

## Signal transduction pathways in FSH regulation of rat Sertoli cell proliferation

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**Riera MF, Regueira M, Galardo MN, Pellizzari EH, Meroni SB, Cigorraga SB.** Signal transduction pathways in FSH regulation of rat Sertoli cell proliferation. *Am J Physiol Endocrinol Metab* 302: E914–E923, 2012. First published January 24, 2012; doi:10.1152/ajpendo.00477.2011.—The final number of Sertoli cells reached during the proliferative periods determines sperm production capacity in adulthood. It is well known that FSH is the major Sertoli cell mitogen; however, little is known about the signal transduction pathways that regulate the proliferation of Sertoli cells. The hypothesis of this investigation was that FSH regulates proliferation through a PI3K/Akt/mTORC1 pathway, and additionally, AMPK-dependent mechanisms counteract FSH proliferative effects. The present study was performed in 8-day-old rat Sertoli cell cultures. The results presented herein show that FSH, in addition to increasing p-Akt, p-mTOR, and p-p70S6K levels, increases p-PRAS40 levels, probably contributing to improving mTORC1 signaling. Furthermore, the decrease in FSH-stimulated p-Akt, p-mTOR, p-p70S6K, and p-PRAS40 levels in the presence of wortmannin emphasizes the participation of PI3K in FSH signaling. Additionally, the inhibition of FSH-stimulated Sertoli cell proliferation by the effect of wortmannin and rapamycin point to the relevance of the PI3K/Akt/mTORC1 signaling pathway in the mitotic activity of FSH. On the other hand, by activating AMPK, several interesting observations were made. Activation of AMPK produced an increase in Raptor phosphorylation, a decrease in p70S6K phosphorylation, and a decrease in FSH-stimulated Sertoli cell proliferation. The decrease in FSH-stimulated cell proliferation was accompanied by an increased expression of the cyclin-dependent kinase inhibitors (CDKIs) p19INK4d, p21Cip1, and p27Kip1. In summary, it is concluded that FSH regulates Sertoli cell proliferation with the participation of a PI3K/Akt/mTORC1 pathway and that AMPK activation may be involved in the detention of proliferation by, at least in part, a decrease in mTORC1 signaling and an increase in CDKI expression.

follicle-stimulating hormone; phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin complex 1; adenosine 5'-monophosphate-activated protein kinase

SERTOLI CELLS provide the structural and nutritional support for germ cell development. Considering that each Sertoli cell is able to support a limited number of germ cells, the final number of Sertoli cells reached during the proliferative periods determines sperm production capacity in adulthood. In the rat, fetal and postnatal periods up to 15 days of age constitute the time period for Sertoli cell mitosis. Around 15 to 18 days of age the blood-testis barrier is formed, and Sertoli cells keep on differentiating to attain the ability to sustain full spermatogenesis. As for the hormonal regulation

of Sertoli cell function, both follicle-stimulating hormone (FSH) and androgens are essential in adulthood. Whereas androgen actions are mostly recognizable by the presence of receptors at puberty (40), FSH actions can be demonstrated during fetal and the whole postnatal life (44). FSH regulates Sertoli cell proliferation during fetal and early postnatal life and differentiation after cessation of mitosis at puberty. This variation in the biological action of FSH led to the search for additional mechanisms that may be involved in the detention of proliferation at puberty (3, 6).

Several signal transduction pathways have been associated with proliferation in different cell types. Among them, the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin complex 1 (mTORC1)-dependent pathway has been mentioned in several cell types (11). PI3K is activated as a result of the activation of tyrosine kinase receptors, G protein-coupled receptors, and integrins. The best-characterized downstream target of PI3K is the kinase Akt, whose activation results in the consequent phosphorylation of a host of other proteins that affect cell growth and entry into cell cycle, such as the serine-threonine kinase mTOR. mTOR does not function in isolation but as part of a complex named mTORC1 that contains other proteins with the ability to regulate downstream responses. Five components have been identified so far in mTORC1: mTOR, mLST8/GβL, Raptor, PRAS40, and DEPTOR (28, 38, 41, 42). mTOR protein, described in relation to the property of the antibiotic rapamycin to inhibit its activity, is the best studied of these proteins. mLST8/GβL is a protein whose biological function in mTORC1 has not yet been identified. Raptor acts as a scaffold to recruit downstream substrates such as 4E-binding protein (4E-BP1) and ribosomal S6 kinase (p70S6K) to mTORC1 (37, 43). When Raptor is phosphorylated, it impairs TORC1 signaling pathway (16). PRAS40 also impairs TORC1 signaling pathway when this protein is not phosphorylated. PRAS40 is a substrate of Akt, and it has been shown that upon phosphorylation by the latter kinase, PRAS40 no longer inhibits mTORC1 signaling (42, 47). Finally, DEPTOR is a constituent of mTORC1 and is considered a negative regulator of mTOR function (24). DEPTOR is phosphorylated and quickly degraded via the ubiquitin-proteasome system pathway (38). Two well-known downstream targets of mTORC1 are p70S6K and 4E-BP1, which once phosphorylated promote the initiation of translation of proteins involved in cell growth and cell cycle progression such as c-Myc and cyclin D1 (2, 15, 48). The protooncogene c-Myc plays an important role in the regulation of cellular proliferation and differentiation. Numerous studies have documented the growth-regulated accumulation of c-Myc mRNA (1, 4, 32), which may result from an increase in c-Myc gene

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transcription and/or an increase in c-Myc mRNA stability (23, 32). In Sertoli cells, it was demonstrated that FSH increases c-Myc mRNA levels in prepubertal and early pubertal rats, and it was suggested that this FSH-dependent regulation of c-Myc is related to the regulation of the Sertoli cell cycle (31). Although it has been shown previously that FSH activates mTOR/p70S6K through a PI3K-dependent pathway (35), the possible participation of the PI3K/Akt/mTORC1-dependent pathway in the mechanism of action utilized by FSH to regulate Sertoli cell proliferation and c-Myc mRNA levels has not been studied.

One of the most intriguing questions in Sertoli cell biology is referred to the signaling pathways that may counteract the proliferating signals from FSH (9). The AMP-activated protein kinase (AMPK), a serine/threonine protein kinase that has been involved in sensing cell energy levels, has been associated with the suppression of cell proliferation (18). In this context, pharmacological activation of AMPK, by means of the AMP-mimetic 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR), has been shown to cause cell cycle arrest in hepatoma HepG2 cells, mouse embryonic fibroblasts, and human aortic smooth muscle cells (20, 21, 22). The above-mentioned reports postulated that the mechanism of cell cycle arrest by AMPK activation involves accumulation of the tumor suppressor protein p53 with the consequent upregulation of p21Cip protein, one of the widely expressed cyclin-dependent kinase inhibitors (CDKIs). It has been shown that detention of Sertoli cell proliferation is accompanied by increased expression of some CDKIs belonging to the Cip/Kip family (3). The participation of AMPK and of other CDKIs in Sertoli cell cycle arrest has not been analyzed.

