhibitor AG 1478 (50 μM; Calbiochem) for 15 min at 35°C [22]. Afterwards, the cells were stimulated with E2 (0.1 nM; Sigma) for 24 h at 35°C. Medium was removed and the cells were washed with ice-cold PBS and 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 ethylene glycol tetraacetic acid, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 2 mM Na3VO4, 50 mM NaF, 10 mM Na4P2O7; Sigma). Total cell lysates (40 μg of protein/lane) were incubated with sample buffer containing β-mercaptoethanol and subjected to 15% SDS/PAGE. Proteins were electrotransferred onto PVDF membranes overnight at 4°C. Membranes were blocked in PBS containing 10% glycerol and 10% nonfat dry milk, pH 7.6, overnight at 4°C. After several washes in PBS, membranes were incubated with a rabbit polyclonal antibody raised against a peptide derived from the N-terminus of human GPER polypeptide (red) was observed in Sertoli cells and MCF-7 (positive control). GPER was not detected in the HEK-293 cells (negative control). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Note that GPER is not localized in the cell nuclei. Bar = 30 μm. The data shown are representative of three independent experiments.
FIG. 2. Effects of ICI 182,780 and G-1 on MAPK3/1 phosphorylation in the Sertoli cells. Cells were incubated in the absence (C, control) and presence of ICI 182,780 (ICI) (1 nM) (A) or G-1 (1 nM) (B) for 5–30 min. Total cell lysates (60 μg of protein/lane) were resolved in 10% SDS/PAGE, transferred to PVDF membrane, and probed with antibody specific for phosphorylated MAPK3/1 (top panel) or with antibody that recognizes total (phosphorylation state-independent) MAPK3/1 proteins (bottom panel). The relative positions of phosphorylated MAPK3/1 and total MAPK3/1 proteins are shown at the right. The data shown are representative of four independent experiments. Right panel: bars represent the densitometric analysis of the Western blot assays. Solid bars = MAPK3; Open bars = MAPK1. Results were normalized to total MAPK3/1 expression in each sample and plotted (mean ± SEM) in relation to control, C (=1). *MAPK3/1 activation significantly greater than that control (P < 0.05, Student t-test).

Protein Assays

Protein concentration was determined with the BioRad protein assay, using BSA as standard (Bio Rad Laboratories).

Statistical Analysis

Data were expressed as mean ± SEM. Statistical analysis was carried out by Student t-test. P values <0.05 were accepted as significant.

RESULTS

The Expression of GPER in the Sertoli Cells

RT-PCR experiments detected the presence of transcript for Gper in cultured Sertoli cells from 15-day-old rats and in testes from 5-, 15-, and 120-day-old rats (Fig. 1A). The identity of the transcript was confirmed by automated sequencing. No PCR products were detected when reverse transcriptase was omitted from the RT-PCR, assuring that the amplified product was not from genomic DNA contamination (data not shown).

GPER was detected by immunofluorescence in the Sertoli cells obtained from 15-day-old rats (Fig. 1B), and in the MCF-7 cells, used as a positive control [5] (Fig. 1B). GPER immunostaining was mostly found in the intracellular region but not in the nucleus of Sertoli cells. Whether GPER is localized on plasma membrane and/or on intracellular organelles is beyond the scope of this study. No immunostaining was observed in the negative control, the HEK-293 cells (Fig. 1B).

The Involvement of GPER on MAPK3/1 Phosphorylation Induced by ICI 182,780 and G-1 in the Sertoli Cells

MAPK3 and MAPK1 activity and expression in Sertoli cell lysates were measured by immunoblotting using phosphorylation state-dependent (top panels) and -independent (bottom panels) antibodies (Fig. 2). The treatment with ICI 182,780 (1 nM) induced a rapid and transient increase in the phosphorylation state of MAPK3/1 in the Sertoli cells. Peak MAPK3/1 phosphorylation levels occurred at 10 min (10- to 18-fold increase). The MAPK3/1 activity returned to control levels by 30 min (Fig. 2A). Similarly, the GPER selective agonist G-1 (1 nM) also induced a rapid and transient increase in the phosphorylation state of MAPK3/1 in the Sertoli cells. Peak MAPK3/1 phosphorylation levels occurred at 10 min (8- to 15-fold increase). The MAPK3/1 activity returned to control levels by 20 min (Fig. 2B).

