Interdependence of Platelet-Derived Growth Factor and Estrogen-Signaling Pathways in Inducing Neonatal Rat Testicular Gonocytes Proliferation¹

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ABSTRACT

We previously found that platelet-derived growth factor (PDGF) and 17 β -estradiol stimulate gonocyte proliferation in a dose-dependent, nonadditive manner. In the present study, we report that gonocytes express RAF1, MAP2K1, and MAPK1/3. Inhibition of RAF1 and MAP2K1/2, but not phosphoinositide-3-kinase, blocked PDGF-induced proliferation. AG-370, an inhibitor of PDGF receptor kinase activity, suppressed not only PDGF-induced proliferation but also that induced by 17 β -estradiol. In addition, RAF1 and MAP2K1/2 inhibitors blocked 17 β -estradiol-activated proliferation. The estrogen receptor antagonist ICI 182780 inhibited both the effects of 17 β -estradiol and PDGF. PDGF lost its stimulatory effect when steroid-depleted serum or no serum was used. Similarly, 17 β -estradiol did not induce gonocyte proliferation in the absence of PDGF. The xenoestrogens genistein, bisphenol A, and DES, but not coumestrol, stimulated gonocyte proliferation in a dose-dependent and PDGF-dependent manner similarly to 17 β -estradiol. Their effects were blocked by ICI 182780, suggesting that they act via the estrogen receptor. AG-370 blocked genistein and bisphenol A effects, demonstrating their requirement of PDGF receptor activation in a manner similar to 17 β -estradiol. These results demonstrate the interdependence of PDGF and estrogen pathways in stimulating *in vitro* gonocyte proliferation, suggesting that this critical step in gonocyte development might be regulated *in vivo* by the coordinated action of PDGF and estrogen. Thus, the inappropriate exposure of gonocytes to xenoestrogens might disrupt the crosstalk between the two pathways and potentially interfere with gonocyte development.

crosstalk, estradiol, estrogens, gametogenesis, growth factors, PDGF, proliferation, signal transduction, testicular gonocytes, testis

INTRODUCTION

Testicular gonocytes are the direct precursors of spermatogonial stem cells (SSCs), the germ cell reservoir from which spermatozoa arise through spermatogenesis. After a first *in utero* mitotic phase followed by several days of quiescence, rat gonocytes become mitotically active between Postnatal Days (PND) 3 and 4, at which time they migrate toward the basement membrane of the seminiferous cord [1, 2]. Gonocytes then differentiate into type A single spermatogonia, believed to correspond to SSCs [3]. In a previous study performed on highly enriched gonocyte cultures from PND3 rats, we found that platelet-derived growth factor (PDGF) and 17 β -estradiol induced *in vitro* gonocyte proliferation [4].

PDGF plays a critical role in developmental processes, where it was shown to regulate the migration, proliferation, and differentiation of different cell types [5]. There are four PDGF polypeptide chains, the A and B chains that can form homo- or heterodimers, and the C and D chains that are only found as homodimers [6]. The PDGF receptors (PDGFR) α and β are transmembrane tyrosine kinases that can form homo- and heterodimers upon ligand binding, triggering downstream signaling cascades, including the extracellular-signal-regulated protein kinases 1 and 2 (ERK1/MAPK3; ERK2/MAPK1) and the phosphatidylinositol-3-kinase (PI3K/PIK3) pathways [6]. PDGF AA binds only PDGFRA homodimers *in vivo* and *in vitro*, whereas PDGF BB was shown to bind both homo- and heterodimers of the receptors *in vitro*, but only PDGFRB dimers *in vivo* [6]. The role of PDGFA and PDGFRA in nervous system, skeletal, lung, skin, retina, and intestine development have been well established, while PDGFB and PDGFRB were found necessary for vasculature, kidney, and cardiac development [5, 6]. Aside from our studies demonstrating the PDGF role in gonocyte development, Sertoli cell-secreted PDGF has been shown to regulate fetal myoid cell migration toward the seminiferous cord and the resulting proliferation, and to control adult-type Leydig cell proliferation [7]. PDGFRB was found to be expressed in human fetal gonocytes [8]. Our work demonstrated that PDGFRA and B are expressed in GD21 and PND3 rat gonocytes and that prenatal estrogen exposure induces changes in PDGFR expression in PND3 gonocytes and testicular somatic cells, suggesting that estrogen and PDGF pathways might interact *in vivo* [4, 9].

The concept that estrogens play a role in testis development has emerged over the past decades [10–12]. Several studies, including ours, have described estrogen receptor β (ER β)/

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ESR2) expression in germ cells [13–16], suggesting that germ cells might be direct targets of estrogens. Moreover, human normal and tumoral testicular germ cells express several isoforms of ESR2 [17]. Yet, *Esr2* knockout mice presented normal fertility, leading to the conclusion that ESR2 does not play a critical role in testis development [18]. Inactivation of ESR2 in mice was shown to affect PND2 gonocyte number in vivo, mainly by decreasing fetal gonocyte apoptosis, suggesting a role of ESR2 in gonocyte development [19, 20]. The ESR2-selective ligand 5 α -androstane-3 β , 17 β -diol (3 β Adiol), a metabolite of dihydrotestosterone produced by Leydig cells, was recently shown to stimulate DNA synthesis in adult rat type A spermatogonia, similarly to 17 β -estradiol [21]. Understanding the role of ESRs in germ cells is further complicated by the existence of alternative subcellular localization of these receptors in several cell types [22, 23]. Thus, the role of estrogen and ESR2 in developing germ cells remains a mystery.

In the present study, we characterized components of the PDGF signaling cascade in gonocytes, examined the relationship between the PDGF and estrogen pathways in gonocyte proliferation using inhibitors and antagonists, and questioned whether exogenous estrogens could mimic the effect of 17 β -estradiol on gonocyte proliferation.

MATERIALS AND METHODS

Animals

Newborn male Sprague Dawley rats were purchased from Charles Rivers Laboratories (Wilmington, MA). PND3 pups were euthanized according to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and following institutional regulations.