In this study we investigate 1) the regulation by FSH of the PI3K/Akt/mTORC1 pathway and its participation in the stimulation of Sertoli cell proliferation and c-Myc expression and 2) a possible role of AMPK activation in Sertoli cell proliferation and in the expression of the CDKIs p19INK4d, p21Cip1, and p27Kip1.

## MATERIALS AND METHODS

**Materials.** Tissue culture media were purchased from Gibco BRL (Life Technologies, Rockville, MD). Ovine FSH (NIH-oFSH-S-16) was obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD. A-769662 was kindly provided by Dr. D. G. Hardie (University of Dundee, Dundee, UK). AICAR was purchased from Calbiochem (San Diego, CA). Methyl- $^3$ H]thymidine (NET027A; 2 Ci/mmol) and  $\alpha$ - $^{32}$ P-deoxy-CTP (BLU513H; 3000Ci/mmol) were purchased from NEN (PerkinElmer, San Jose, CA). H89 (PKA inhibitor) and wortmannin (W; PI3K inhibitor) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Antibodies and rapamycin (R; mTOR inhibitor) were obtained from Cell Signaling Technology (New England Biolabs). All other drugs were purchased from Sigma-Aldrich (St. Louis, MO).

**Sertoli cell isolation and culture.** Sertoli cells from 8-day-old Sprague-Dawley rats were isolated as described previously (10). Animals were housed and used according to the guidelines recommended by the National Institutes of Health; our experiments were approved by the Institutional Ethics Committee at Hospital de Niños R. Gutiérrez. Animals were euthanized by asphyxiation with CO<sub>2</sub> and decapitated. Briefly, testes were removed, decapsulated, and incubated in culture medium containing 0.03% collagenase and 0.003%

soybean trypsin inhibitor for 5 min at room temperature. Culture media consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium supplemented with 0.1% BSA, 100 IU/ml penicillin, 2.5  $\mu$ g/ml amphotericin B, and 1.2 mg/ml sodium bicarbonate. After the initial dispersion, seminiferous tubules were sedimented, and supernatant was discarded to remove interstitial cells. Seminiferous tubules were submitted to 1 M glycine-2 mM EDTA (pH 7.4) treatment to remove peritubular cells. After several washes, a second collagenase treatment was performed. Tubules were treated for 10 min at room temperature with a solution of 0.03% collagenase and 0.003% soybean trypsin inhibitor and 0.03% DNase. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium described above with the following additions: 10  $\mu$ g/ml transferrin, 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml vitamin E, and 4 ng/ml hydrocortisone. Sertoli cells were cultured in six- or 24-multiwell plates (15  $\mu$ g DNA/cm<sup>2</sup>) at 34°C in a mixture of 5% CO<sub>2</sub>-95% air.

**Culture conditions.** Sertoli cells were allowed to attach for 48 h in culture medium mentioned above. A medium change was performed at this time (*day 3*) with fresh medium without insulin. As indicated in the figure legends, treatments with 100 ng/ml FSH in the absence or presence of different doses of H89, W, and R, 1 mM A769662, 1 mM AICAR, or a combination of FSH and A769662 or AICAR, were started on *day 4*. Cells harvested on *day 5* were used to evaluate thymidine and bromodeoxyuridine (BrdU) incorporation, to perform cell proliferation assay (MTS), to count cell number, or to perform Northern blot analysis. For Western blot analysis, cells maintained under basal conditions until *day 4* and treated as indicated in the figure legends were used.

**Cell extracts and Western blot analysis.** Sertoli cells cultured in six-multiwell plates were washed once with PBS at room temperature. Then, 200  $\mu$ l of PBS containing 2  $\mu$ l of protease inhibitor cocktail (P-8340; Sigma-Aldrich), 1 mM NaF, 1 mM EGTA, 1 mM EDTA, 50 nM okadaic acid, and 2 mM PMSF was added to each well. Cells were then placed on ice and disrupted by ultrasonic irradiation. A 200- $\mu$ l volume of 2 $\times$  Laemmli buffer (4% wt/vol SDS, 20% vol/vol glycerol, 10% vol/vol 2-mercaptoethanol, 0.004% wt/vol bromophenol blue, and 0.125 M Tris-HCl, pH 6.8) was added and thoroughly mixed (29). Samples were immersed in a boiling water bath for 5 min and then immediately settled on ice. Proteins were resolved in 5 or 10% SDS-PAGE (5 or 10% acrylamide-bisacrylamide for the resolving gel and 4.3% acrylamide-bisacrylamide for the stacking gel) in a Mini Protean 3 Cell (Bio-Rad, Hercules, CA). After SDS-PAGE, gels were equilibrated in transfer buffer for 10 min and electrotransferred at 100 V for 60 min onto polyvinylidene difluoride membranes (Hybond-P; Amersham Pharmacia Biotech) using a Mini Trans-Blot Cell (Bio-Rad). Membranes were probed with commercial antibodies that allow specific recognition of the phosphorylated forms of Akt (Ser<sup>473</sup>), p70S6K (Thr<sup>389</sup>), mTOR (Ser<sup>2448</sup>), PRAS40 (Thr<sup>246</sup>), Raptor (Ser<sup>792</sup>), AMPK (Thr<sup>172</sup>), and acetyl-CoA carboxylase (ACC; Ser<sup>79</sup>) or with antibodies that recognized the nonphosphorylated form of these proteins. To evaluate apoptosis, antibodies that recognize cleaved caspase-3 (Asp<sup>175</sup>) and  $\beta$ -actin were used (Cell Signaling Technology, Danvers, MA); 1:1,000 dilutions of primary antibodies were used. The intensities of the autoradiographic bands were estimated by densitometric scanning using NIH Image (Scion, Frederick, MD) software.

**Thymidine incorporation assay.** Sertoli cell monolayers were incubated with 1  $\mu$ Ci/ml methyl- $^3$ H]thymidine (NET027A; 2 Ci/mmol) for 18 h. After this incubation period, monolayers were washed, harvested with Tripsin-EDTA, and centrifuged for 2 min at 400 g. The cell pellet was resuspended in PBS and precipitated with 10% TCA on ice. The suspension was allowed to stand for 30 min and then passed through Whatman GF/C filters. Filters were washed twice with cold 10% TCA. The radioactivity retained in the filter was determined in a liquid scintillation spectrophotometer.

