Embryonic Testis Cord Formation and Mesonephric Cell Migration Requires the Phosphotidylinositol 3-Kinase Signaling Pathway

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ABSTRACT

Mesonephric cell migration and seminiferous cord formation are critical processes in embryonic testis development at the time of male sex determination. Extracellular growth factors shown to influence seminiferous cord formation such as neurotropin-3 utilize in part the phosphotidylinositol 3-kinase (PI3K) signal transduction pathway. The current study investigates the hypothesis that the PI3K pathway is critical in seminiferous cord formation and testis development. The role of the PI3K signaling pathway in testicular cord formation was examined using an Embryonic Day 13 organ culture system and a PI3K-specific inhibitor LY294002. The actions of a mitogen-activated protein (MAP) kinase-specific inhibitor PD98059 was also examined. The PI3K inhibitor blocked cord formation or reduced the number of cords in a concentration-dependent manner. The actions of LY294002 were found to have a developmental stage specificity in that cord inhibition was observed in organs from embryos with 16–17 tail somites, while organs from embryos with 19 or more tail somites had no block in cord formation and only a small reduction in cord number. In contrast, the MAP kinase inhibitor PD98059 did not block cord formation and only caused a slight reduction in cord number. Neither PI3K or MAP kinase inhibitor altered apoptotic cell number, suggesting apoptosis was not the reason for the inhibition of cord formation. Embryonic testis cell migration assays showed that the PI3K inhibitor LY294002 blocked mesonephros cell migration into the testis, while the MAP kinase inhibitor had no effect. Observations suggest the interference of cell migration is the cause for the inhibition of cord formation. Western blot analysis confirmed that LY294002 and PD98059 inhibited phosphorylation of Akt and ERK1/ERK2, respectively. Combined observations demonstrate that the PI3K signaling pathway is involved in embryonic testis cord formation and mesonephros cell migration.

INTRODUCTION

Seminiferous cord formation occurs on Embryonic Day 13 (E13) in the rat and is the initial morphological event in testis development and male sex determination. Organ culture experiments have shown that Sertoli cell differentiation [1] and mesonephric cell migration [2] are critical for normal cord formation. Cell-cell interactions are critical in this process, as has been shown in other cell migrations.

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diated by PI3K, MAP kinase, and STAT pathways, respectively [21–24]. The PDGFs also act through their specific PDGF receptors [25]. Actions of PDGFs are mediated through several signaling pathways, including PI3K, MAP kinase, and PLC-γ, which influence cell mobility, cell growth, and cell differentiation, respectively [26–29]. PI3K also mediates the antiapoptotic cell survival response when activated by PDGF [30].

A signal transduction pathway common to all the above growth factors is PI3K. The multiple isoforms of PI3K can be divided into three classes [31]. Class I PI3Ks are heterodimers made up of a 110-kDa catalytic subunit and an adapter 85-kDa subunit that mediates intracellular signaling through tyrosine kinase family receptors [31]. PI3Ks activate phosphoinositide metabolism, which acts as a second messenger and influences diverse cellular target molecules [32]. Through these molecules, PI3K affects many cellular processes such as cell survival, differentiation, chemotaxis, protein trafficking, and glucose homeostasis [33]. To investigate the actions of PI3K, a PI3K inhibitor, LY294002, [34] was used. LY294002 is a specific competitive inhibitor at the ATP site of the catalytic subunit of PI3K [34]. LY294002 inhibits all p110 isoforms of class I PI3K [35]. As an alternative signaling pathway, the role of MAP kinase in cord formation was investigated with a specific MAP kinase inhibitor, PD98059 [36]. The current study was designed to examine the importance of the PI3K pathway in embryonic testis cord formation.