Gonocyte Isolation

Isolated gonocytes were obtained from the testes of 30–40 pups per experiment as previously described [4]. Briefly, decapsulated testes were submitted to sequential enzymatic tissue dissociation and overnight differential adhesion, in which dissociated cells from the tubules were incubated at 36°C in the presence of 5% serum to allow somatic cell adhesion. The next morning, nonadherent cells were collected and their pellet resuspended in medium without serum, and gonocytes were further separated on a 2%–4% bovine serum albumin (BSA) gradient in the absence of serum, producing $\sim 0.3 (\pm 0.1) \times 10^6$ gonocytes at 75%–85% purity. In most experiments, the cells (10000 cells per 500 μ l) were cultured for 20 h in 4% CO₂ atmosphere at 36°C in RPMI 1640 containing 2.5% fetal bovine serum (FBS), antibiotics, and factors to test. In some experiments, regular FBS was substituted with charcoal-stripped or PDGF-depleted serum or no serum was added. Sertoli/myoid cell extracts were prepared after the overnight differential adhesion step by collecting the adherent cells remaining after removal of the nonadherent gonocytes and washing of the monolayers [4].

Protein Analysis

Immunoblot analyses were performed as previously described [9] on several cell preparations solubilized in Laemmli buffer [24] right after separation on BSA gradient (~ 15000 cells per sample; no serum added) by SDS-PAGE electrophoresis on 4%–20% gels and transfer onto nitrocellulose membranes. After blocking with 5% milk, proteins were identified using specific antibodies, including anti-RAF1 (cRaf), MEK1 (MAP2K1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Erk1/2 (MAPK3/1; Chemicon/Millipore, Temecula, CA), and PI3-kinase (PIK3; BD Biosciences, San Jose, CA), and sequential incubations with biotin-conjugated secondary antibodies, streptavidin-horseradish-peroxidase (Zymed, Invitrogen Corp., Carlsbad, CA), and a chemiluminescent peroxidase substrate (ECL plus; Amersham Biosciences, Piscataway, NJ). Actin or tubulin used as loading controls were visualized with specific antibodies (Neomarkers Inc., Fremont, CA). All antibodies were diluted (1:250 to 1:1000 for the primary antibodies, 1:10000 for the secondary antibodies) in PBS + 0.05% Tween 20. Nonspecific reaction with rabbit and mouse IgG were tested on some of the membranes prior to using primary antibodies, and they showed only background signals.

Immunohistochemistry

RAF1, MAP2K1, MAPK3/1, PIK3, and AMH (MIS; R&D, Minneapolis, MN) protein expression were determined on 5- μ m paraffin sections of PND3 testes as previously described [9]. In brief, following treatment with DakoCytomation Target Retrieval solution (DAKO North America Inc., Carpinteria, CA), the sections were incubated with PBS containing 10% goat serum + 1% BSA + 0.02% Triton X100 for 1 h to block nonspecific interactions. Then sections were incubated with primary antibodies diluted in PBS + 1% BSA + 0.02% Triton X100 at 1:100 (for RAF1, MAP2K1, MAPK3/1, and AMH) and 1:25 to 1:50 (for PIK3) overnight at 4°C, followed by incubation with Alexa Fluor 568-coupled secondary antibodies (1:300; Invitrogen) for 1 h at room temperature. Nuclei were detected using nucleic acid stain Hoechst 33342 (1:5000, Invitrogen). Negative control slides were prepared by incubating them with nonspecific mouse or rabbit IgG, and others were directly incubated with secondary antibody. The slides were mounted with ProLong Antifade reagent (Invitrogen) and examined with a BX40-Olympus microscope coupled to a DP70 digital camera (Olympus America Inc., Melville, NY), using the same settings and time of exposure for slides incubated with specific antibodies and their corresponding negative controls. Several samples were analyzed, and representative pictures are shown.

Immunocytochemistry

Protein expression of RAF1, MAP2K1, MAPK3/1, AMH, and PIK3 in gonocytes was examined by immunocytochemistry right at the end of the cell isolation procedure (after one night coculture with Sertoli/myoid cells followed by enrichment on a BSA gradient in the absence of serum) and after one day in culture as isolated cells. A method similar to that described above was used, except that cells were fixed with 4% paraformaldehyde in PBS for 5 min, centrifuged, washed, collected on microscopic slides by cytospin centrifugation, dried, and treated with acetone:methanol (60:40) prior antigen retrieval treatment. The slides were incubated overnight with antibodies (1:50 to 1:100). Negative controls were done by incubating some samples with nonspecific mouse IgG, rabbit IgG, or secondary Ab directly. 4',6'-Diamidino-2-phenylindole (DAPI; Invitrogen) was used to label nuclei.

In Situ Hybridization

The mRNA expression of *Raf1*, *Map2k1*, *Mapk3*, and *Pik3* in PND3 testes, fixed right after tissue collection, was examined by in situ hybridization as previously described [9]. Briefly, probes were synthesized using a rat testis cDNA Library (Marathon-Ready cDNA kit; Advantage, Clontech, Mountain View, CA) using the following set of primers (Biosynthesis Inc., Lewisville, TX): for *Raf1*: 491–509, 5'-GCAAAGGACTGTGGTCAAT-3'; 1524–1503, 5'-CCATGAACAGCAGGATATTAAC-3'; for *Map2k1*: 173–192, 5'-AGAAGGTGGGAGAGTTGAAG-3'; 1087–1066, 5'-TCTTGATGAAAGCATGTACCAT-3'; for *Mapk3*: 196–216, 5'-AAGACCAGAGTGGCTATCAAG-3'; 985–964, 5'-AGTACTGTTCCAGGTAAGGGTG-3'; and for *Pik3*: 3593–3611, 5'-AGCCTACAACATTGTGAGG-3'; 4574–4556, 5'-GCAGAGCTGAATGTTAACC-3'; and PCR kits (Clontech). PCR fragments were purified, cloned, sequenced, and linearized; antisense, sense, and nonsense probes were labeled using ³⁵S-CTP. In situ hybridization reactions were performed on paraffin sections of PND3 testes. Nonsense and sense probes used as controls gave low signals as compared to antisense probes. Hematoxylin-eosin (H&E) staining was performed on all slides. A bright field image is presented for each dark field picture to facilitate the localization of positive signals with regard to the seminiferous cords. Representative pictures are shown.

