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Regulation of Sertoli cell activin A and inhibin B by tumour necrosis factor α and interleukin 1α: Interaction with follicle-stimulating hormone/adenosine 3′,5′-cyclic phosphate signalling

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ABSTRACT

Regulation of crucial events during spermatogenesis involves dynamic changes in cytokine production and interactions across the cycle of the seminiferous epithelium. Regulation of activin A and inhibin B production by the inflammatory cytokines, tumour necrosis factor α (TNFα) and interleukin 1α (IL1α), alone and in conjunction with FSH or a cAMP analogue (dibutyryl cAMP), was examined in cultures of Sertoli cells from 20-day old rats. Both TNFα and IL1α stimulated activin A secretion and expression of its subunit (β_A) mRNA, and suppressed inhibin B secretion and expression of its subunit (α and β_B) mRNAs. The actions of TNFα and IL1α were opposed by FSH and dibutyryl cAMP. Both cytokines inhibited FSH/dibutyryl cAMP-stimulated inhibin B secretion and mRNA expression as well as stem cell factor mRNA expression. Both cytokines also inhibited FSH-induced cAMP production, and reduced baseline FSH receptor mRNA expression. These data highlight the reciprocal relationship that exists between FSH/cAMP signalling and inflammatory cytokine signalling pathways in the control of Sertoli cell function, and production of activin A/inhibin B in particular. It is anticipated that these interactions play important roles in the fine control of events during the cycle of the seminiferous epithelium and in the inhibition of spermatogenesis during inflammation.

1. Introduction

The production of cytokines by the Sertoli cell is regulated across the cycle of the seminiferous epithelium, indicating a role for these cytokines in localised control of spermatogenesis (O'Bryan and Hedger, 2008). In particular, critical roles in controlling the seminiferous epithelium have been established for activin A and its homologous antagonist inhibin B, involving autocrine/paracrine actions in the case of activin A, and endocrine control of pituitary secretion of FSH in the case of inhibin B (de Kretser et al., 2004). Consequently, understanding the control of activin and inhibin production by the Sertoli cell is essential for understanding the complexity of the cycle of the seminiferous epithelium. The principal driver of inhibin B is FSH, but production is modulated by intratesticular factors (Kaipia et al., 1991; Pineau et al., 1990). Production of activin A by the Sertoli cell, on the other hand, is less well characterised, but is not primarily under endocrine control.

The pro-inflammatory cytokine, interleukin 1 (IL1) is a potent stimulator of activin A in many cell types, including Sertoli cells (Okuma et al., 2005). Two IL1 forms, IL1α and IL1β, are typically produced by activated monocytes and macrophages during inflammation (Dinarello, 1996), but IL1α is also produced by the Sertoli cell in response to inflammatory stimuli and the presence of spermatogenic cells and residual cytoplasm (Gérard et al., 1992; Jonsson et al., 1999; Winnall et al., 2009, 2011). Numerous *in vitro* studies have established that both IL1 isoforms have complex regulatory effects on both Sertoli cell and spermatogenic cell function (O'Bryan and Hedger, 2008). These data suggest a role for IL1α in controlling spermatogenesis in both the normal and inflamed testis. Accordingly, FSH and IL1α act in a reciprocal manner to control the production of activin A and inhibin B by the Sertoli cell across the cycle of the seminiferous epithelium (Okuma et al., 2005, 2006). However, the complex pattern of production of IL1α, activin A and inhibin B across the cycle indicates that other local factors, such as developmental changes in the spermatogenic cells, also exert an influence (O'Bryan and Hedger, 2008).

Tumour necrosis factor α (TNFα) is another pro-inflammatory cytokine produced by activated monocytes and macrophages, and many regulatory activities of TNFα have been shown to over-

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lap with the actions of the IL1s in other systems (Basak and Hoffmann, 2008). Although IL1α and TNFα act through separate initial receptor/adaptor systems, they trigger parallel regulatory pathways via different TNF-receptor associated factors (TRAFs) leading to phosphorylation and degradation of IκB and activation of the inflammatory transcription factor NFκB, as well as activation of the stress-activated MAP kinases (JNK and p38) and transcription factor AP-1 (Basak and Hoffmann, 2008; Chung et al., 2002). Notably, both MAP kinase signalling and AP-1 have been implicated in the regulation of activin A (Ardekani et al., 1998; Myskiw et al., 2009; Scicchitano et al., 2008; Tanimoto et al., 1996).