MATERIALS AND METHODS

Gonadal Organ Cultures

Timed pregnant Sprague-Dawley rats were bred in the institutional vivarium at Washington State University. Institutional Animal Care and Use Committee Guidelines were followed in all procedures. At 0800 h on E13 (sperm-positive vaginal smear date = E0), gonads were dissected with the mesonephros attached. At this stage of embryonic development, the average number of tail somites is 16–17. If embryos had 19 or higher tail somites, they were cultured but excluded from the statistical analysis. The organs were cultured in drops of medium on Millicell CM filters (Millipore, Bedford, MA) floating on the surface of 0.4 ml of CMRL 1066 media (Gibco BRL, Gaithersburg, MD) supplemented with penicillin-streptomycin, insulin (10 μg/ml), transferrin (10 μg/ml), 0.01% BSA, and 30 μM l-glutamine [9]. PI3K inhibitor- or MAP kinase-specific inhibitor, LY294002 or PD98059, were dissolved in ethanol (EtOH) or dimethyl sulfoxide (DMSO) and used to treat one of the gonads, and the contralateral gonad served as a control. Control organs were treated with vehicle control (final concentrations of DMSO or EtOH were ≤0.1% v/v). The medium was changed on the second day of culture. E13 gonads and mesonephros were typically maintained for 3 days in culture, at which point seminiferous cords were developed in the controls [8]. Images of whole organs were obtained using a digital-image analysis system (Diagnostic Instruments Inc., Sterling Heights, MI). Cord number per testis was determined using whole-organ images. Data were confirmed using hematoxylin and eosin-stained sections. Organ culture studies involved at least three separate experiments for each treatment group and each experiment contained 4–6 pairs of male gonads. The sex of embryos was determined using polymerase chain reaction (PCR) for Sry as previously described [8].

Hematoxylin and Eosin Staining, Morphometry, and Cell Apoptosis

Following imaging of the whole organs, the tissue was fixed in Bouin fixative (Sigma) for 2 h and stored in 70% ethanol until embedding in paraffin. Paraffin-embedded tissues were serially sectioned. The tissue sections closest to the largest cross-sectional area of the organs were used in the analysis. One of the serial sections from each experiment was stained with hematoxylin and eosin (HE) using standard procedures. Stained sections were imaged using light microscopy. One of the serial sections from each experiment was used to detect the presence of apoptotic cells. To detect apoptotic cells, an Apoptosis Detection System (Promega, Madison, WI) was utilized as originally described [37]. This system measures fragmented DNA from apoptotic cells by catalytically incorporating fluorescein-12-dUTP at the 3′ DNA end using the enzyme terminal deoxynucleotidyl transferase (TdT), which forms a polymeric tail using the principal of the TdT-mediated dUTP nick-end labeling (TUNEL) assay. The fluorescent cells in the entire testis cross-section were counted. The average number of fluorescent cells/testis area from one experiment was used as a replicate in the statistical analysis.

Cell Migration Assay

A cell migration assay was utilized to assess mesonephros cell migration into the testis. The migration assay involves E13 gonads dissected from embryos at the 16–17 tail somite stage. The mesonephros is cut away and separated from the gonad. Gonads and mesonephros were separately placed on a Millicell CM filters floating on the surface of 0.4 ml of CMRL 1066 media supplemented with penicillin-streptomycin, insulin (10 μg/ml), transferrin (10 μg/ml), 0.01% BSA (w/v), and 350 μM l-glutamine. Gonads were kept on ice while mesonephros were incubated with 20 μM 5(6)-carboxyfluorescein diacetate (CFDA) (Molecular Probes, Eugene, OR) for 30–40 min at 37°C. CFDA has been used in cell tracking experiments involving many cell types, including hepatocytes, which retain the dye up to 3 wk [38]. The mesonephros were washed by incubating in a fresh culture medium with no CFDA for 30–40 min at 37°C. The mesonephros were transferred next to its respective gonad in a manner that cut sides were together. The mesonephros and gonad aggregates were cultured in a similar manner to the E13 organs for 3 days and treated with vehicle, 10 μM LY294002, or 10 μM PD98059 daily. Each treatment included 13 XY organ pairs. If the cut sites of mesonephros and gonad were placed together and the tissues were reasonably intact at the end of manipulations, mesonephros and gonad pairs were included in the analysis. Approximately 95% of the pairs were included in the analysis. The XX gonad and mesonephros pairs were also analyzed; however, they showed minimal or no labeled cells in the gonad (data not shown). Cocultured organs were fixed in Histochoice (Sigma Chemical Co., St. Louis, MO) overnight. The organs were imaged using a Bio-Rad MRC 1024 laser scanning confocal microscope equipped with a krypton/argon laser (Bio-Rad, Hercules, CA). The images were obtained by constructing a z-series three dimensionally using 13–15 optical sections covering 15–20 μm from the most central portion of the fixed organ using LaserSharp software 3.1 (Bio-Rad).