**BrdU incorporation.** Sertoli cell monolayers were incubated with BrdU (200  $\mu$ M) for 24 h on *day 4* in culture. After the incubation period, cultured cells were fixed with 4% paraformaldehyde for 10 min and permeabilized by microwave irradiation. For BrdU incorporation assay, the slides were incubated for 30 min with DNase (45 U/ $\mu$ l) at 37°C to break DNA strands and to allow anti-BrdU antibody to interact with incorporated BrdU. Then, cells were blocked for 90 min with 10% horse serum in PBS-0.2% Triton and then incubated with mouse anti-BrdU antibody (1:200) overnight. The next day, slides were incubated with a FITC-conjugated anti-mouse secondary antibody (1:200). Control slides were incubated with normal serum instead of primary antibodies. Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing 4,6-diamidino-2-phenylindole dihydrochloride for DNA staining and visualized in a fluorescent light microscope (Axiophot; Carl Zeiss, Jena, Germany). The percentage proliferating (BrdU-positive) Sertoli cells was calculated as (BrdU-positive cells/total Sertoli cells)  $\times$  100.

**MTS assay.** A commercial kit, Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), was used.

**Cell count assay.** The monolayers were washed twice with PBS, and then cells were recovered by trypsinization. Cell suspension was then centrifuged for 5 min at 400 g, and the cell pellet was recovered. Cells were resuspended in fresh medium, and 0.4% trypan Blue was added. Cells that excluded the colorant (viable) and those that were stained (nonviable) were counted in a Neubauer chamber.

**Northern blot analysis.** Total RNA was extracted from Sertoli cells cultured in six-multiwell plates by the guanidinium isothiocyanate method (5). The amount of RNA was estimated by spectrophotometry at 260 nm. About 10  $\mu$ g of total RNA was electrophoresed on a 1% agarose-10% formaldehyde gel. After migration, RNAs were transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, Buenos Aires, Argentina) by capillary transfer with  $10\times$  SSC (1.5 M NaCl and 0.15 M sodium citrate, pH 7.4) and fixed with UV Stratalinker (Stratagene Cloning Systems, La Jolla, CA). cDNA probes (mouse p19INK4d, 0.5 kb, *Bam*HI-*Eco*RI insert, rat c-Myc, and 18S oligonucleotide) were labeled with [ $\alpha$ - $^{32}$ P]deoxy-CTP using a random-primed labeling kit (Prime-a-Gene Labeling System; Promega). Blots were prehybridized for 3 h at 42°C in 50% formamide, 0.75 M NaCl, 20 mM sodium phosphate (pH 7.5), 1 mM EDTA,  $5\times$  Denhart's solution, 10% dextran sulfate, 0.5% SDS, and 100  $\mu$ g/ml herring sperm DNA. Hybridization was then performed overnight at 42°C in the same hybridization buffer containing  $1-4 \times 10^6$  counts $\cdot$ min $^{-1}$  $\cdot$ ml $^{-1}$   $^{32}$ P-labeled probe. Membranes were washed utilizing different stringency conditions depending on the probe utilized. Membranes were exposed to Kodak X-Omat S films (Eastman Kodak, Rochester, NY). The intensities of the autoradiographic bands were estimated by densitometric scanning using NIH Image (Scion, Frederick, MD) software. The 18S signal was used to standardize mRNA content.

**Real-time PCR.** Total RNA was isolated from Sertoli cells cultured in six-multiwell plates using the Trizol Reagent (Invitrogen, Carlsbad, CA). The amount of RNA was estimated by spectrophotometry at 260 nm. Reverse transcription was performed on 2  $\mu$ g of RNA at 42°C for 50 min using 200 U of SuperScript II reverse transcriptase enzyme (Invitrogen) containing 125 ng of random primer and 0.5 mM dNTP Mix. Real-time PCR was performed using the Step One Real-Time PCR System (Applied Biosystems, Warrington, UK). The specific primers for real-time PCR are 5'-gTCTTgCACTCTggTgTCTCA-3' and 5'-gCACTTCAgggCTTCTCTT-3' for p21, 5'-TTCgACgCCA-gACgTAAAC-3' and 5'-TTCAATggAgTCAgCgATATg-3' for p27, and 5'-TggCACCACACTTTCTACAAT-3' and 5'-ggTACgACCA-gAggCATACA-3' for  $\beta$ -actin. Amplification was carried out as recommended by the manufacturer: 25- $\mu$ l reaction mixture contained 12.5  $\mu$ l of SYBR Green PCR Master mix (Applied Biosystems), the

appropriate primer concentration, and 1  $\mu$ l of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The data were normalized to  $\beta$ -actin. The amplification program included the initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The comparative  $\Delta\Delta C_T$  method was used to calculate relative gene expression.

**Statistical analysis.** All experiments were run in triplicate and repeated three times. Results were expressed as means  $\pm$  SD. One-way ANOVA followed by Tukey-Kramer test using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA) was performed. Probabilities of  $<0.05$  were considered statistically significant.

## RESULTS

**FSH regulates Sertoli cell proliferation in a PI3K/Akt/mTORC1-dependent manner.** To analyze whether FSH modulates the PI3K/Akt/mTORC1 signal transduction pathway in 8-day-old Sertoli cells, the following set of experiments were performed. Sertoli cell cultures were stimulated for variable periods of time (15, 30, and 60 min) with 100 ng/ml FSH, a concentration that has been shown previously to elicit maximal biological response (34). The levels of phosphorylated Akt (p-Akt), phosphorylated mTOR (p-mTOR), phosphorylated PRAS40 (p-PRAS40), and phosphorylated p70S6K (p-p70S6K) were analyzed by Western blot. Figure 1A shows that FSH produced a time-dependent increase in p-Akt, p-mTOR, p-PRAS40 and p-p70S6K levels. By pooling data obtained in three independent experiments, the following results were obtained: p-Akt  $3.2 \pm 0.2$ , p-PRAS40  $1.8 \pm 0.4$ , p-mTOR  $1.7 \pm 0.2$ , and p-p70S6K  $3.5 \pm 0.9$  (means  $\pm$  SD,  $P < 0.05$  vs. basal) fold stimulation at the times when maximal values were observed (p-Akt: 15 min; p-PRAS40: 30 min; p-mTOR and p-p70S6K: 60 min).

We next examined the participation of PI3K in the regulation of the stimulatory effects of FSH on the Akt/mTORC1 pathway. For this purpose, we analyzed the effect of the inhibitor wortmannin on the observed effects of FSH on p-Akt, p-mTOR, p-PRAS40 and p-p70S6K levels. Cells were preincubated for 15 min with the inhibitor and then stimulated with 100 ng/ml FSH for periods of time where maximal stimulation had been observed. Figure 1B shows that wortmannin decreased the ability of FSH to increase the levels of p-Akt, p-mTOR, p-PRAS40, and p-p70S6K.

On the other hand, Fig. 2 shows the participation of different signal transduction pathways in Sertoli cell proliferation. Figure 2A shows that, as expected, H89, a PKA inhibitor, inhibited FSH-stimulated [ $^3$ H]thymidine incorporation. Figure 2A also shows that W, a PI3K inhibitor, and R, a mTOR inhibitor, decreased FSH-stimulated [ $^3$ H]thymidine incorporation. Proliferation was also evaluated by BrdU incorporation into DNA. Figure 2, B and C, shows that W and R also inhibited FSH-stimulated BrdU incorporation.