Mouse pachytene spermatocytes and round spermatids produce TNFα (De et al., 1993), while Sertoli cells possess high affinity TNFα receptors (De et al., 1993). TNFα regulates aromatase expression in spermatogenic cells (Bourguiba et al., 2003), promotes spermatogenic cell survival (Pentikäinen et al., 2001; Suominen et al., 2004), and stimulates production of several important Sertoli cell products involved in supporting spermatogenic cell development, including lactate, transferrin, nitric oxide and glial cell-derived neurotrophic factor (Nehar et al., 1997; Sigillo et al., 1999; Simon et al., 2007). However, TNFα also disrupts the Sertoli tight junctions (Li et al., 2006), and has been shown to inhibit the ability of FSH to stimulate Sertoli cell aromatase and lactate production (Mauduit et al., 1993). Evidence that TNFα plays a role in activin/inhibin regulation comes from the observation that TNFα stimulates activin A in several cell types, including bone marrow stromal cells, monocyte/macrophages and placental cells (Keelan et al., 1998; Shao et al., 1992), and antagonises FSH and cAMP analogue stimulation of inhibin α-subunit and β_B-subunit mRNA and inhibin B production by porcine Sertoli cells (Le Magueresse-Battistoni et al., 1995; Mauduit et al., 1993). These data suggest that TNFα may exert a similar pattern of effects to those exerted by IL1α on activin A and inhibin B production in the Sertoli cell.

In the following study, the ability of TNFα to reproduce the biological effects of IL1α on production of activin A, inhibin B and the FSH signalling pathway in the Sertoli cell, was investigated. Overlapping control of several related Sertoli cell activities by IL1α/TNFα and FSH/cAMP was also investigated: these were production of cAMP, expression of the FSH receptor (FSH-R), and expression of stem cell factor (SCF), which is a cAMP-regulated cytokine involved in the control of spermatogonial development (Rossi et al., 1993; Taylor et al., 1996; Yan et al., 1999). Finally, responses to ceramide, which is an intermediate in a stress response signalling pathway activated by hydrolysis of membrane sphingomyelin to ceramide and phosphorylcholine by sphingomyelinase, and which is active in Sertoli cells (Grataroli et al., 2000; Meroni et al., 1999), were examined. Ceramide is stimulated by both IL1α and TNFα, and activates the stress-regulated MAP kinases, but acts independently of the central IL1α and TNFα (*i.e.* TRAF-mediated) signalling pathways (Kolesnick and Golde, 1994; Ruvolo, 2001; Schütze et al., 1994). These studies have important implications for understanding the local control mechanisms whereby activin A and inhibin B production, as well as responses to FSH, are modulated throughout the cycle of the seminiferous epithelium, and the inhibition of spermatogenesis during inflammatory disease.

2. Materials and methods

2.1. Isolation and culture of Sertoli cells

Sertoli cells were prepared from immature (18–22 days old) male Sprague–Dawley rats (Central Animal House, Monash University, Victoria, Australia), as previously described (Okuma et al., 2005). Briefly, Sertoli cells (approximately 1.0×10^6 cells/ml) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Invitrogen, Carlsbad, CA) containing 0.1% bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO) in Falcon 24-well or 6-well culture plates (Becton–Dickinson Labware, Bedford, MA) at 37 °C, pH 7.2–7.3 for 24 h, after which the medium was removed and the cells washed to remove unattached germ cells and dead cells. Fresh medium containing the following test substances was added to triplicate or quadruplicate wells: human recombinant (hr)IL1α (160,000 U/μg; R and D Systems, Minneapolis, MN), hrTNFα (R and D Systems), dibutyryl adenosine 3′,5′-cyclic phosphoric acid (dbcAMP; Sigma), ovine FSH (NIH oFSH-20, 4453 IU/mg; NIDDK, Betheseda, MD), and N-acetylsphingosine (C2 ceramide) and its inactive analogue, D-*erythro*-N-acetylsphingosine (C2 dihydroceramide) (BIOMOL Research Laboratories, Plymouth Meeting, PA). The choice of the doses of hrIL1α and hrTNFα was based on those employed in previous studies (Le Magueresse-Battistoni et al., 1995; Mauduit et al., 1993; Okuma et al., 2005). After a further 2–48 h of culture, the attached cells were immediately treated for extraction of mRNA. Even at the highest cytokine doses, there was no evidence of increased cell death in the cultures. Medium was collected at 48 h of culture for ELISAs, and stored at −20 °C prior to assay.