Western Blot Analysis

E13 gonads were cultured and treated as described in the organ culture section above. After 72 h in culture, organs positive for an Sry PCR product were lysed with 1× cell lysis buffer (Cell Signalling Technology, Beverly, MA). The protein concentration in the supernatants were estimated using Bradford protein assay (Bio-Rad). Approximately 100 μg of protein in sample-loading buffer was boiled for 5 min and electrophoresed on a 5–20% SDS gradient gel (Bio-Rad). The protein was subsequently transferred onto nitrocellulose membrane and probed with specific antibodies to phospho-Akt, Akt, phospho MAP/ERK kinase (Cell Signalling Technology), and pan ERK (BD Transduction Laboratories, San Jose, CA). The specific antigen-antibody complex was visualized using an alkaline phosphatase chemiluminescent detection kit (Bio-Rad) and viewed by autoradiography. The autoradiograms were scanned with a densitometer (Molecular Dynamics, Sunnyvale, CA). The relative density of phosphorylated proteins was determined as the ratio of phosphorylated protein density to total protein density. The relative density data from two separate experiments using 3–7 E13 male gonads were combined for analysis.

Statistical Analysis

The data from cord number per testis, cell apoptosis, and migration assay were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The values were expressed as the mean ± SEM. Statistical analysis was performed and the difference between the means of treatments and respective controls were determined using one-way ANOVA followed by a Tukey multiple comparison. A statistically significant difference was confirmed at P < 0.05.

RESULTS

Effects of the PI3K inhibitor LY294002 on cord formation were assessed using E13 organ cultures. For com-
FIG. 1. Effect of PI3K inhibitor LY294002 or MAP kinase inhibitor PD98059 on cord formation in E13 testis organ cultures. Pairs of E13 gonads plus mesonephros were isolated and each of the pairs was separately cultured for 3 days. One contralateral testis was treated with vehicle (control), the other was treated with inhibitors daily. A representative control testis (A), 10 μM LY294002-treated testis (B), and 5 μM LY294002-treated testis (C). Tests treated with 10 μM PD98059 (D). Tests and mesonephros are labeled (T) and (M), respectively. Data are representative of at least three separate experiments with each inhibitor, using 4–6 testis pairs in each experiment.

FIG. 2. Effect of LY294002 or PD98059 on cord number/testis in cultured E13 gonads. All visible testicular cords/testis were counted using whole-organ images. The analysis was confirmed in HE section from the same organ. Data is the mean ± SEM and are representative of at least three experiments using 4–6 organ pairs in each experiment. Control represents combined data from all three group of control testis pairs where the other pairs were treated with 5 μM or 10 μM LY294002 or 10 μM PD98059. *Statistically significant difference compared with the control at P < 0.05; **statistically significant difference compared with controls at P < 0.01.
The 10 μM LY294002 inhibited cord formation in E13 gonads. The sections from cultured E13 gonads were stained with HE. Light micrographs of sections from control organs (A, B), 10 μM LY294002-treated organs (C, D), 5 μM LY294002-treated organs (E, F), and organs from embryos that had a more advanced stage (19 or higher tail somite) embryo were treated with 10 μM LY294002 (G, H). Original magnifications are ×100 (A, C, E, G) or ×400 (B, D, F, H). Testis, mesonephros, and cords were labeled (T), (M), and (arrow), respectively. Data are representative of at least three separate experiments using 4–6 individual testis pairs in each experiment.

