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Intracellular signaling pathways involved in the relaxin-induced proliferation of rat Sertoli cells

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ABSTRACT

Regulation of Sertoli cell number is a key event to determine normal spermatogenesis. We have previously shown that relaxin and its G-protein coupled receptor RXFP1 are expressed in rat Sertoli cells, and that relaxin stimulates Sertoli cell proliferation. This study examined the mechanisms underlying the mitogenic effect of relaxin in a primary culture of Sertoli cells removed from testes of immature rats. Stimulation with exogenous relaxin increased Sertoli cell number and the expression of the proliferating cell nuclear antigen (PCNA), but did not affect the mRNA level of the differentiation markers cadherins 1 and 2. Relaxin-induced Sertoli cell proliferation was blocked by inhibition of MEK/ERK1/2 or PI3K/AKT pathways, but not by inhibition of PKC or EGFR activity. Relaxin induced a rapid and transient activation of ERK1/2 phosphorylation, which was MEK and SRC-dependent, and involved upstream activation of G_i. AKT activation could be detected 5 min after relaxin stimulation, and was still detected after 24 h of stimulation with relaxin. Relaxin-induced AKT phosphorylation was G_i- but not PKA-dependent, and it was blocked by both PI3K and MEK inhibitors. In conclusion, the mitogenic effect of relaxin in Sertoli cell involves coupling to G_i and activation of both MEK/ERK1/2 and PI3K/AKT pathways.

1. Introduction

Relaxin is a member of the family of insulin-related peptides, first recognized for its important role during pregnancy and parturition, but now known to have several additional functions (Sherwood, 2004; Dschietzig et al., 2006). Relaxin is structurally similar to insulin, but binds to RXFP1 (relaxin family peptide receptor 1), a G-protein coupled receptor (GPCR) that belongs to the subfamily of leucine-rich repeat containing GPCRs (LGRs), which also includes the receptors for the glycoprotein hormones TSH, FSH and LH (Hsu et al., 2002).

The role of relaxin in male reproduction is still unclear (Ivell et al., 2011). It was initially thought that relaxin was produced by the prostate and released to the seminal fluid to affect sperm motility (Sasaki et al., 2001; Sherwood, 2004). More recently, relaxin has been shown to influence sperm functionality (Ferlin et al., 2011; Miah et al., 2011). Although the major production of relaxin in the male reproductive tract occurs in the prostate, testes are also a source of relaxin (Cardoso et al., 2010; Gunnersen et al., 1995; Kohsaka et al., 2009). Studies with relaxin gene *knockout* animals ($Rln^{-/-}$) revealed that relaxin plays a role in the growth and development of the male reproductive tract (Samuel et al., 2003). The $Rln^{-/-}$ mice have smaller testis, decreased sperm maturation, and increased apoptosis (Samuel et al., 2003, 2005).

Relaxin mRNA levels are higher in the testis of immature than adult rats, and Sertoli cells of immature rats represent an important source of relaxin mRNA (Cardoso et al., 2010). Relaxin induces proliferation of cultured rat Sertoli cells, suggesting an autocrine or paracrine role (Cardoso et al., 2010; Filonzi et al., 2007). Sertoli cells play an essential role in several stages during life. They are important for the expression of the male phenotype and to support spermatogenesis during puberty (Sharpe et al., 2003). The number of Sertoli cells will determine the extension of sperm production.

There are several evidences that interplay between Ras/Raf/MEK/ERK and PI3K/AKT pathways may be necessary for normal cell proliferation to occur (rev. Chambard et al., 2007), and both pathways are important for Sertoli cell function (Meroni et al., 2002; Cheng et al., 2007; Fix et al., 2004; Royer et al., 2012). GPCR agonists may promote cell proliferation and differentiation through the activation of these pathways (rev. Rozengurt, 2007).