Preparation of PDGF-Depleted FBS

FBS was incubated with antibodies against PDGF AA (Biodesign Intl., Saco, MA) and PDGF Ab-1 (recognizing PDGF BB and AB; Oncogene Research Products, San Diego, CA) overnight at 4°C under slow agitation. The solution was then passed through a protein A HiTrap column (Amersham Biosciences), and fractions were collected. Western blots were performed using the same antibodies, showing that two bands were detected in regular FBS while only a faint band remained in PDGF-depleted FBS, confirming the removal of most of the PDGF from the serum (data not shown).

In Vitro Gonocyte Proliferation

Gonocyte proliferation was examined using BrdU incorporation as previously described [4]. Briefly, cells were incubated for 20 h at 36°C with

30 µg/ml BrdU and 3 µg/ml 5-fluoro-2'-deoxyuridine (Amersham) $\pm$ 10 ng/ml PDGF (type BB, rat recombinant), 17 β -estradiol, genistein (synthetic, $\geq$ 98%, HPLC purified), diethylstilbestrol (DES), or coumestrol, all from Fluka/Sigma-Aldrich Corp. (St. Louis, MO), or bisphenol A (4,4'-isopropylidenediphenol; BPA; ICN/Millipore Biomedicals, Solon OH), at concentrations ranging from 10^{-15} to 10^{-6} M; testosterone and progesterone (Sigma-Aldrich Corp.) used at 10^{-6} M; 100 µM ICI 182780 (Tocris, Ellisville, MO); 100 µM AG-370 (Biomol Research Laboratories, Plymouth Meeting, PA); 10 µM RAF1 kinase Inhibitor 1 (IRAF1), 10 µM UO126, 25 µM PD98059 (Calbiochem, San Diego, CA), or 1 µM Wortmannin (Sigma-Aldrich), in the presence of either 2.5% FBS, 2.5% charcoal-stripped FBS (Biomed, Foster City, CA), 2.5% PDGF-depleted FBS, or no serum. The concentration of 10 ng/ml PDGF was selected because it was found to induce the highest proliferation rate in a dose-response study performed earlier on enriched gonocyte suspensions ($\geq$ 90% purity) that showed a dose-dependent effect of PDGF BB on gonocyte proliferation [4]. In some experiments, cell viability was determined using trypan blue exclusion, showing no effect of the compounds on gonocyte viability, except for AG-370, which slightly decreased cell number (data not shown). BrdU-positive cells were identified by immunocytochemistry using an anti-BrdU antibody (Neomarkers, Fremont, CA) or a BrdU kit from Exalpha (Shirley, MA). Pictures (25–30 per slide) were taken and visualized using Photoshop (Version 6.0; Adobe), and the number of positively stained gonocytes was determined and expressed as a percent of the total number of gonocytes ($\sim$ 1000 cells per sample were scored). In some experiments, PCNA immunoreaction was used to determine the percent of proliferative cells in comparison to BrdU using an anti-PCNA antibody from Santa Cruz, a horseradish-peroxidase-coupled secondary anti-mouse IgG (BD Biosciences), an AEC colorimetric solution (Zymed, Invitrogen Corp., Carlsbad, CA), and hematoxylin counterstaining. The slides were coated with Crystal-Mount (Biomed, Foster City, CA) and examined by bright-field microscopy. The measurement of gonocyte proliferation rate using PCNA immunoreactivity produced similar results to those obtained using BrdU incorporation. The results represent the means ($\pm$ SEM) of three to six individual experiments, in which each condition was tested in duplicate or triplicate.

Statistical Analysis

Statistical analysis was performed by the two-tailed unpaired *t*-test, in which case single comparisons were carried out between the control condition and each treatment, as well as by one-way ANOVA analysis in dose-response experiments. Analyses were done by the statistical program GraphPad Prism (version 4.02; GraphPad, Inc., San Diego, CA).

RESULTS

Identification of Downstream Components of PDGF Pathway in PND3 Testes and Gonocytes

We examined the *in vivo* mRNA and protein expression of several genes known to be associated with PDGF pathway in PND3 testes fixed immediately after tissue collection as well as in freshly isolated gonocytes, and after one day in culture. *In situ* hybridization analysis of testis sections showed that *Mapk3* transcript was the most abundant in the seminiferous cords, especially at the center of the cords corresponding to gonocyte location, followed by *Raf1*, *Map2k1*, and *Pik3* in order of signal intensity (Fig. 1A). Comparison of the specific (antisense probes) and nonspecific (sense probes) signals showed that *Raf1* transcripts were also abundant in the interstitium (Fig. 1A). Protein expression of RAF1, MAP2K1, MAPK3/1, and PIK3 examined by immunohistochemistry showed higher expression inside the seminiferous cords than in the interstitium, more precisely in Sertoli cell and gonocyte cytoplasm, which is in agreement with the transcripts location (Fig. 1B). All antibodies, including that against AMH, gave a positive signal in the cytosol of the Sertoli cells surrounding the gonocytes. The anti-PIK3 antibody used in the present study gave a weaker signal than previously found in gonocytes on polyester wax sections of PND3 rat testes [4], suggesting either a weaker antigenicity of PIK3 in paraffin versus polyester wax-fixed tissues or differences in the antibodies used. Although the immunoreactive signals found in gonocytes were usually