(data not shown). In contrast, treatment by 10 μM PD98059 did not block cord formation (Fig. 1D) and treated organs appeared to be similar to the controls (Fig. 1A). Twenty micromolar PD98059 was used in two experiments containing a total of 10 pairs of gonads. Organs treated with 20 μM PD98059 showed cord number and morphology similar to the control organs treated with vehicle alone (data not shown). Some cords treated with PD98059 had irregular morphology. These data demonstrated that 10 μM LY294002 reversibly and specifically inhibited cord formation in a developmentally stage-specific manner.

Analysis of cord number in control and treated organs indicated that 10 μM LY294002 treatment significantly reduced cord number (P < 0.01). The 5 μM LY294002 treatment reduced cord number (P < 0.05) but did not block cord formation (Fig. 2). Cord number per testis in 10 μM PD98059-treated organs was not significantly different than control. Cord number analysis in whole organ images was confirmed in HE sections from the same organ. These data suggest that inhibition of the PI3K signaling pathway inhibits cord formation in a dose-dependent manner. In contrast, inhibition of MAP kinase did not significantly affect cord number.

Cord formation and morphology following treatment with LY294002 or PD98059 was further analyzed in HE-stained organ culture sections using light microscopy. Morphology of the cords in sections from control organs was normal with distinct cord borders, and most of the cords spanned the entire width of the testis (Fig. 3, A and B).
Sections from organs treated with 10 μM LY294002 showed no visible cord formation (Fig. 3, C and D). Sections from organs that were treated with 5 μM LY294002 had visible cords, but cord numbers were reduced and the morphology was irregular (Fig. 3, E and F). The organ sections from embryos with 19 or more tail somites had cords even following 10 μM LY294002 treatment; however, cord morphology was irregular and the cord number was decreased (Fig. 3, G and H). Sections from organs that were treated with 10 μM PD98059 (Fig. 4, B and D) had cords, but morphology was slightly irregular and the cord number was slightly reduced compared with control organ sections (Fig. 4, A and C). These data confirmed the observations on cord number obtained from whole-organ images and provided further insight into cord morphology in treated organs.

Apoptotic cell number in cultured testis was determined to examine whether apoptosis played a role in inhibition of cord formation by the PI3K inhibitor LY294002. The MAP inhibitor PD98059 (10 μM) was used for comparison. LY294002 (10 μM) slightly increased apoptosis, but the increase was not statistically significant (Fig. 5). Apoptotic cell number in 10 μM PD98059-treated organs also slightly increased, but the increase was not statistically significant (Fig. 5). The data suggest that inhibition of the cord formation by LY294002 was not due to an increase in cell apoptosis in the developing gonads.

FIG. 4. Effect of MAP kinase inhibitor PD98059 on cord formation in E13 gonads. The sections from cultured E13 gonads were stained with HE. Control sections (A, C). Testis histology of 10 μM PD98059-treated testis (B, D). Original magnifications are ×100 (A, B) or ×400 (C, D). Testis, mesonephros, and cords were labeled (T), (M), and (arrows), respectively. Data are representative of four separate experiments using 4–6 individual testis pairs in each experiment.