As mentioned in the INTRODUCTION, FSH increases c-Myc mRNA levels in prepubertal and early pubertal rats. It has been suggested that this FSH-dependent regulation of c-Myc is related to the regulation of the Sertoli cell cycle, although the signal transduction pathways participating in these biological effects have not been evaluated. Considering that a

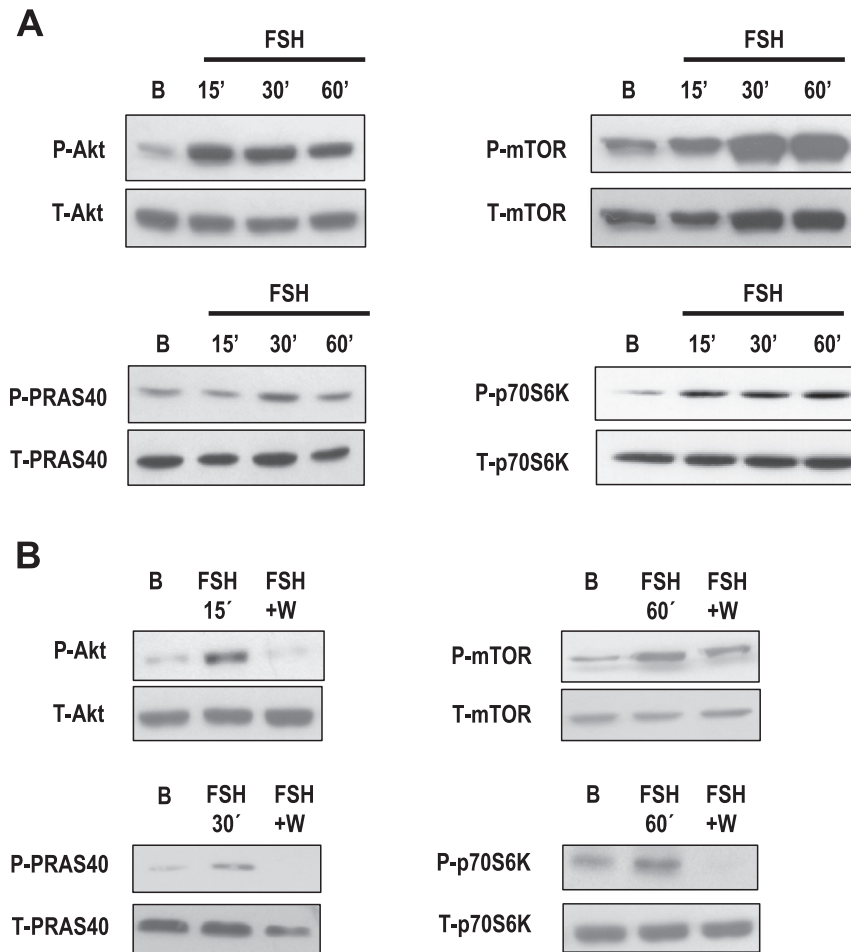


Fig. 1. FSH regulates phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) complex 1 (mTORC1) pathway in Sertoli cells. *A*: Sertoli cell monolayers were incubated under basal (B) conditions or stimulated with 100 ng/ml FSH for 15, 30, or 60 min. Western blot analysis was performed utilizing antibodies for phosphorylated Akt (p-Akt), total Akt (t-Akt), p-mTOR, t-mTOR, p-PRAS40, t-PRAS40, p-p70S6K (ribosomal S6 kinase), or t-p70S6K. *B*: Sertoli cell monolayers were incubated under B conditions or stimulated with 100 ng/ml FSH in the absence or presence of 100 nM wortmannin (W) for those periods of time where maximal stimulation had been observed. Western blot analysis was performed utilizing the antibodies described above. Results are representative of 3 independent experiments/treatment group.

PI3K/Akt-dependent pathway is apparently involved in FSH-stimulated Sertoli cell proliferation, we decided to analyze a possible participation of a PI3K/Akt-dependent pathway on FSH-stimulated c-Myc mRNA levels. Figure 3A shows that FSH increased c-Myc mRNA levels in a time-dependent manner, and Fig. 3B shows that W decreased this stimulation.

*AMPK activation regulates PI3K/Akt/mTORC1-dependent pathway in Sertoli cells.* Taking into account that AMPK activation has been shown to induce cessation of proliferation in several cell types and that the PI3K/Akt/mTORC1 signaling pathway is involved in Sertoli cell proliferation, we decided to analyze whether the PI3K/Akt/mTORC1 signaling pathway is affected by AMPK activation in these cells. To achieve this goal, cultures were treated with the compound A-769662 (A76), a cell-permeable activator of AMPK that has been shown to regulate this kinase both in vivo and in vitro (7, 12). Figure 4A shows that treatment with A76 increased phosphorylated levels of AMPK (p-AMPK) and of acetyl-CoA carboxylase (p-ACC), a downstream target of AMPK. We then analyzed whether the PI3K/Akt/mTORC1 signal transduction pathway was regulated under conditions of AMPK activation. Figure 4B shows that A76 did not modify p-Akt or p-mTOR levels, increased p-Raptor levels, and decreased p-p70S6K levels. By pooling data obtained in three independent experiments, the following results were obtained:

p-Akt  $1.1 \pm 0.1$ , p-mTOR  $0.8 \pm 0.2$ , p-Raptor  $1.8 \pm 0.2$ , and p-p70S6K  $0.3 \pm 0.2$  (means  $\pm$  SD,  $P < 0.05$  vs. basal) fold variation at the times when maximal variations were observed (p-Raptor: 30 min; p-Akt, p-mTOR, and p-p70S6K: 60 min). These changes in the levels of phosphorylated proteins in conditions of AMPK activation are consistent with an inhibition of the mTORC1 activity.