distinguishable from those in Sertoli cells, we confirmed whether the proteins were indeed present in gonocytes by performing immunocytochemical analysis on cells fixed right after the cell isolation procedure (Day 0). These cells were maintained in the presence of Sertoli cells overnight prior to enrichment by passage on a BSA gradient, staying a few hours in the absence of Sertoli cells prior to fixation, and these cells correspond to the earliest time one can obtain isolated gonocytes. As shown in panel Day 0 of Figure 1C, gonocytes at the end of the enrichment procedure expressed RAF1, MAP2K1, and MAPK3/1. There were clearly two types of remaining contaminated small cells, with some cells strongly positive and others totally negative for the signaling molecules. Similarly, AMH was strongly expressed in most, but not all, small cells, presumably corresponding to Sertoli cells, whereas the less abundant negative cells were probably myoid cells. Surprisingly, some but not all gonocytes showed a weak positive cytosolic signal for AMH, suggesting that AMH produced by Sertoli cells might have been taken up by gonocytes. We then examined the expression of RAF1, MAP2K, MAPK3/1, and PIK3 in gonocytes maintained for 1 day in culture in order to verify that the signaling molecules were still expressed during the time frame used to study proliferation. All four proteins appeared as cytoplasmic rings, with MAPK3/1 and MAP2K1 presenting the most pronounced signals, confirming their retention under these culture conditions (Fig. 1C, panel Day1). Some somatic cell contaminants in the cultures were negative for the signaling molecules, whereas others showed weak positive immunoreactivity. RAF1, MAP2K1, and MAPK3/1 proteins were also identified by immunoblot analysis in gonocytes solubilized directly after isolation, as well as in Sertoli/myoid cell extracts (Fig. 1D). Several cell preparations were examined, showing that the strength of the immunoreactive bands seen was proportional to the amount of total protein loaded onto the gels (Fig. 1D). In general, the Sertoli/myoid protein extracts presented stronger immunoreactive bands than gonocytes for a similar amount of total protein, in agreement with the immunocytochemistry data of somatic cells right after cell isolation. Densitometry analysis of immunoblots from several experiments, normalized to actin or tubulin levels, showed that MAPK3 (p44) was 10-fold more abundant than MAPK1 (p42) in gonocytes (data not shown). In view of these data, the effects of inhibitors specific for RAF1, MAP2K1/2, and PIK3 on gonocyte proliferation were examined to determine if these proteins were involved in the transduction of PDGF signaling.

MAPK Pathway Is Involved in PDGF-Induced Neonatal Gonocyte Proliferation

We first showed that AG-370, a specific inhibitor of PDGFR kinase activity [25], significantly suppressed PDGF-BB-induced proliferation (Fig. 2A), confirming that functionally active PDGFRs are required to transduce PDGF mitotic signal in gonocytes. Wortmannin, a specific inhibitor of PIK3 [26], did not significantly decrease PDGF-induced proliferation (Fig. 2B), ruling out the possibility that PIK3 is implicated in PDGF-induced proliferation in gonocytes. However, wortmannin alone induced a small (50% above control values) but significant increase of the basal proliferation rate found in the presence of 2.5% FBS. By contrast, RAF1 inhibitor and the MAP2K1/2 inhibitors PD98059 and UO126 significantly suppressed PDGF-induced gonocyte proliferation (Fig. 2, C and D), demonstrating that RAF1 and MAP2K/MAPK are elements of the PDGF signaling cascade in gonocytes.

FIG. 1. Identification of PDGFR-associated molecules in PND3 testes and isolated gonocytes. **A** The mRNA expression of potential downstream elements of the PDGFR signaling cascade were examined in PND3 testes sections by in situ hybridization, using antisense ³⁵S-labeled probes for specific labeling (top panels) and sense probes for background signals (bottom panels). ISH signals and corresponding H&E staining are shown. **B** Protein expression was examined in the testes by immunohistochemistry. Nuclear labeling (Hoechst), specific immunoreactions (Ab), merged images, and nonspecific signals obtained by using either a mouse or rabbit IgG or by omitting primary antibodies (NS) are presented. Arrows indicate representative gonocytes. **C** Expression of the signaling molecules was examined by immunocytochemistry in gonocytes fixed right after cell isolation (Day 0) or after 1 day in culture following cell isolation (Day 1) and collection by cytospin centrifugation. Specific immunoreaction, DAPI staining of nuclei, and merged images are presented, as well as nonspecific stainings obtained with either a mouse or rabbit IgG or by omitting primary antibodies (2d Ab). Bars = 20 μm (**A**, **B**) and 10 μm (**C**). Representative pictures are shown. **D** Protein expression was determined by immunoblot analysis of immunoblot preparations are shown.

FIG. 2. Role of PDGF signaling cascade in gonocyte proliferation. Isolated gonocytes were incubated overnight in the presence of BrdU used as a proliferation marker in 2.5% FBS $\pm$ 10 ng/ml PDGF-BB and $\pm$ the following inhibitors: 100 μ M AG-370 (A); 1 μ M wortmannin (B); 10 μ M RAF1 kinase Inhibitor 1 (iRAF1; C); 10 μ M UO126 or 25 μ M PD98059 (D). The cells were collected by cytospin centrifugation, and BrdU incorporation was determined by immunocytochemistry. The percents of BrdU-positive versus total cell numbers (proliferation rate) were determined. The histograms represent the means $\pm$ SEM calculated from three independent experiments in which duplicate or triplicate samples were used. * P < 0.05; ** P < 0.01; *** P < 0.001.

Interdependence of PDGF and 17 β -Estradiol Pathways in Stimulating Gonocyte Proliferation

Our previous studies showing no additivity in the effects of PDGF and 17 β -estradiol on proliferation suggested the existence of a crosstalk between their pathways in gonocytes. This possibility was explored by combining inhibitors of one pathway with the stimulatory agent of the other pathway. First, we examined whether the ER antagonist ICI 182780 had an effect on 17 β -estradiol-induced gonocyte proliferation similar to that of ICI 164384, the ER antagonist used in our previous studies [4]. Both ICI 182780 and ICI 164384 have been shown to act as full antagonists on ESR2, but to act as partial agonist/antagonist on ESR1 in a cellular context [27, 28]. Indeed, ICI 182780 significantly blocked 17 β -estradiol-induced proliferation (Fig. 3A), confirming that 17 β -estradiol requires binding onto ER to transduce its mitotic effect in gonocytes. Moreover, ICI 182780 significantly blocked the stimulatory effect of PDGF on proliferation (Fig. 3B), suggesting that the estrogen present in the serum interacted with ER and that the PDGF effect required the concomitant ER activation. In some experiments, ICI 182780 alone induced a small increase in proliferation (40%–50% above control values; Fig. 3, A and B), lower than the 2- to 3-fold increases induced by PDGF and 17 β -estradiol (Figs. 3–6), whereas in other experiments, ICI 182780 did not have any effect when added alone (Fig. 5C). Thus, ICI 182780 exerted consistently inhibitory effects on PDGF- and 17 β -estradiol-induced proliferation, but it had

variable effects when added alone. It should be noted that the ER antagonist ICI 164384 used in previous studies did not induce any stimulatory effect by itself [4].