To analyze whether these effects were also observed in the absence of the classical estrogen receptors ESR1 and ESR2, the cells were incubated with a high concentration of E2 (100 μM) for 12 h. As can be observed in Figure 3, after this strategy both receptors were no longer observed in the Sertoli cell by immunofluorescence (Fig. 3A). The Western blot analysis confirmed the down-regulation of ESR1 and ESR2 (Fig. 3B).

G-1 (1 nM), E2, at physiological concentrations (0.1 nM; Fig. 4A and [22]), or ICI 182,780 (1 nM), for 10 min, rapidly induced an increase in the phosphorylation state of MAPK3/1...
FIG. 3. Effects of high concentration of E2 on ESR1 and ESR2 expression in the Sertoli cells. A) Detection of ESR1 and ESR2 in primary culture of Sertoli cells by immunofluorescence. Specific immunostaining for ESR1 (using rabbit anti-ESR1 antibody, MC20) and ESR2 (using goat anti-ESR2 antibody, Y19) were observed in Sertoli cell nuclei (red) under basal conditions (control). After incubation with E2 (100 μM, 12 h), ESR1 and ESR2 were not detected in the Sertoli cells. Negative controls were performed using the primary antibody preadsorbed with the respective blocking peptide (inserts). Bar = 30 μm. B) Detection of ESR1 and ESR2 in primary culture of Sertoli cells by Western blot. Total protein extracts (50 μg protein/lane) obtained from Sertoli cells were subjected to 7.5% SDS-PAGE. Immunoblotting using the anti-ESR1 and anti-ESR2 antibodies revealed specific bands (C, control). After incubation with E2 (100 μM, 12 h), ESR1 and ESR2 were down-regulated in Sertoli cells. The relative positions of ESR1 and ESR2 were determined from molecular weight standards, which are shown at the left side. The data shown are representative of three to five independent experiments.

FIG. 4. Effects of E2, G-1, and ICI 182,780 on MAPK1/2 phosphorylation after down-regulation of ESR1 and ESR2 in the Sertoli cells. A) Cells were incubated in the absence (C, control) and presence of G-1 (1 nM), E2 (0.1 nM), and ICI 182,780 (ICI) (1 nM) for 10 min. B) Cells were pretreated with E2 (100 μM, 12 h) and incubated in the absence (C, control) and presence of G-1 (1 nM), E2 (0.1 nM), and ICI (1 nM) for 10 min. Total cell lysates (60 μg of protein/lane) were resolved in 10% SDS/PAGE, transferred to PVDF membrane, and probed with antibodies specific for phosphorylated MAPK3/1 (top panels) or with antibodies that recognize total (phosphorylation state-independent) MAPK3/1 proteins (bottom panels). The relative positions of phosphorylated MAPK3/1 and total MAPK3/1 proteins are shown at the right. The data shown are representative of four independent experiments. Right panels: bars represent the densitometric analysis of the Western blot assays. Solid bars = MAPK3; open bars = MAPK1. Results were normalized to total MAPK3/1 expression in each sample and plotted (mean ± SEM) in relation to control, C (=1). *MAPK3/1 activation significantly greater than control (P < 0.05, Student t-test).
in the Sertoli cells (Fig. 4A). These effects were observed even after the down-regulation of ESR1 and ESR2 (Fig. 4B). The down-regulation of both receptors did not induce any changes in the basal phosphorylation state of MAPK3/1. Taken together, these experiments suggested that GPER plays a role in MAPK3/1 phosphorylation.

**Signaling Pathways Involved in MAPK3/1 Phosphorylation Induced by G-1-GPER in the Sertoli Cells**

The activation of MAPK3/1 induced by a 10-min treatment with G-1 (1 nM) was blocked by pretreatment with selective inhibitor of the SRC family of protein tyrosine kinases inhibitor PP2, metalloprotease inhibitor GM6001 (GM), MAP2K1/2 inhibitor U0126, or pertussis toxin PTX (Fig. 5). Similar results were observed when cells were pretreated with these inhibitors and treated with ICI 182,780 (1 nM, 10 min; Supplemental Fig. S1, available online at www.biolreprod.org). The treatment with PP2, GM6001, U0126, or PTX for 30 min, in the absence of G-1 or ICI 182,780, did not have any effects on MAPK3/1 phosphorylation (data not shown).

To further confirm the involvement of EGFR, the EGFR kinase inhibitor AG 1478 was tested and markedly decreased both G-1 and ICI 182,780-induced MAPK3/1 phosphorylation (Fig. 5B, Supplemental Fig. S1). EGF alone was used as a positive control and strongly activated MAPK3/1, and this effect was blocked by AG 1478, as previously reported [22]. PTX, PP2, and GM6001 did not block the effect induced by EGF (data not shown).