*Interplay between FSH and AMPK activation in the regulation of Sertoli cell proliferation.* Considering that FSH stimulates PI3K/Akt/mTORC1 and cell proliferation and that AMPK activation induces inhibition of mTORC1 activity, a relationship between FSH biological effects and the state of activation of AMPK was postulated. To evaluate this hypothesis, the ability of FSH to regulate AMPK signaling was explored. Sertoli cell cultures were stimulated for variable periods of time (15, 30, and 60 min) with 100 ng/ml FSH, and the levels of p-AMPK and p-ACC were evaluated. Figure 5A shows that FSH decreased AMPK activity in a time-dependent manner. The latter assertion is based on the decreased levels of p-AMPK and p-ACC observed. A possible inhibition of FSH-stimulated Sertoli cell proliferation by AMPK activation was then examined. Considering that FSH inhibited AMPK activation, it was important to evaluate whether A76 and AICAR were able to stimulate AMPK under FSH challenge. Figure 5B shows that the combination of A76 or AICAR with FSH for 60 min resulted in an increase in p-ACC levels, indicating that activation of AMPK was occurring in the presence of FSH. A

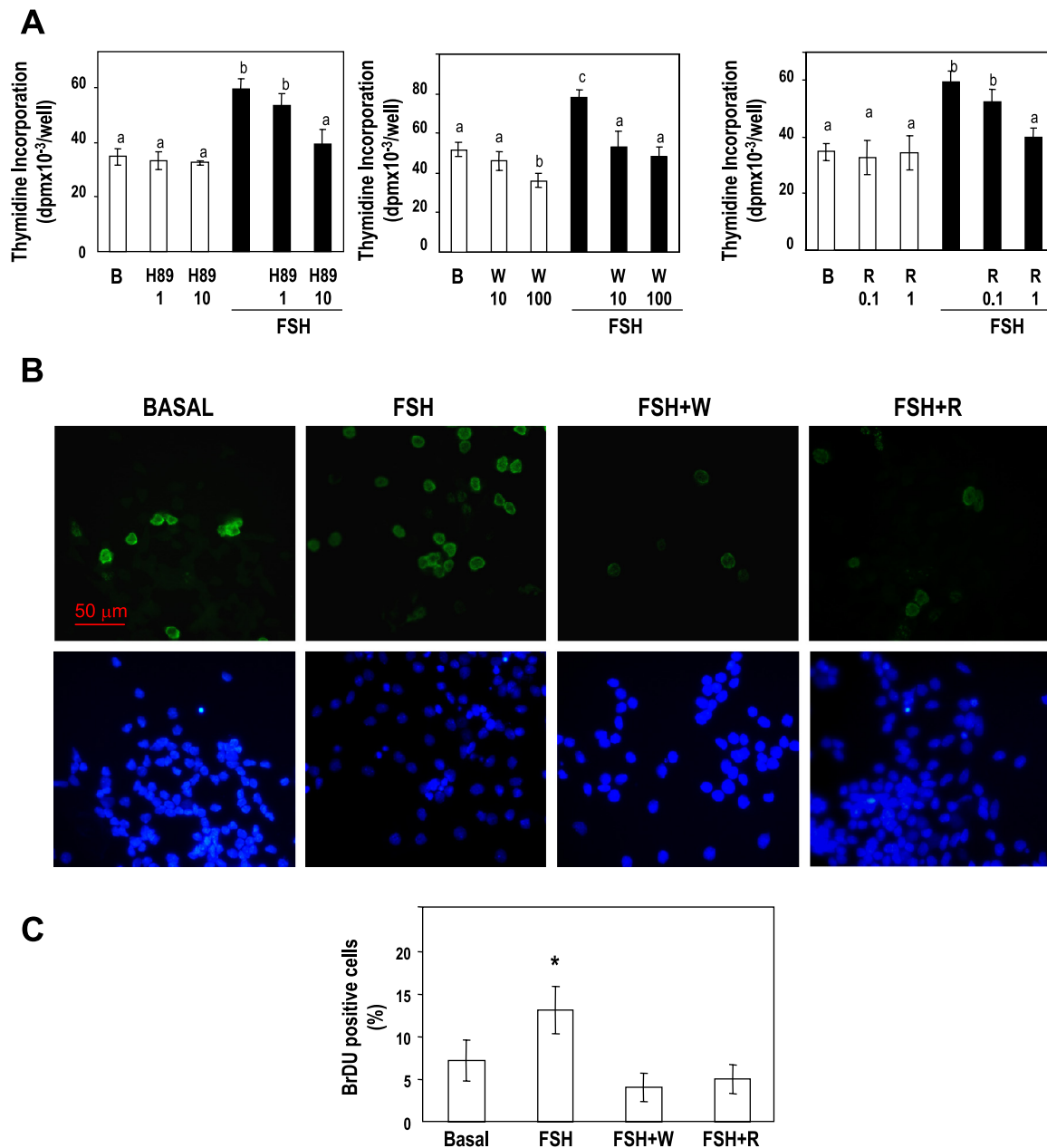


Fig. 2. FSH regulates Sertoli cell proliferation in a PI3K/Akt/mTORC1-dependent manner. *A*: Sertoli cell monolayers were incubated under B conditions or stimulated for 24 h with FSH (100 ng/ml) in the absence or presence of H89 (1 and 10  $\mu$ M), W (10 and 100 nM), and rapamycin (R; 0.1 and 1 nM). At the end of the culture period, [ $^3$ H]thymidine incorporation was determined. Results are presented as means  $\pm$  SD of triplicate incubations in 1 representative experiment out of 3. Different letters indicate statistically significant differences ( $P < 0.05$ ). *B*: Sertoli cell monolayers were incubated under B conditions or stimulated for 24 h with FSH (100 ng/ml) in the absence or presence of W (100 nM) and R (1 nM). Bromodeoxyuridine (BrdU) incorporation was evaluated by fluorescence microscopy. Representative images of Sertoli cells showing immunoreactivity for BrdU (green) counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (blue). Scale bar, 50  $\mu$ m. *C*: each bar represents %BrdU-positive cells (1,000 cells/group). Results are presented as means  $\pm$  SD of triplicate incubations in 1 representative experiment out of 3. \* $P < 0.05$ .

thymidine incorporation assay could not be used to evaluate cell proliferation because AICAR competes with thymidine for the nucleoside transporter. For this reason, Sertoli cell proliferation in the presence of AICAR or A76 was evaluated by counting cells in a Neubauer chamber or by the use of a MTS proliferation assay. Sertoli cell monolayers were incubated for 24 h with 100 ng/ml FSH in the absence or presence of 1 mM A76 or AICAR. Figure 6, *A* and *B*, shows that AMPK activation decreased FSH-stimulated Sertoli cell proliferation. Fur-

thermore, Fig. 6*C* shows that FSH-stimulated BrdU incorporation was also inhibited in the presence of A76.

AICAR and A76 did not modify cell viability in 24-h incubations, as evaluated by a trypan blue exclusion test (FSH:  $23 \pm 4$ ; FSH + A76:  $27 \pm 4$ ; FSH + AICAR:  $21 \pm 3$ , no. of trypan blue stained cells  $\times 10^{-3}$ /well). In addition, Fig. 6*D* shows that treatment of Sertoli cell cultures with A76 or AICAR did not induce cleavage of caspase-3, indicating that these drugs did not induce apoptosis.