Similarly to the cross-interaction of ICI 182780 on both PDGF and estrogen pathways, the presence of AG-370 suppressed the effect of 17 β -estradiol on gonocyte proliferation (Fig. 3C), suggesting that PDGFR activation was needed for estrogen action and that PDGF present in serum was sufficient to allow this interaction to occur in the absence of added PDGF. To examine whether the activation of downstream elements of the PDGF pathway was required for the 17 β -estradiol effect, we added RAF1 or MAP2K1/2 inhibitors with 17 β -estradiol. These experiments revealed that these inhibitors prevented 17 β -estradiol-induced proliferation (Fig. 3, D and E), implying that activation of a MAPK was required for 17 β -estradiol to stimulate gonocyte proliferation.

To verify that the serum added in the culture medium provided sufficient amounts of both PDGF and 17 β -estradiol to allow each agent added alone to optimally stimulate proliferation, we performed experiments in which regular FBS was replaced by serum depleted either of estrogen or PDGF. When charcoal-stripped serum was used, to insure the absence of steroids including 17 β -estradiol, PDGF was no longer able to stimulate gonocyte proliferation (Fig. 4A). However, the stimulatory effect of PDGF was recovered by adding back 10^{-6} M 17 β -estradiol to charcoal-stripped serum. Moreover, the addition of 10^{-9} M 17 β -estradiol to charcoal-stripped serum was also efficient at rescuing PDGF response (data not

FIG. 3. Interdependence of PDGF and estrogen pathways in the stimulation of gonocyte proliferation. The possibility of interdependence between the PDGF and estrogen pathways in gonocyte proliferation was examined by incubating isolated gonocytes overnight in the presence of BrdU in 2.5% FBS with or without the following compounds: 1 μ M 17 β -estradiol (A, C–E); 100 μ M ICI 182780 (A, B); 10 ng/ml PDGF-BB (B, C); 100 μ M AG-370 (C); 10 μ M iRAF1 (D); 10 μ M UO126 (E); or 25 μ M PD98059 (E). The histograms represent the means $\pm$ SEM of proliferation rates calculated from three independent experiments in which duplicate or triplicate samples were used. * P < 0.05; ** P < 0.01; *** P < 0.001.

shown). Experiments performed with PDGF-depleted serum showed that 10^{-6} M 17 β -estradiol was not capable of stimulating proliferation in the absence of PDGF. However, when PDGF was added with PDGF-depleted serum to the medium, the 17 β -estradiol-induced gonocyte proliferation was recovered (Fig. 4B). Similar results were found using 10^{-9} M 17 β -estradiol, which did not induce any change when added alone to PDGF-depleted serum, but induced a 2-fold increase when added with PDGF, further confirming the nonadditivity of their effects (Fig. 6C). These results confirmed that the small amount of serum (2.5% FBS) added in the culture medium contained both PDGF and 17 β -estradiol at levels sufficient to allow the action of each of them added alone, further corroborating our findings that the concomitant activation of PDGF and estrogen receptor pathways are required to stimulate gonocyte proliferation.

Comparative Effects of Estrogenic Compounds on Gonocyte Proliferation

Since our experiments indicated that 17 β -estradiol stimulated gonocyte proliferation in conjunction with PDGF, and in view of reports describing various effects of exogenous estrogens on rat neonatal testis [12, 29], we investigated whether exogenous estrogens could mimic the effects of 17 β -estradiol on gonocyte proliferation using the same culture conditions as above. Dose-response experiments using 17 β -estradiol, the synthetic estrogens DES and BPA, or the phytoestrogens genistein and coumestrol revealed that DES, BPA, and genistein induced gonocyte proliferation in a dose-dependent manner (Fig. 5, A, B, and D). By contrast, coumestrol did not have an effect on gonocyte proliferation

(Fig. 5E). Inhibition of genistein, BPA, or DES responses by addition of ICI 182780 indicated that activation of gonocyte proliferation by these compounds required their interaction with ER (Fig. 5, A, B, and D). Furthermore, the addition of AG-370 with genistein or BPA significantly abrogated the effects of these compounds on proliferation (Fig. 5, A and B), suggesting that their effects required concurrent activation of the PDGF pathway, in a manner similar to 17 β -estradiol. Because exposure to more than one exogenous estrogen might occur in the environment, we examined whether femtomolar concentrations of genistein and BPA, shown to have no effect on gonocyte proliferation when added alone, could act in synergy on gonocyte proliferation. The results obtained show that addition of both compounds at such low concentrations in the presence of 2.5% FBS induced a significant increase in gonocyte proliferation (Fig. 5F). To examine whether other types of steroid hormones would exert the same effect on gonocyte proliferation as did estrogens, cells were incubated with either 10^{-6} M testosterone or progesterone in the presence or absence of 2.5% FBS. In both cases, neither progesterone nor testosterone was able to increase gonocyte proliferation above basal levels (Fig. 6A), suggesting that the effect observed is limited to estrogenic compounds and not other steroid hormones. Because genistein and BPA increased gonocyte proliferation at picomolar concentrations in the presence of FBS, in contrast to 17 β -estradiol, which showed significant effects starting at one nanomolar (Fig. 5), and despite their reported lower affinity for ERs, we performed additional experiments in the absence of serum to examine whether the presence of serum could explain this discrepancy. Though PDGF alone did not elicit any change in gonocyte proliferation, the addition of increasing concentrations of

genistein, BPA, and 17 β -estradiol to PDGF induced similar dose-dependent responses, reaching 2- to 3-fold increases above basal proliferation levels in the presence of 10⁻⁶ M estrogen (Fig. 6B). When PDGF-depleted serum was used instead of the regular FBS, the addition of genistein, BPA, or 17 β -estradiol alone at 10⁻⁹ M did not induce any change in proliferation, whereas the addition of PDGF to the estrogens showed increases similar to those observed with PDGF alone, confirming that genistein and BPA acted in a nonadditive manner with PDGF, similar to that of 17 β -estradiol (Fig. 6C). Genistein or BPA added at 10⁻⁹ M to charcoal-depleted serum induced a 2- to 3-fold increase in proliferation over the basal level, in a manner similar to 17 β -estradiol (data not shown). Thus, genistein and BPA showed dose-response curves and fold-changes similar to those of 17 β -estradiol under these conditions.