No differences were observed in total MAPK3/1 protein expression under any of the tested conditions (bottom panels of Figs. 2, 4, and 5).

**G-1-GPER Did Not Stimulate CCND1 Expression in the Sertoli Cells**

G-1 (1 nM, 24 h) did not change the CCND1 expression in the Sertoli cells when compared to control (absence of G-1; Fig. 6A). On the other hand, E2 at physiological concentrations (0.1 nM, 24 h), caused an increase in CCND1 expression (Fig. 6A). This effect was blocked by pretreatment with ICI 182,780 (Fig. 6B), suggesting that ESRs are upstream components regulating Sertoli cell proliferation. Furthermore, the effect of E2 was blocked by pretreatment with EGFR kinase inhibitor AG 1478 and MAP2K1/2 inhibitor U0126 (Fig. 6B), suggesting a role for EGFR and MAPK3/1 in the effect induced by E2-ESRs.

**The Involvement of GPER in BAX and BCL2 Expression Induced by G-1 in the Sertoli Cells**

G-1 (1 nM, 24 h) induced a decrease in BAX expression (Fig. 7A) and an increase in BCL2 expression (Fig. 7B) in the Sertoli cells when compared to control (absence of G-1). These
FIG. 6. Effects of E2 and G-1 on CCND1 expression in the Sertoli cells. A) Cells were incubated in the absence (C, control) and presence of E2 (0.1 nM) or G-1 (1 nM) for 24 h. B) Cells were untreated or pretreated with EGFR kinase inhibitor (AG 1478, 50 μM, 15 min) or MAP2K1/2 inhibitor (U0126, 20 μM, 30 min). Afterwards, cells were stimulated with E2 (0.1 nM, 24 h). Total cell lysates (40 μg of protein/lane) were resolved in 15% SDS/PAGE, transferred to PVDF membrane, and probed with antibody specific for CCND1. Results were normalized to ACTB expression in each sample and plotted (mean ± SEM) in relation to control, C (¼100%). *CCND1 expression significantly different from control (P < 0.05, Student t-test).

FIG. 7. Effects of G-1 on BAX and BCL2 expression in the Sertoli cells. A) Cells were incubated in the absence (C, control) and presence of G-1 (1 nM) for 24 h. B) Cells were untreated or pretreated with EGFR kinase inhibitor (AG 1478, 50 μM, 15 min) or MAP2K1/2 inhibitor (U0126, 20 μM, 30 min). Afterwards, cells were stimulated with G-1 (1 nM, 24 h). Total cell lysates (40 and 60 μg of protein/lane, respectively for BAX and BCL2) were resolved in 15% SDS/PAGE, transferred to PVDF membrane, and probed with antibody specific for BAX and BCL2. Results were normalized to ACTB expression in each sample and plotted (mean ± SEM) in relation to control, C (¼100%). *BAX and BCL2 expression significantly different from control (P < 0.05, Student t-test).
effects were blocked by pretreatment with EGFR kinase inhibitor AG 1478 and MAP2K1/2 inhibitor U0126 (Fig. 7), suggesting a role for EGFR and MAPK3/1 in the effects induced by G-1.

E2 (0.1 nM, 24 h) induced a similar result on BAX (Fig. 8A) and BCL2 expression (Fig. 8B). These effects were also blocked by pretreatment with EGFR kinase inhibitor AG 1478 and MAP2K1/2 inhibitor U0126 (Fig. 8, A and B). The pretreatment with ICI 182,780 did not block the effect of E2 on BAX and BCL2 expression (Fig. 8C), suggesting that the effect of E2 is not through ESRs.

**DISCUSSION**

Previous studies from our laboratory have shown that the treatment of Sertoli cells with a physiological concentration of E2 for 10 min induces a translocation of ESR1 and ESR2 to the plasma membrane and a concomitant phosphorylation of MAPK3/1. Both effects were blocked by pretreatment with ICI 182,780 at concentration of 1 nM for 30 min [22].