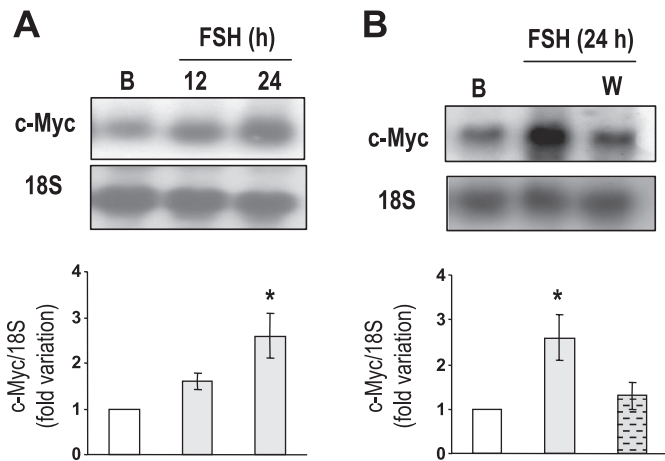


Fig. 3. FSH regulates c-Myc expression in a PI3K-dependent manner. *A*: Sertoli cell monolayers were incubated under B conditions or stimulated for 12 or 24 h with 100 ng/ml FSH. *B*: Sertoli cell monolayers were incubated under B conditions or stimulated for 24 h with 100 ng/ml FSH in the absence or presence of W (100 nM). Northern blot analysis was performed. Membranes were hybridized with labeled cDNA probes for c-Myc and 18S. *Top*: 1 representative experiment out of 3. *Bottom*: pooled data of 3 independent experiments, indicating fold variation in c-Myc mRNA levels (ratio c-Myc mRNA to 18S in each sample) relative to basal. Results are expressed as means  $\pm$  SD, \* $P$  < 0.05 vs basal.

AMPK activation regulates Sertoli cell p19INK4d, p21Cip1, and p27Kip1 mRNA levels. Considering that AMPK activation decreased cell proliferation and that the expression of CDKIs may participate in the cessation of Sertoli cell

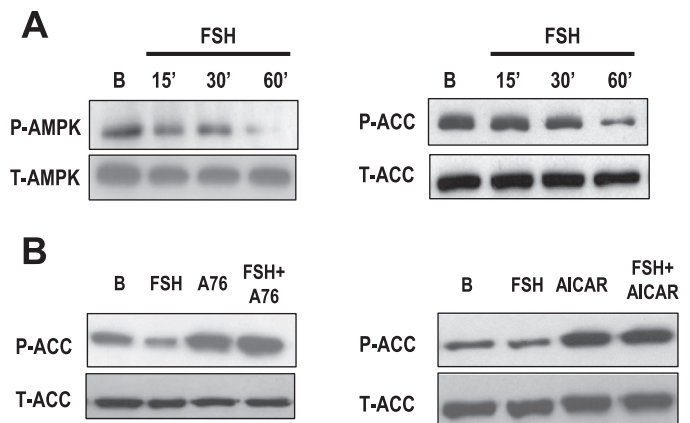


Fig. 5. FSH regulates AMPK activity in Sertoli cells. *A*: Sertoli cell monolayers were incubated under B conditions or stimulated with FSH (100 ng/ml) for 15, 30, or 60 min. Western blot analysis was performed utilizing antibodies for p-AMPK, t-AMPK, p-ACC, or t-ACC. *B*: Sertoli cell monolayers were incubated under B or FSH-stimulated conditions for 60 min in the presence or absence of A76 (1 mM; *left*) or 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribose (AICAR) (1 mM; *right*). Western blot analysis was performed utilizing antibodies for p-ACC or t-ACC. Results are representative of 3 independent experiments/treatment group.

proliferation, we decided to analyze a possible regulation by AMPK activation of the mRNA levels of cell cycle inhibitors p19INK4d (CDKI not studied previously in these cells), p21Cip1, and p27Kip1. Sertoli cell monolayers were incubated under basal or FSH-stimulated conditions in the absence or presence of A76 (1 mM) for 24 h. Figure 7, *A* and *B*, shows that AMPK activation significantly increased p19INK4d and p21Cip1 mRNA levels in FSH-treated cultures. Figure 7*C* shows that FSH treatment decreased p27Kip1 levels and that AMPK activation reversed this inhibition.

DISCUSSION

Sertoli cell mitotic capacity declines during the neonatal period and ceases by about postnatal day 15. Each Sertoli cell supports a fixed number of germ cells, and consequently, Sertoli cell number is the major factor that establishes the magnitude of sperm production. Deciphering the factors that regulate Sertoli cell proliferation and establishment of an adequate adult cell population constitutes one of the most critical questions in testicular biology. It is well known that FSH is the major Sertoli cell mitogen; however, little is known about the signal transduction pathways that participate in the proliferation of Sertoli cells. In this context, Griswold et al. (13) have shown that FSH or dbcAMP stimulates the incorporation of [<sup>3</sup>H]thymidine into DNA of cultured immature Sertoli cells, indicating the participation of a cAMP pathway in proliferation. Crépieux et al. (8) showed that FSH induces p-ERK levels and that activation of this kinase is related to Sertoli cell proliferation. In this investigation, we pursued the search for mechanisms participating in the regulation of Sertoli cell proliferation.

mTORC1 has been involved in the proliferation of several cell types. The activity of this complex is regulated by a wide variety of cellular signals, including mitogenic growth factors, hormones, nutrients, energy levels, and stress con-

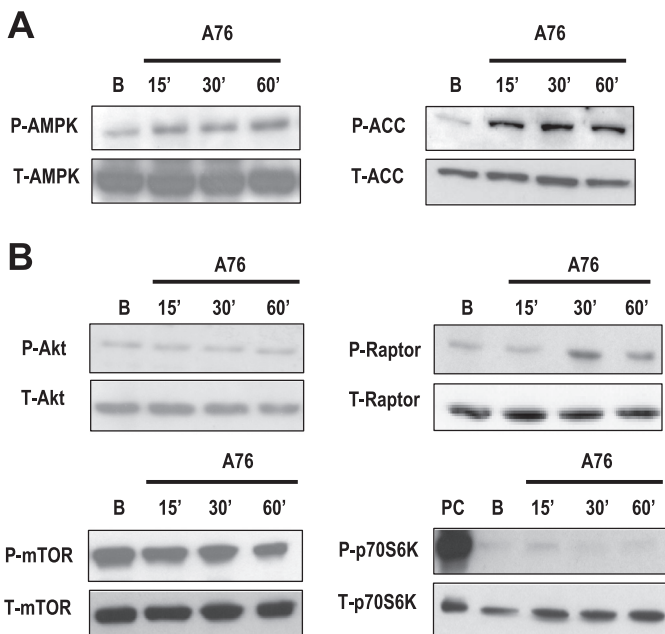
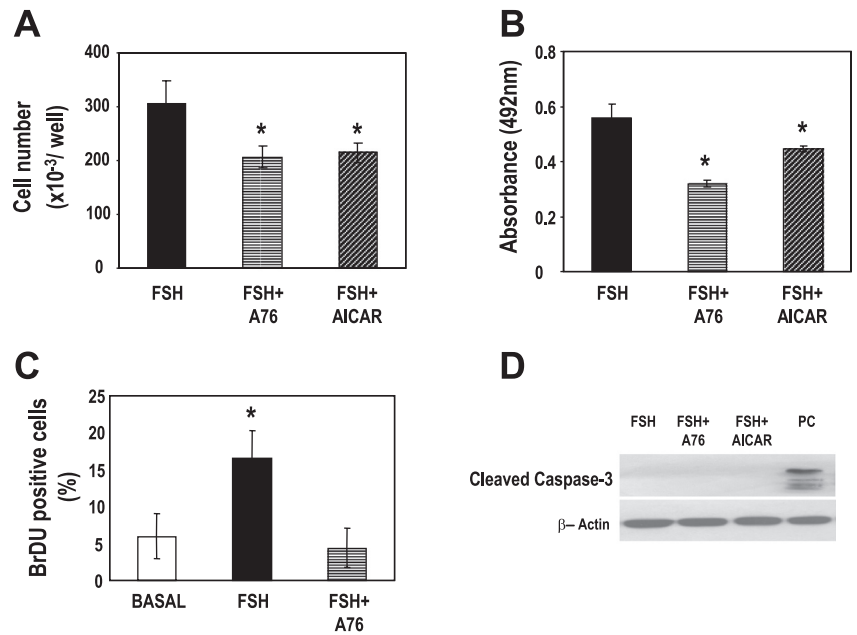