DISCUSSION

Identifying the cellular and molecular mechanisms regulating gonocyte development is essential to comprehend how SSCs are formed and may provide information on the etiology of carcinoma in situ and testicular germ cell tumors, which are believed to result from a failure of gonocytes to differentiate to SSCs [30]. The goal of the present study was to identify downstream components of the PDGF pathway involved in gonocyte proliferation and to determine whether PDGF and 17 β -estradiol, previously shown to stimulate gonocyte proliferation [4], act in an independent or interdependent manner in gonocytes. Here, we found that RAF1, MAP2K1, and MAPK3/1 are expressed at higher levels in gonocytes and Sertoli cells than in the interstitium. The presence of these proteins has been previously reported in perinatal myoid and immature Sertoli cells [7, 31]. We confirmed that PIK3, a lipid kinase known to activate the AKT pathway downstream of PDGF [6], is present in gonocytes, as earlier reported [4]. To determine which of these pathways was mediating the PDGF-BB proliferative effect, we examined the effects of MAPK and PIK3 pathways on PDGF-induced gonocyte proliferation. To eliminate the possibility of observing indirect effects mediated by somatic cells of the testis expressing PDGFR-associated proteins, we used enriched gonocyte populations instead of the more commonly used somatic/germ cell co-cultures. Despite its limitations, due to the loss of germ cell-somatic cell interactions, the isolated gonocytes paradigm has the advantage of allowing for the investigation of direct effects of compounds in short term studies, contrary to more physiological systems such as organ cultures and co-cultures, where the presence of somatic cell-secreted products precludes the identification of any direct effects on gonocytes.

The inhibition of PDGF-induced proliferation by the tyrphostin AG-370, an inhibitor of PDGFR tyrosine kinase activity, confirmed that functional PDGFRs were required in this process. Our studies did not determine whether PDGF-BB activated homodimers of PDGFRB or PDGFRA-B heterodimers, which are both expressed in neonatal rat gonocytes [9]. However, a study published during the preparation of this manuscript reported that PDGFRB proteins were involved in mouse gonocyte proliferation, using *in vivo* treatments of neonatal mice with the tyrosine kinase inhibitor imatinib [32], suggesting that it might also be the case in neonatal rat. Both PDGFRA and B have been shown to be required in testis development, with PDGFRA being involved primarily in fetal and adult Leydig cell development, while PDGFRB is involved in mesonephric cell migration, cord formation, and vascularization [5–8, 33–37]. To bypass the limitations due to the

FIG. 4. Role of FBS in the effects of PDGF and 17 β -estradiol on gonocyte proliferation. PDGF- or steroid-depleted sera were used in order to determine whether serum was a source of PDGF and 17 β -estradiol in amounts sufficient to permit each compound to stimulate proliferation when added alone in the culture medium. Isolated gonocytes were incubated overnight in the presence of BrdU with 2.5% of normal FBS (A, B), charcoal-stripped FBS (A), or PDGF-depleted FBS (B) $\pm$ 10 ng/ml PDGF-BB $\pm$ 1 μ M 17 β -estradiol (A, B). The histograms represent the means $\pm$ SEM of proliferation rates calculated from three independent experiments in which duplicate or triplicate samples were used. * P < 0.05; ** P < 0.01; *** P < 0.001.

embryonic lethality of the ligand and receptor KO models, investigators have explored the role of PDGFRs in development through a series of tyrosine mutations at amino acid positions required for the activation of specific downstream cascades in either PDGFRA or B [38]. Although none of these transgenic mice presented male infertility, they did not recapitulate the full scale of PDGFR downstream pathways, and thus one cannot exclude the requirement of PDGFRs in germ cell development. Moreover, a partial redundancy between PDGFRs and other related receptors was revealed in studies using constructs of receptor chimera and cytoplasmic domain swapping [38], suggesting that the disruption of a pathway by a nonfunctional mutant PDGFR could be partially

FIG. 5. Comparative effects of xenoestrogens and 17 β -estradiol on gonocyte proliferation. The effects of genistein (A, F), BPA (B, F), DES (D), and coumestrol (E) on gonocyte proliferation were compared to that of 17 β -estradiol (C) in dose-response experiments performed on isolated gonocytes. The cells were incubated overnight in the presence of BrdU in 2.5% FBS using concentrations of estrogens ranging from 10^{-15} to 10^{-6} M, with or without 100 μ M ICI 182780 (A–E) or 100 μ M AG-370 (A, B). The histograms represent the means $\pm$ SEM of proliferation rates calculated from three independent experiments in which duplicate or triplicate samples were used. * P < 0.05; ** P < 0.01; *** P < 0.001.

compensated by the coexistence in the same cells of the other normal PDGFR type. Nevertheless, these studies demonstrated that the cytoplasmic domain of PDGFRA was not interchangeable with that of PDGFRB to sustain PDGFRB function, which required an intact kinase activity. Moreover, these studies suggested that PIK3 pathway is critical for the transduction of PDGFRA signals, whereas it is not essential to support PDGFRB developmental functions. Our finding that PIK3 activation is not required for PDGF-induced gonocyte proliferation is in agreement with PDGFRB mediating this process. However, the finding that addition of wortmannin alone slightly increased the basal rate of proliferation observed in the presence of 2.5% FBS suggests that PIK3 might be involved in other aspects of the cell cycle regulation of gonocytes. By contrast, experiments performed in the presence of inhibitors of the MAPK pathway showed that active RAF1 and MAP2K1/2 were required for PDGF effect, indicating that the PDGF proliferative signal is transduced through the activation of RAF1 and MAP2K1/2 and, by deduction, MAPK3/1 activation in gonocytes. Although the MAP2K inhibitor PD98059 can act on molecules other than MAP2K1/2, due in part to its flavonoid nature [39], the fact that the

nonflavonoid MAP2K inhibitor UO126 [40] showed similar inhibitory effects supports the conclusion that both inhibitors blocked PDGF-induced proliferation by inhibiting MAP2K/MAPK activation. Nevertheless, one cannot at this point conclude which MAPK is involved in this process between MAPK3 and MAPK1, despite the higher expression levels of total MAPK3 compared to MAPK1 in gonocytes.