Short-term treatment of Sertoli cells with 1 nM ICI 182,780 (ESR1 and ESR2 antagonist, GPER agonist) or 1 nM G-1 (selective GPER agonist) did not induce translocation of ESR1 and ESR2 to the plasma membrane (data not shown), but rapidly activated MAPK3/1, even after down-regulation of both ESR1 and ESR2. These results suggest that GPER also is involved in the rapid signaling pathways in these cells, as previously reported in different cell types (reviewed in [5, 7–9, 30]) and in breast cancer metastasis [49]. In fact, GPER (mRNA and protein) is present in cultured Sertoli cells from...
tyrosine kinases SRC or PTK2B protein tyrosine kinase 2 beta, as GPCR agonists or inhibitors have shown that signalling pathways involving EGFR by SRC-family tyrosine kinases are incompletely understood (reviewed in [44]). In Sertoli cells, experiments with pertussis toxin led to activation of MAPK3/1 signaling pathway [56]. In Sertoli cells, the EGFR kinase inhibitor AG 1478 markedly decreased G-1 or ICI 182,780-induced MAPK3/1 activation, confirming the involvement of SRC and EGFR in this action. The mechanisms of GPCR-induced activation of SRC and of transactivation of EGFR by SRC-family tyrosine kinases are incompletely understood (reviewed in [44]). In Sertoli cells, experiments with pertussis toxin have shown that MAPK8 and MAPK14, two proteins related to apoptosis, in the Sertoli cells. This effect was blocked by EGFR kinase inhibitor and MAP2K1/2 inhibitor. The interdependence and interplay of GPER and ERS1 activates the rapid EGFR/MAPK/FOS signaling cascade, which in turn induces cell proliferation [33]. These results may be dependent of the cell context, and GPER could play a significant role in rapid E2 effects and steroid-regulated proliferation in other cell types.

The stimulation of various GPCRs may result in the activation of a number of signalling pathways [44, 51, 56]. The mechanisms whereby metacaspases are activated through GPCR-induced signals include Gβγ subunits of the Gαi/o and Gαq11 family, the nonreceptor tyrosine kinases Src or PTK2B protein tyrosine kinase 2 beta, Ca^2+ signaling pathway [57–59]. In Sertoli cells, the metalloprotease inhibitor GM6001 reduced the MAPK3/1 phosphorylation induced by G-1 or ICI 182,780. Thus, the activation of GPER by G-1 or ICI 182,780 in the Sertoli cells involves EGFR transactivation through Gβγ subunits of G proteins that promote SERCA-mediated metalloprotease-dependent cleavage and release of EGFR ligands from the cell surface and binding to EGFR, and MAPK3/1 activation, as previously reported in breast cancer cells [3, 43]. Unlike EGF treatment, MAPK3/1 activation in response to G-1 or ICI 182,780 was sensitive to PTX, PP2, and GM6001, indicating that this effect is upstream of EGFR.

MAPK3/1 activation plays several important roles in Sertoli cell physiology. MAPK3/1 phosphorylation induced by FSH is involved in the regulation of Sertoli-Sertoli junctions, Sertoli-cell matrix anchoring junctions, and the tight junction barrier [60]. MAPK3/1 also plays a role in adhesion between Sertoli and germ cells, especially at the apical ectoplasmic specialization, where MAPK3/1 and phosphorylated MAPK3/1 have been observed [61, 62]. In fact, rat seminiferous tubules at spermiation present higher levels of MAPK3/1 phosphorylation than tubules before or after spermiation [61]. Recently, our laboratory has shown that E2 activates EGFR/MAPK3/1 and Sertoli cell proliferation through SRC-mediated translocation of ESRRs to the plasma membrane [22].