Fig. 4. A-769662 (A76) regulates AMP-activated protein kinase (AMPK) activity and PI3K/Akt/mTOR pathway in Sertoli cells. *A*: Sertoli cell monolayers were incubated under B conditions or stimulated with A76 (1 mM) for 15, 30, or 60 min. Western blot analysis was performed utilizing antibodies for p-AMPK, t-AMPK, p-ACC (acetyl-CoA carboxylase), and t-ACC. *B*: Sertoli cell monolayers were incubated under B conditions or stimulated with A76 (1 mM) for 15, 30, or 60 min. Western blot analysis was performed utilizing antibodies for p-Akt, t-Akt, p-Raptor, t-Raptor, p-mTOR, t-mTOR, p-p70S6K, and t-p70S6K. Results are representative of 3 independent experiments/treatment group. PC, positive control for p-p70S6K provided by Cell Signaling Technology.

Fig. 6. AMPK activation regulates FSH-stimulated proliferation of Sertoli cells. Sertoli cell monolayers were incubated for 24 h with FSH (100 ng/ml) in the absence or presence of 1 mM A76 or 1 mM AICAR. The number of Sertoli cells was estimated by counting viable cells in a Neubauer chamber (A) or by the use of an MTS assay (B). Results represent means  $\pm$  SD of triplicate incubations in 1 representative experiment out of 3 ( $*P < 0.05$  vs FSH). C: Sertoli cell monolayers were incubated under B conditions or stimulated for 24 h with FSH (100 ng/ml) in the absence or presence of 1 mM A76. BrdU incorporation was evaluated by fluorescence microscopy. Each bar represents %BrdU-positive cells (1,000 cells/group). Results represent means  $\pm$  SD of triplicate incubations in 1 representative experiment out of 3 ( $*P < 0.05$  vs basal). D: Sertoli cell monolayers were incubated for 24 h with FSH (100 ng/ml) in the absence or presence of 1 mM A76 or 1 mM AICAR. Western blot analysis was performed utilizing antibodies for cleaved caspase-3 or  $\beta$ -actin (Jurkat cells treated with 1  $\mu$ M staurosporine). The experiment was repeated twice.



ditions. One of the most important pathways that signal through mTORC1 is the PI3K/Akt signal transduction pathway, which is critically involved in the mediation of cell survival and proliferation. In this context, FSH signaling through the PI3K/Akt pathway has been shown previously in Sertoli cells (27, 34). As mentioned in the INTRODUCTION, mTORC1 is composed of five known constituents, mTOR, mLST8/Gbl, PRAS40, Raptor, and DEPTOR, and its activity can be regulated by at least phosphorylation of mTOR, PRAS40, Raptor, and DEPTOR. Activation of mTORC1 results in phosphorylation of several downstream targets such as p70S6K. In proliferating Sertoli cells, Musnier et al. (35) demonstrated that the main pathway required for FSH to stimulate p70S6K is the PI3K/Akt pathway. Recent observations from two independent laboratories have demonstrated that Grb10, the growth factor/receptor-bound protein 10, is also phosphorylated by mTORC1 and as a result is stabilized, which leads to inhibition of the PI3K/Akt pathway (19, 50). The results presented herein show that FSH, in addition to increasing p-Akt, p-mTOR, and p-p70S6K levels, raises the levels of phosphorylation of PRAS40, which may also contribute to improve mTORC1 signaling. Furthermore, the decrease in p-Akt, p-mTOR, p-p70S6K, and p-PRAS40 levels in the presence of wortmannin reinforces the participation of PI3K in FSH signaling. Additionally, the inhibition of FSH-stimulated Sertoli cell proliferation by wortmannin and rapamycin, the inhibitors of PI3K and mTOR respectively, indicates the relevance of the PI3K/Akt/mTORC1 signaling pathway in the mitotic activity of FSH.

As mentioned before, Crépieux et al. (8) showed that FSH induces p-ERK levels. These authors also showed ERK-related expression of cyclin D1, which is associated with cell proliferation. Additional mechanisms that may be involved in FSH-regulated Sertoli cell proliferation have been proposed. Lim and Hwang (31) showed that FSH increases c-Myc mRNA levels, and these authors postulated that expression of c-Myc was responsible for the FSH effects on

proliferation. We have observed that, in agreement with Lim and Hwang (31), FSH increases c-Myc mRNA levels. Additionally, we have observed that this increase in c-Myc mRNA levels is inhibited by wortmannin, which again reinforces the participation of a PI3K/Akt pathway in FSH-stimulated Sertoli cell proliferation.

AMPK functions as a key energy-sensing kinase by virtue of its exquisite sensitivity to the cellular AMP/ATP ratio. Increments in this ratio promote AMPK phosphorylation and activation by the upstream kinase liver kinase B1, a human tumor suppressor mutated in Peutz-Jeghers syndrome. Recently, the role of AMPK in cell growth and proliferation has captured attention. It has been demonstrated that AMPK activation causes G<sub>1</sub>/S phase cell cycle arrest in several cell lines and also that mTORC1 signaling can be downregulated by AMPK (21, 39, 45).