A study in which several PDGF target genes were mutated reported that the disruption of two of the targeted genes, sphingosine phosphate lyase 1 (*Sgpl1*) and pleckstrin homology domain family A1 (*Plekha1*), led to male sterility in mice [41]. Though both genes were expressed in testicular cords as well as in the interstitium, testis morphology was reported to appear normal until PND7, suggesting that *Sgpl1* and *Plekha1* did not play a role in the early steps of germ cell development. Because Leydig cell steroidogenic function was greatly reduced in the Leydig cells of the mutant mice, it was suggested that the germ cell phenotypes of *Sgpl1* and *Plekha1* mutant mice were secondary to alterations in Leydig cell steroidogenesis. Alternatively, it is possible that despite their normal appearance, the spermatogonia of the mutant mice carried alterations that precluded them from differentiating

FIG. 6. Effects of steroid hormones, xenoestrogens, and 17 β -estradiol on gonocyte proliferation in the absence of serum or the presence of FBS or PDGF-depleted FBS. **A**) The effects of 10⁻⁶ M testosterone and progesterone on proliferation were examined in the absence or presence of 2.5% FBS. **B**) Comparison of the dose-response curves of genistein, BPA, and 17 β -estradiol added with PDGF to gonocytes in the absence of serum. **C**) Effects of genistein, BPA, and 17 β -estradiol added with PDGF-depleted FBS with or without PDGF to gonocyte suspensions. The histograms represent the means $\pm$ SEM of proliferation rates calculated from two to three independent experiments in which duplicate or triplicate samples were used. * P < 0.05; *** P < 0.001.

properly, as suggested by the phenotypes observed at older ages. Considering that gene expression was observed in the tubules and that gonocytes express PDGFRs, it will be interesting to examine whether *Sgpl1* and *Plekha1* are expressed in germ cells.

Using the ER antagonist ICI 182780, we confirmed previous results obtained with the ER antagonist ICI 164384 showing that 17 β -estradiol acts via binding onto ER [4]. However, in contrast to ICI 164384, which did not have any significant effect by itself on gonocyte proliferation, ICI 182780 induced small increases in proliferation when added alone to gonocytes in some experiments. It should be noted that the gonocyte suspensions likely represent heterogeneous cell populations because they are isolated from pups that may be half a day apart in age at the time of euthanasia, a time lapse sufficient to make a difference in gonocytes, which do not develop in a synchronized fashion [42, 43]. Considering that estrogens have a proapoptotic effect in fetal but not neonatal gonocytes [19, 44] and that ICI 182780 has been found to act as an ER agonist and to activate the MAPK pathway in several cell systems [45, 46] as well as induce ER-independent responses [47], one cannot exclude that ICI 182780 alone exerts different effects on the gonocyte subpopulations present in the suspensions. Nonetheless, ICI 182780 blocked estrogen-induced proliferation, indicative of an overall antagonistic effect in this system. Moreover, its inhibitory effect on PDGF-induced proliferation, together with the finding that PDGF effect was estrogen-dependent, supports the idea that it also behaved as an ER antagonist in this condition.

A critical finding of this study is that PDGF and 17 β -estradiol work in concert through activation of their respective receptors to stimulate gonocyte proliferation, and blocking one of the two pathways abolishes the action of the other one on proliferation. Experiments where FBS was replaced by serum depleted of either PDGF or 17 β -estradiol or omitted clearly showed that the concomitant action of both compounds is required for in vitro gonocyte proliferation. Taken together with our observation that a basal level of proliferating gonocytes is found after 1 day in culture independently of the presence of serum, these results suggest that the combination of PDGF and 17 β -estradiol is able to recruit a

new group of gonocytes into the mitotic cycle. By contrast, our results suggest that testosterone and progesterone do not increase neonatal gonocyte proliferation. The finding that RAF1 and MAPK1/2 inhibition blocked the effect of 17 β -estradiol on proliferation further demonstrated the involvement of MAPK activation in the crosstalk between PDGF and estrogen pathways in gonocytes. At present, the exact nature of this interaction is not known. Because both molecules were added together to the cells for 20 h, one cannot determine whether the activation of these pathways needs to be simultaneous or successive, and whether it involves a single or several molecular targets. Complex interactions between estrogen and growth factor pathways have been documented, including the activation of ER by MAPK-dependent phosphorylation in breast and prostate cancer cells [48] and the activation of MAPK by 17 β -estradiol and/or ER activation [49]. Genomic and nongenomic actions of estrogen have been implicated in crosstalks leading to the activation of cell-cycle molecules and transcription factors in testis and other tissues [50, 51]. 17 β -estradiol was shown to induce MAPK activation in the mouse GC-1 type B spermatogonia cell line and mouse pachytene spermatocytes [52]. The ability of estrogens to stimulate cell proliferation through rapid, nongenomic activation of MAPK3/1 and PKA via a membrane nonclassical ER was described in human testicular JKT-1 cancer cells [53], recently characterized as embryonal carcinoma cells [54]. Another possible site of interaction is HSP90AA1, a chaperone molecule required for the stability and activity of both ERs and RAF1 [55] that could shuttle between the two pathways. Considering that PDGFRs and ESR2 are present in gonocytes when the cells are quiescent and still unresponsive to PDGF or 17 β -estradiol [4], the existence of a crosstalk and requirement of a concerted activation of the two pathways could represent the mechanism ensuring the proper timing of the reentry of gonocytes into the cell cycle.