FIG. 5. Effect of LY294002 on apoptotic cell numbers in E13 testis in vitro. Serial organ culture sections were used for determination of apoptotic cell number in the testis using the TUNEL assay. Each group includes data from at least 12 separate sections from organ pairs. Control-1 and -2 represent data from one of the testis pair where the other pairs were treated with 10 μM LY294002 or 10 μM PD98059, respectively. The mean ± SEM is presented, and no statistical difference was detected.
The role of PI3K signaling on mesonephros cell migration into the testis was assessed due to the importance of this cell migration to cord formation and testis development. Cell migration assays showed that fluorescently labeled cells migrated from the mesonephros into the control testis (Fig. 6A). The PI3K inhibitor LY294002 (10 μM) blocked labeled mesonephric cell migration into the testis (Fig. 6B). In contrast, the MAP kinase inhibitor PD98059 (10 μM) did not block mesonephric cell migration (Fig. 6C). In control and MAP kinase cultures, cords were observed, but after LY294002 treatment, no cords were detected (data not shown). The fluorescently labeled cells that migrated into the testis were counted. The mean ± SEM of the cells per 0.1 mm² area of testis were 48.6 ± 3.9, 10.1 ± 1, or 43.3 ± 5.2 for control, 10 μM LY294002-, or 10 μM PD98059-treated organs, respectively. The reduction in the number of migrated cells in LY294002-treated organs was significant (P < 0.05). These data suggest that the PI3K signaling pathway plays a role in mesonephros cell migration into the embryonic testis while the MAP kinase pathway is not critical for this process.

To confirm that the PI3K inhibitor LY294002 and MAP kinase inhibitor PD98059 inhibit phosphorylation of respective substrates Akt or ERK1/2, a Western blot experiment for phospho-Akt and phospho-ERK1/2 was performed. Embryonic E13 testes were incubated in the absence or presence of the inhibitors and a protein extract obtained for analysis. The PI3K inhibitor LY294002 (10 μM) or MAP kinase inhibitor PD98059 (10 μM) dramatically reduced phosphorylation of Akt or ERK1/2, respectively (Fig. 7). The Western blot analysis also demonstrated that total Akt or ERK1/2 protein levels were not affected.
by the treatments (Fig. 7). The densitometric analysis of the Western blot data showed that the relative levels of phospho-Akt and phospho-ERK1/2 were approximately 1/4 and 1/20 of the controls, respectively. Observations demonstrate that LY294002 inhibited both the PI3K signaling pathway and cord formation in E13 male gonad cultures. In contrast, PD98059 inhibited the MAP kinase signaling pathway, but it did not affect cord formation.

**DISCUSSION**

Mesonephros cell migration takes place during gonadal differentiation and is a developmental stage-dependent process. Organ culture experiments in mice have shown that mesonephros cell migration takes place between embryonic 11.5–16.5 days [39]. In the current study, the PI3K inhibitor blocked cord formation in organs from 16–17 tail somite embryos. However, the PI3K inhibitor did not block cord formation in the organs from more advance stage embryos (e.g., 19 or higher tail somites). It is speculated that the testsis of embryos that are at a 19 or more tail somite stage have limited cell migration prior to exposure to the inhibitor, which leads to partial cord formation.

Involvement of PI3K in cell migration processes has been shown in various systems [17, 21, 27]. Therefore, we assessed whether the inhibition of cord formation was due to a reduced mesonephros cell migration using the cell migration assay. Cell migration data showed that migrating cell numbers in PI3K inhibitor-treated organs were significantly reduced. This suggests that inhibition of testicular cord formation by LY294002 was likely due to inhibition of mesonephros cell migration. Previous work has shown that mesonephros cell migration potentially involves a chemoattractive agent from the gonad [39]. One potential chemoattractive agent is NT3. Prior to cord formation, the TrkC and p75 receptors are localized in the mesonephros, and during cord formation, are localized in the migrating mesonephros cells [5, 9]. The TrkC receptor ligand NT3 is localized to testicular cords and is expressed by Sertoli cells [5, 9]. Blocking TrkC signaling by specific inhibitors inhibits cord formation [5], possibly through inhibiting mesonephros cell migration into the testis (unpublished observations). Neurotropins are involved in migration of other non-neuronal cell migration events through the PI3K signaling pathway [17, 40]. It is postulated that the PI3K signaling involved in mesonephros cell migration into the testis involves the inhibition of NT3 actions or TrkC signaling in the migrating mesonephros cells. Inhibition of cell migration and cord formation by LY294002 is a specific event in that inhibition of the MAP kinase signaling pathway had no significant effect on cord formation or cell migration.