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Ras/Raf/MEK/ERK and PI3K/AKT pathways may be activated by $G_{\alpha s}$, $G_{\alpha q}$ or $\beta\gamma$ subunits released from G_i or G_0 (Yart et al., 2002; Stork and Schmitt, 2002). GPCRs may also activate the ERK1/2 and PI3K/AKT pathways through the transactivation of growth factor receptors (Schlessinger, 2000; Ohtsu et al., 2006; Rozengurt, 2007; Lucas et al., 2008), and through G protein-independent and arrestin-dependent mechanisms (Lefkowitz and Shenoy, 2005).

The mechanism by which relaxin stimulates proliferation and differentiation is not yet clear. When overexpressed in HEK293T cells, RXFP1 couples with G_s, G_{0B}, and G_{i3}, affecting cyclic AMP accumulation in a complex manner (Halls et al., 2006, 2007). Endogenous expression of RXFP1 allows a more selective coupling to G proteins, depending on the cellular context (rev. in Halls, 2012). This study investigated the pathways involved in the relaxin-stimulated proliferation of rat Sertoli cells, which endogenously express RXFP1 (Filonzi et al., 2007).

2. Material and methods

2.1. Primary cell culture

The experimental procedures were approved by the Research Ethical Committee from UNIFESP-EPM (CEP0001/09).

We used 15-day old male Wistar rats that were housed in the Animal Facility at the Instituto de Farmacologia e Biologia Molecular (INFAR), Escola Paulista de Medicina-Universidade Federal de São Paulo (EPM-UNIFESP), and maintained on a 12 h light/12 h dark lighting schedule at 23 °C, with food and water freely available. The testes were removed and decapsulated, and Sertoli cells were isolated as previously described (Grima et al., 1995; Lucas et al., 2004, 2008). Cells were plated at a density of approximately 4×10^6 cells/100 mm dish (about 5×10^4 cells/cm^2), in phenol-red free Ham's F12/Dulbecco's Modified Eagle Medium (F12/DMEM 1:1, Gibco, Invitrogen, Grand Island, NY, USA) containing 0.02 g/l gentamicin (Sigma Chemical Co., St Louis, MO, USA), pH 7.2–7.4, and supplemented with 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml sodium selenite and 10 ng/ml epidermal growth factor (Sigma). Cells were grown in a humidified atmosphere of 5% CO_2–95% air at 35 °C for 48 h, treated with 20 mM Tris–HCl, pH 7.4, to lyse residual germ cells (Galdieri et al., 1981), and allowed to grow for another 24 h. The culture was analyzed by morphological and immunocytochemical techniques (Lucas et al., 2008), confirming that the large majority of the population was Sertoli cells. Culture medium was replaced by another one without supplements 20 h before the experiments. At this stage, cells were 90–95% confluent and the viability, as determined by trypan blue exclusion, was higher than 90%. For proliferation assays, Sertoli cells were prepared as described above, plated at a low density, and at the day of experiments the confluence was 50–60%.