2.2. Activin A ELISA

Activin A was measured using a specific enzyme linked immunosorbent assay (ELISA) (Knight et al., 1996) according to the manufacturer's instructions (Oxford Bio-Innovations, Oxfordshire, UK) with some modifications (Okuma et al., 2005). The standard used was human recombinant activin A as described previously (Robertson et al., 1992).

2.3. Inhibin B ELISA

Inhibin B was measured using a specific enzyme linked immunosorbent assay (Groome et al., 1994; Okuma et al., 2005) according to the manufacturer's instructions (Oxford Bio-Innovations) with some modifications (Okuma et al., 2005). The standards used were WHO 96/784 inhibin B reference standard (NIBSC, Potter's Bar, UK).

2.4. Quantitative RT-PCR (qRT-PCR)

Inhibin and activin mRNA subunit expression levels were measured by qRT-PCR, as described previously (Okuma et al., 2005). Briefly, total RNA was extracted from Sertoli cells with Trizol® Reagent (Life Technologies, Rockville, MD). Extracts were treated with DNase I (Ambion, Austin, TX) to eliminate potential genomic DNA contamination. Reverse transcription (RT) was performed using Superscript II (Life Technologies) according to the manufacture's instruction primed with Oligo d(T)15 (Pharmacia, Uppsala, Sweden). Oligonucleotide primers (Sigma Genosys, Castle Hill, NSW, Australia) for activin/inhibin α-, β_A- and β_B-subunit and for β-actin were as previously described (Okuma et al., 2005). Primers for SCF were 5′-CAAAACTGGTGGCGAATCTT-3′ (forward) and 5′-GCCACGAGGTCATCCACTAT-3′ (reverse) to produce a 217 bp product. These primers span a region of the mRNA that is common to both membrane-bound and soluble variants of SCF (Martin et al., 1990). Primers for the FSH-R were 5′-AAACAGGAGCAGGCTGTGAT-3′ (forward) and 5′-TCTTGGTGTCGCTTGATGAG-3′ (reverse) to produce a 399 bp product (Sprengel et al., 1990). Reactions were performed using a real-time fluorimetric capillary based thermocycler (LightCycler, Roche, Basel, Switzerland). In each reaction, a standard curve was prepared from serial dilutions of an arbitrary standard. PCR products were quantified using the LightCycler software. Values were controlled for RT efficiency and cDNA loading by normalizing with an endogenous control (β-actin). It was confirmed that the expression levels of β-actin mRNA in immature Sertoli cell were not significantly affected by treatment with IL1α, TNFα or FSH/cAMP at the doses and culture times used (data not shown). Normalised values were then calibrated against values for untreated control Sertoli cells to give a relative expression (fold change) value.

2.5. Assay of cAMP production

Production of cAMP was measured using a Bridge-It™® cAMP fluorescence assay (Mediomics, St. Louis, MO), according to the manufactures protocol with minor modifications (Heyduk et al., 2003). Briefly, after plating down in a 24-well plate at 1×10^6 cells/well, immature Sertoli cells were washed and preincubated for 30 min with or without 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and then stimulated with 500 ng/ml FSH with or without 20 U/ml IL-1α, 20 ng/ml TNFα and 50 μM C_2-ceramide for 15 min. All experiments were terminated by the aspiration of the buffer and addition of ice-cold 10 mM HCl–ethanol. After incubation for 30 min with 10 mM HCl–ethanol on ice, the cells were dried under vacuum. Reaction mixture was added to the dried samples and incubated at room temperature for 30 min. The cAMP content was determined by fluorometry (FLUOstar, BMG Labtechnologies, Mount Eliza, Victoria, Australia) with excitation 485 nm and emission 520 nm.

2.6. Statistical analysis

All experiments were performed at least twice to confirm reproducibility. Data were analysed using one-way or two-way analysis of variance (ANOVA) following appropriate transformation to normalise data and equalize variance, where necessary. Mean values were compared using either Student–Newman–Keuls multiple