Western blot analysis showed that phosphorylation of Akt in the embryonic testis organ culture was significantly inhibited by 10 μM LY294002. Akt is one of the main downstream signaling molecules in the PI3K pathway and mediates cellular responses such as cell survival [41]. Therefore, cell apoptosis was examined, and it was demonstrated that apoptotic cell number was not affected by LY294002 treatment. Western blot analysis also demonstrated that 10 μM PD98059 inhibited the MAP kinase signaling pathway in that the phosphorylation of ERK 1/2 was completely blocked. The Western blot data demonstrated that the inhibitors used in the current study blocked their respective signaling pathways in the organ cultures.

Mesonephros cell migration into the testis was found to require the PI3K signaling pathway. PI3K effects on cell motility involve several signaling molecules, including rac and Cdc42. Rac and Cdc42 are small GTP-binding proteins that have roles in cell mobility. Rac influences generation of lamellipodia/membrane ruffling [42], and Cdc42 stimulates formation of microspikes and filopodia [43]. It has been shown that PI3K regulates both rac and Cdc42 in their actions in cell mobility [44, 45]. Rac and Cdc42 are likely regulatory molecules involved in mesonephros cell migration into the embryonic testis.

PI3K signaling via Akt influences cell survival in numerous cell types [41]. Observations demonstrated that inhibition of the PI3K pathway by LY294002 did not significantly alter apoptotic cell number in the cultured testis. This indicates that decreased cell survival (e.g., cytotoxicity) was not the reason for lack of cord formation in LY294002-treated organs. The TUNEL assay also demonstrated that inhibition of the MAP kinase pathway by PD98059 did not significantly affect cell apoptosis. Apoptosis and cytotoxicity [46] do not appear to have significant roles at this stage of embryonic testis development.

In rodents and humans, Akt has three isoforms, Akt1, Akt2, and Akt3 [47]. While Akt1 and Akt2 are widely expressed, expression of Akt3 is more restricted and is primarily found in the brain and testis [47]. Deletion of Akt1 by homologous recombination causes growth retardation [48, 49], increased apoptosis in testis, and attenuation of spermatogenesis [49]. Although a significant number of Akt1 (−/−) pups die during the first 3 days of birth, surviving Akt1 (−/−) animals live into adulthood and are fertile [48]. Akt2-deficient mice show impaired ability to lower blood glucose by insulin in liver and skeletal muscle [50]. There is no information available on deletion of Akt3. LY294002, which reduced Akt phosphorylation dramatically, reduced cell migration and blocked cord formation. These observations suggest the actions of the PI3K inhibitor involve, in part, the downstream target Akt and its likely role in cell migration.

**FIG. 7.** Western blot analysis of phospho-ERK1/2 and phospho-Akt following LY294002 or PD98059 treatment. Western blot for phosphorylated Akt (phospho-Akt), total Akt protein (Akt), phosphorylated ERK1/2 (phospho-ERK1/2), and total ERK protein (ERK1/2) following treatment with LY294002 (LY) or PD98059 (PD). The data are representative of two separate experiments using 3–7 cultured E13 male gonad in each experiment.
be worthwhile in the future to examine whether casein kinase-2 is affected by LY294002 in E13 testis testis organ culture.

In summary, inhibition of the PI3K signal transduction pathway inhibited cord formation in a dose-dependent and developmental stage-specific manner. The inhibition was reversible and involved a reduction in mesonephros cell migration but not an increase in apoptotic cell number. Observations suggest that the PI3K signaling pathway is required for embryonic testis development through a mechanism involving mesonephros cell migration.

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