To analyze whether AMPK activation had any role in Sertoli cell proliferation, the ability of A-769662, a direct and specific activator of AMPK, to regulate signal transduction and proliferation was tested. A-769662 increased p-AMPK and p-ACC levels, proving activation of AMPK. It was observed that A-769662 decreased p-p70S6K levels, indicating a reduction in the activity of the mTORC1 pathway. In the cell line human embryonic kidney-293, it has been shown that AMPK-mediated phosphorylation of Raptor induces binding of the 14-3-3 protein and inhibition of mTORC1 activity (16). Therefore, it may be speculated that the decrease in the mTORC1 signaling observed in this study is due, at least in part, to an increase in p-Raptor levels. The results obtained may indicate that AMPK activation elicits signals that are opposite to those evoked by FSH and suggest that AMPK may have a role in counteracting the proliferative effects of FSH. In this context, it was not surprising to find that FSH inhibits AMPK activity. It is worth mentioning that similar results were reported in granulosa cells (25). In these cells, it has also been shown that FSH activates mTOR and inhibits AMPK activation and that this inhibition leads to a reduced p27Kip1 and an

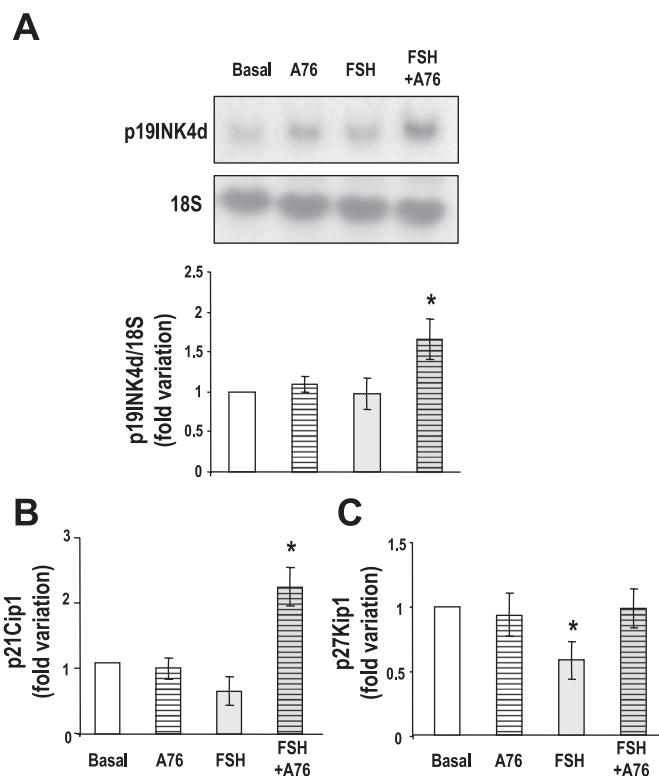


Fig. 7. AMPK activation regulates Sertoli cell p19INK4d, p21Cip1, and p27Kip1 mRNA levels. Sertoli cell monolayers were incubated under basal or FSH-stimulated conditions for 24 h in the presence or absence of A76 (1 mM). RNA was isolated, and Northern blot analysis or real-time PCR was performed to analyze mRNA for cyclin-dependent kinase inhibitors. A: membranes were hybridized with labeled cDNA probes for p19INK4d and 18S. One representative Northern blot experiment out of 3 is shown. The bar graph shows pooled data of 3 independent experiments, indicating the fold variation in p19INK4d mRNA levels (ratio p19INK4d mRNA to 18S in each sample) relative to basal. B and C: p21Cip1 and p27Kip1 mRNA levels were analyzed by real-time PCR. Results are expressed as means  $\pm$  SD of 3 independent experiments. \* $P$  < 0.05 vs. basal.

increased cyclin D2 mRNA expression, which are key molecules involved in granulosa cell proliferation (25). Similar results have been observed in other cell types. For example, it has been demonstrated that fibronectin stimulates phosphorylation of Akt and inhibits AMPK in the lung cell line NSCLC, promoting in this way activation of mTOR and downstream targets such as p70S6K, the outcome of which increases NSCLC proliferation *in vitro* (17). There is also evidence that IGF-I participates in the proliferation of the vascular smooth muscle cell line by suppressing AMPK activation (36).

The results presented herein show that AMPK activation reduced FSH-stimulated Sertoli cell proliferation. In some cell types, it was observed that stimulation of AMPK causes cell apoptosis (14, 25, 32). It may be argued that the effects of AMPK activation on Sertoli cell number may be a consequence of the latter biological phenomenon and not a picture of the role of AMPK on the fine-tuning of cell proliferation. Considering that neither changes in the number of nonviable Sertoli cells nor the presence of the cleaved activated form of caspase-3 were observed after activation of AMPK, a role of AMPK in the regulation of cessation of proliferation of this cell type is proposed. The molecular

mechanisms associated with AMPK activation and its anti-proliferative effects were analyzed in some cell types. AMPK has been found to reduce the levels of mRNAs encoding cell cycle regulators such as cyclin D and cyclin A1 (46, 51). Other reports support the hypothesis that the mechanism of cell cycle arrest by AMPK activation involves accumulation of the tumor suppressor protein p53 by phosphorylation and an increase in p21Cip1 and p27Kip1 protein levels (21, 39). In Sertoli cells, we observed that FSH reduces p27Kip1 mRNA levels and that AMPK activation, which decreased Sertoli cell proliferation, reverses this inhibition. As mentioned before, similar results were reported in granulosa cells (25). Additionally, activation of AMPK in FSH-stimulated conditions increases p19INK4d and p21Cip1 mRNA levels. The regulation of p21Cip1 and p27Kip1 protein levels in response to retinoic acid, testosterone, and thyroid hormone has previously been related to the detention of proliferation in FSH-stimulated Sertoli cells (3); however, a relationship with AMPK activation was not studied. Noticeably, thyroid hormone and retinoic acid activate AMPK in several cell lines (30, 49). In this context, it is possible to speculate that AMPK activation, which may be involved in the mechanism of action of thyroid hormone and/or retinoic acid, can regulate the transition of Sertoli cells from the mitotic to the postmitotic state during early postnatal development.

In summary, the results presented herein show that FSH regulates the PI3K/Akt/mTORC1 pathway in proliferating Sertoli cells and that this pathway is involved in FSH stimulation of c-Myc expression and Sertoli cell proliferation. On the other hand, these investigations show that AMPK activation may participate in the detention of Sertoli cell proliferation by, at least in part, a decrease in the activity of mTORC1 and an increase in CDKI expression.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

M.F.R., S.B.M., and S.B.C. did the conception and design of the research; M.F.R., M.R., M.N.G., and E.H.P. performed the experiments; M.F.R., M.R., E.H.P., and S.B.M. analyzed the data; M.F.R., M.N.G., E.H.P., S.B.M., and S.B.C. interpreted the results of the experiments; M.F.R. prepared the figures; M.F.R. and S.B.M. drafted the manuscript; M.F.R., S.B.M., and S.B.C. edited and revised the manuscript; M.F.R., M.R., M.N.G., E.H.P., S.B.M., and S.B.C. approved the final version of the manuscript.



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