This study also revealed that the xenoestrogens genistein, BPA, and DES stimulated in vitro gonocyte proliferation in a dose-dependent manner. The finding that genistein and BPA presented similar dose-response curves as 17 β -estradiol in the absence of serum but appeared to act at lower concentrations in the presence of serum could be due to differences in the

binding affinities of these compounds and 17 β -estradiol for proteins present in serum, such as steroid binding proteins and albumin, resulting in higher amounts of free molecules available for binding to ER. However, at 10⁻⁹ M and 10⁻⁶ M, these compounds induced the same fold-changes as 17 β -estradiol, and their effects clearly involved interaction with ER since they were suppressed by ICI 182780. ER β being the major ER expressed in gonocytes [15], it is most likely the ER involved in their proliferation. These results also showed that genistein and BPA induced gonocyte proliferation at similar concentrations as 17 β -estradiol despite their lower expected potencies as determined by in vitro ESR2 and ESR1 receptor-binding assays and reporter gene transcriptional assays [28, 56, 57]. This discrepancy might reflect differences in the ability of their ligand-receptor complexes to interact with ER-associated proteins in gonocytes, as it was found in other models where xenoestrogens had differential effects on the recruitment of coactivators by ERs [58–60]. Genistein was shown to recruit coactivators to ESR2 at concentration ranges similar to 17 β -estradiol despite a 10-times-lower affinity in receptor binding assays. However, DES was shown to be less potent than 17 β -estradiol at inducing coactivator recruitment to ERs in spite of their similar receptor binding affinities. Furthermore, BPA and genistein were shown to exhibit selective estrogen receptor mediator-like activities and were shown to behave either as estrogenic or antiestrogenic compounds in the function of the cell/tissue and promoter involved [61, 62]. Considering that both genistein and BPA required the presence of PDGF and active PDGFRs to stimulate gonocyte proliferation, it is also possible that the difference in potency between estrogens could reside in the ability of their ligand-receptor complexes to interact with the PDGFR pathway in these cells. By contrast, the phytoestrogen coumestrol did not induce proliferation. We previously found that prenatal exposure to coumestrol induced changes in PDGFRs and ER-associated protein expression in PND3 testes and gonocytes, similarly to genistein and BPA [9, 15]. The difference between the lack of in vitro effect of coumestrol on gonocytes and its in vivo effects suggests that gonocytes are not its primary target cells in vivo. Altogether, our results showed that xenoestrogens can function in a manner similar to 17 β -estradiol in gonocytes. Moreover, the finding that the combination of femtomolar concentrations of genistein and BPA, which were ineffective by themselves, led to a significant increase in gonocyte proliferation suggests that there might be a threshold in the number of activated ERs required and thus in the amount of estrogenic molecules present in the cells to trigger the signaling cascades leading to proliferation.

The present finding of a positive effect of estrogens on isolated PND3 gonocyte proliferation differs from that of a study performed on rat gonocytes either co-cultured with somatic cells or in organ cultures, reporting that 10⁻⁸ and 10⁻⁶ M 17 β -estradiol and DES had no effect on the numbers of PND3 gonocytes, while they both induced a decrease in fetal (Postcoital Day 16.5) gonocyte numbers [19]. The lack of effect of added 17 β -estradiol at PND3 was not surprising considering that Sertoli cells produce 17 β -estradiol and PDGF, and thus their presence with gonocytes would most likely provide sufficient amounts of both compounds to maintain maximal levels of gonocyte proliferation in the absence of additional factors. This interpretation is supported by the fact that 40% of gonocytes were BrdU-positive in the above-mentioned study in the absence or presence of estrogen, similar to the maximal level of BrdU-positive cells found in the present study using isolated gonocytes stimulated by a combination of PDGF and 17 β -estradiol. Another study reported that rat pups

treated by daily subcutaneous injection of 17 β -estradiol presented at PND3 a 2-fold higher number of gonocytes than control pups, supporting a stimulatory effect of 17 β -estradiol on PND3 gonocyte proliferation in vivo [63]. However, continuous 17 β -estradiol treatment until PND8–PND16, corresponding to the period of spermatogonial differentiation, resulted in decreased germ cell numbers and concomitant increase in germ cell apoptosis, indicating differential effects of estrogen as a function of the time of exposure [63]. The finding that *Esr2* knockout mice presented a 2- to 3-fold decrease in apoptotic gonocytes and a small increase in the number of BrdU-labeled gonocytes at PND2 led to the suggestion that endogenous estrogen stimulates apoptosis and inhibits germ cell growth in the male [19]. However, these effects appear to be age dependent, since 17 β -estradiol was reported to act as a germ cell survival factor in adult human testis [64], and soy isoflavones partially prevented spermatogenesis disruption in aromatase knockout mice [65]. Thus, estrogens appear to have different and sometimes opposite functions throughout germ cell development. Furthermore, these effects involve interactions with other signaling pathways that themselves may vary in the function of the germ cell stages and ages examined.

Although ESR2 is expressed in human and rodent germ cells [11–14] and estrogens have been shown to exert various effects on germ cells in a number of studies [4, 19–21, 52], two independent studies have shown that male *Esr2* knockout mice are fertile, indicating that ESR2 is not essential for germ cell development [18, 66]. Moreover, ESR2 was reported to be expressed in testicular germ cell tumors where its expression was decreased as compared to normal germ cells [67]. Taken together with our results, these data suggest that the role of ESR2 activation in germ cells might be to promote or modulate other signaling pathways rather than to act directly on essential cellular functions. In this regard, it should be noted that fertility can be achieved even in suboptimal conditions for spermatogenesis. Thus, it is possible that the role of 17 β -estradiol in gonocytes is to initiate or support PDGF-induced effects on proliferation.

In conclusion, this study shows that the concomitant activation of PDGF and estrogen pathways is required to induce neonatal gonocyte proliferation in vitro, suggesting that the existence of such crosstalk between the two pathways provides a way for Sertoli cells to tightly regulate the timing and amplitude of gonocyte reentry into mitosis in vivo. Moreover, the finding that xenoestrogens can mimic 17 β -estradiol effects on gonocyte proliferation in vitro implies that environmental estrogens might have a direct effect on gonocyte function in vivo.

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