E2 promotes cell proliferation in both normal and transformed epithelial cells by modifying the expression of hormone-responsive genes involved in the cell cycle and/or programmed cell death. The principal mechanism by which E2 stimulates growth is through the induction of G1→S-phase transition. This induction is associated with up-regulation of Myc, which controls CCND1 expression along with activation of cyclin-dependent kinase and phosphorylation of retinoblastoma protein (reviewed in [37]). In rat Sertoli cell, CCND3 was not detected during testicular development by immunohistochemistry studies [63]. Nuclear immunoreactivity of CCND2 in rat Sertoli cells seems to start at Day 10, is evident on Day 15, and peaks during puberty around Day 35, but is undetectable in the adult. Stage-specific up-regulation of CCND2 expression was observed by blockade of androgen action [64]. Furthermore, acute treatment with E2 was unable to suppress CCND2 immunoreactivity that occurs after ethane dimethane sulfonate-induced testosterone withdrawal in adult rats for 6 days [64]. CCND1, the most studied member of the D-type CCN family, was high in testes of 7- and 14-day-old rats, and decreased and remained at a constant levels thereafter [63]. Immunohistochemical localization of CCND1 in 1-wk-old and adult rat testis revealed expression in both Leydig and Sertoli cells [65]. The present data show that the mitogenic effect of E2 also triggers up-regulation of CCND1 in the Sertoli cells. This effect was blocked by EGFR kinase inhibitor and MAP2K1/2 inhibitor. The interdependence and cooperation of growth factor and E2 signaling pathways for proliferation have been shown in different cell types (reviewed in [36]). ICI 182,780 also blocked the effect of E2 on CCND1 expression, indicating that ESRs are upstream components regulating this effect. On the other hand, G-1 did not change CCND1 expression in Sertoli cells. Although GPER may play a role in certain biological processes or even proliferation of certain cell types, E2-stimulated proliferation of Sertoli cells has not been associated with this protein. In mouse spermatogonial CG-1 cell, E2 through a cross talk between GPER and ESR1 activates the rapid EGFR/MAPK/FOS signaling cascade, which in turn induces cell proliferation [29]. On the other hand, in MCF-7 breast cancer cells, E2 has stimulated cell proliferation via sequential activation of IGFR1 and EGFR, and knockdown or blockade of ESR1, but not of GPER, has blocked E2-induced MAPK activation [33]. These results may be dependent of the cell context, and GPER could play a significant role in rapid E2 effects and steroid-regulated proliferation in other cell types.

Since recent studies have evidenced a role for GPER in antiapoptotic effects [38], we further investigated whether the activation of this receptor would affect the expression of BAX and BCL2, two proteins related to apoptosis, in the Sertoli cells. In fact, G-1 increased the expression of the antiapoptotic...
protein BCL2 about 78% and decreased the expression of the pro-apoptotic protein BAX by 42%, which is consistent with an antiapoptotic effect. Moreover, these effects were blocked by EGFR kinase inhibitor and MAP2K1/2 inhibitor, illustrating that EGFR transactivation and MAPK3/1 are involved in the G-1-induced effects. E2 also induced similar effects on BAX and BCL2 expression. The pretreatment with ICI 182,780 did not block the effect of E2, suggesting that ESRs are not upstream components regulating this effect. These effects were also blocked by pretreatment with EGFR kinase inhibitor and MAP2K1/2 inhibitor. Taken together, these results indicate that GPER plays a role modulating gene expression involved with apoptosis. Several lines of evidence demonstrate that the function of BCL2 family is controlled by growth factor signaling pathways, including the PI3K/AKT, the JAK/STAT, and the RAS/MAPK pathways. Phosphorylation and dephosphorylation of the members of the BCL2 family of proteins by these pathways regulate stabilization of mitochondrial homeostasis (reviewed in [37]). In the present study, we showed that the RAS/MAPK pathway is involved in the regulation of BAX and BCL2 in the Sertoli cells; whether others pathways are also involved remain to be determined.

In cultured immature rat Sertoli cells, the regulatory mechanisms that control cell proliferation (activation of ESRs) and antiapoptotic effect (activation of GPER) are remarkably overlapping through activation of EGFR-MAPK3/1. Considering that Sertoli cells control the intratubular environment, these effects are certainly important for normal testis development and homeostasis. There are reports showing that GPER can collaborate with rapid signaling pathways induced by ESRs (reviewed in [10]). Few details are known about how GPER potentially collaborates with ESRs in or near the plasma membrane, and this issue is not addressed in this study. Previous studies from our laboratory confirmed the expression of ESR1 and ESR2 in testes from 15-, 28-, and 120-day-old rats [22], and the present study demonstrates the expression of GPER in testes from 5-, 15-, and 120-day-old rats. The role of each receptor in animal models remains to be determined.

In conclusion, these results indicate that in Sertoli cells: 1) GPER-mediated MAPK3/1 activation occurs via EGFR transactivation through G protein-coupled receptor (activation of GPER) and MAP2K1/2 inhibitor, which bind to EGFR and to MAPK3/1 phosphorylation; 2) E2-ESRs play a role on Sertoli cell proliferation; 3) E2-GPER may regulate gene expression involved with apoptosis. ESR and GPER may mediate actions important for Sertoli cell function and maintenance of normal testis development and homeostasis.

ACKNOWLEDGMENTS

We thank Dr. Edward J. Filardo for the kind gift of antibody anti-GPER and Esperlita M. J. Silva Santos for technical assistance.

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