2.2. [Methyl-^{3}H] thymidine incorporation assays

Incorporation of [methyl-^{3}H] thymidine into cell DNA was estimated as described by Guizzetti et al. (1996). Previous studies in our laboratory indicated that incorporation of [methyl-^{3}H]thymidine (2 μCi/ml, specific activity 79.0 Ci/mmol, GE) in cultured Sertoli cells was time-dependent and linear from 2 to 10 h of incubation (Lucas et al., 2004). Therefore, primary Sertoli cell cultures on culture day 4 were initially incubated with 2 μCi/ml [methyl-^{3}H]thymidine for 6 h at 35 °C. Afterwards, cells were incubated in the absence or presence of the MEK1/2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]-butadiene (U0126, 20 μM, 30 min, Cell Signaling Technology, Danvers, MA), the PI3K inhibitor wortmannin (100 nM, 30 min, Sigma), the PKA inhibitor N-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinoline sulfonamide dihydrochloride (H89, 2 μM, 2 h, Sigma), the selective inhibitor of EGFR kinase, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478, 1 μM, 15 min, Calbiochem., San Diego, CA, USA) or the PKC inhibitor GF 109203X (5 μM, 30 min, Sigma). Incubation was continued in the absence or presence of these inhibitors and in the absence or presence of recombinant human relaxin H2 (RLN; Phoenix Pharmaceuticals Co., Burlingame, CA, USA), 50 ng/ml, for 24 h at 35 °C. The reaction was stopped by cooling the cells at 0 °C. The medium was aspirated and the cells rinsed twice with ice-cold PBS and 5% trichloroacetic acid (Sigma). The cells were then solubilized with 0.5 N NaOH, collected with cotton-swabs, and transferred to 5 ml OptiPhase HiSafe-3 scintillation liquid (PerkinElmer Life Science Products, Boston, MA, USA). Bound radioactivity was determined in a scintillation β counter (LS 6000 IC, Beckman Colter Inc., Palo Alto, CA, USA). Results were expressed in relation to control, basal levels of [methyl-^{3}H] thymidine incorporation (absence of relaxin and inhibitors)

2.3. Determination of cadherins 1 and 2 mRNA levels by real-time qRT-PCR

2.3.1. RNA extraction and cDNA synthesis

Cells were incubated with 50 ng/ml of relaxin at 35 °C for 2 and 4 h. The reaction was stopped on ice, and cells were washed with cold sterile phosphate-buffered solution (PBS). Total RNA was extracted with the TRIzol reagent (Invitrogen) according to the standard protocol (Chomczynski and Sacchi, 1987). RNA concentration was measured by spectrophotometry, and OD 260:280 nm ratios between 1.8 and 2.1 were obtained for all RNA samples. Ribosomal RNA integrity was checked by agarose gel electrophoresis, and a sharp and clear 2:1 ratio of ethidium bromide stained 28S:18S rRNA bands was observed for all samples. Two micrograms total RNA were used to synthesize the first strand cDNA at 50 °C with the Superscript III first strand synthesis Supermix (Invitrogen) and the oligo-dT primer supplied with this kit.

2.3.2. Quantitative PCR

For the qPCR, we used the SYBR Green system (Applied Biosystems, Foster City, CA, USA). The following oligonucleotides were used to amplify fragments of cadherin 1 (*Cdh1*), cadherin 2 (*Cdh2*) and the endogenous control β-actin (*Actb*): *Cdh1*: 5′-ACCGGCATCACCACAGAGACC-3′ (forward) and 5′- CCGGGCAGTT-GATGG GAGGG-3′ (reverse); *Cdh2*: 5′-GCGGCCTTGCTTCAGGCATC-3′ (forward) and 5′-CTGGCCTTCGTGCACGTCCT-3′ (reverse); *Actb*: 5′- GTAGCCATCCAGGCTGTG TT-3′ (forward) and 5′-CCCTCATA-GATGGGCACAGT-3′ (reverse). The primers (Invitrogen) were designed with the Primer3 program (Rozen and Skaletsky, 2000) and spanned exon–exon boundaries whenever possible. Primers for target genes and beta-actin were designed to have approximately the same amplification efficiency (=1).

Controls without cDNA or without primers were included in each assay. Samples were run in a 7500 Real-Time PCR System (Applied Biosystems), using default conditions of amplification (50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min). The dissociation curves were obtained at the end to confirm specificity of the amplification. Each sample was run in triplicate. The average cycle threshold (C_T) was determined with Applied Biosystems software.

Data were analyzed by the comparative $\Delta\Delta C_T$ method (ABI PRISM User Bulletin #2, Applied Biosystems). The control values were always used as a calibrator in each experiment. Data are expressed as mean ± S.E.M. of the $2^{-\Delta\Delta CT}$ from 3 different cDNAs (from three different cultures). At the end of the experiments,