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NGF induces adult stem Leydig cells to proliferate and differentiate during Leydig cell regeneration

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ABSTRACT

Nerve growth factor (NGF) has been reported to be involved in male reproductive physiology. However, few reports have described the activity of NGF during Leydig cell development. The objective of the present study was to examine the role of NGF during stem-Leydig-cell (SLC) regeneration. We investigated the effects of NGF on Leydig-cell (LC) regeneration by measuring mRNA levels in the adult rat testis after ethane dimethanesulfonate (EDS) treatment. Furthermore, we used the established organ culture model of rat seminiferous tubules to examine the regulation of NGF during SLC proliferation and differentiation using EdU staining, real-time PCR and western blotting. Progenitor Leydig cells (PLCs) and immature Leydig cells (ILCs) were also used to investigate the effects of NGF on LCs at different developmental stages. NGF mRNA levels changed significantly during Leydig-cell regeneration *in vivo*. In vitro, NGF significantly promoted the proliferation of stem Leydig cells and also induced steroidogenic enzyme gene expression and 3β-HSD protein expression. The data from PLCs and ILCs showed that NGF could increase *Cyclin D1* and *Hsd 17b3* mRNA levels in PLCs and *Cyclin D1* mRNA levels in ILCs. These results indicate that NGF may play an important role during LC regeneration by regulating the proliferation and differentiation of LCs at different developmental stages, from SLCs to PLCs and from PLCs to ILCs. The discovery of this effect of NGF on Leydig cells will provide useful information for developing new potential therapies for PADAM (Partial Androgen Deficiency in the Aging Male).

1. Introduction

Adult Leydig cells are the testosterone-producing cells of the adult testis. These cells arise from stem Leydig cells (SLCs) and undergo four stages of differentiation [1]. Populations of cells at each of the following developmental stages are present in the testis: SLCs, progenitor Leydig cells (PLCs), immature Leydig cells (ILCs) and adult Leydig cells (ALCs). Previous studies in postnatal day 7 male rats showed strong evidence that SLCs may localize on the outer surface of the seminiferous tubules [2,3]. LC maturation involves a complex process of proliferation and differentiation under the control of endocrine and paracrine signals [4,5]. In adult rats, when a critical mass of mature LCs is achieved, the LC population becomes less mitotically active such that fully differentiated ALCs no longer divide. However, when adult male rats were treated with ethylene dimethanesulfonate (EDS), an alkylating toxicant that is selectively cytotoxic for differentiated LCs, ALCs were depleted, and the process of LC development began again, leading to the formation of a new generation of LCs [6,7].

Nerve growth factor (NGF), the first-identified neurotrophic protein, regulates the survival, growth and differentiation of neurons [8]. However, considerable evidence has accumulated to indicate additional activities in certain non-neuronal tissues and cells [9,10]. NGF mediates its cellular effects through interactions with two distinct receptors in Leydig cells, designated TrkA and p75NTR [11,12]. NGF binds preferentially to TrkA, and p75NTR can bind to NGF and also acts as a co-receptor for TrkA. p75NTR can increase the affinity and specificity of TrkA for NGF [13,14]. Peritubular myoid cells, Sertoli cells and germ cells have been identified as potential testicular NGF sources [15,16]. Many studies have focused on the NGF receptors; however, to date, the role of NGF during Leydig-cell development has not been determined. A

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study using rat embryonic tissues [17] reported the presence of p75NTR mRNA in the mesenchymal cells surrounding the epithelia of the developing seminiferous tubules. Immunohistochemical analyses showed that p75NTR-expressing cells were located in the intertubular compartment in the embryonic testis and that during postnatal development these cells become organized in a cellular layer that surrounds the myoid cells of the seminiferous tubules [18]. Consistent with these results, both TrkA and p75NTR gene products were detected in immature rat testes, with maximal expression in 10- and 20-day-old rats. However, the expression of TrkA and p75NTR was barely detectable in 90-day-old adult rats [19]. Furthermore, Leydig cells were positively stained for NGF, as well as for TrkA and p75NTR [20], and the embryonic testes of TrkA knockout mice were found to be developmentally delayed when compared with their wild-type counterparts [21].

The present study was designed to investigate the role of NGF in SLCs proliferation and differentiation during LC regeneration. We examined changes in the mRNA and protein levels of key steroidogenic components after NGF and LH treatment in isolated EDS-treated seminiferous tubules *in vitro*. Furthermore, we isolated PLCs and ILCs to further examine the effects of NGF at different developmental stages.

2. Materials and methods

2.1. Chemicals and kits

EDS was provided by Dr. Jiyan Pang (Sun Yat-Sen University, China). A radioimmunoassay kit for testosterone was purchased from Beijing North Institute of Biological Technology. A Click-iT EdU HCS Assays kit (C10350) was purchased from Life Technology Corporation.

2.2. Animals and treatment

Sprague–Dawley rats were purchased from the Experimental Animal Center of Guangdong Province. The rats received a single, i.p. injection of EDS (75 mg/kg body weight). On days 7, 28, 42 and 63, the testicles of a subset of the rats were removed under deep chloral hydrate anesthesia for RNA extraction, and blood samples were collected to examine serum testosterone. The animal protocol was approved by the Institutional Animal Care and Use Committee of Jinan University.

2.3. Isolation and culture of rat seminiferous tubules

The animals were treated 1 week prior to the beginning of experimentation with ethane dimethanesulfonate (EDS, 75 mg/kg body weight, by i.p. injection). The seminiferous tubules were isolated as previously described [22]. These tubules were transferred to 24-well plates and cultured in basic DMEM/F12 medium for 24 h.

2.4. SLC proliferation and differentiation assay in rat seminiferous tubules

The isolated tubules were cultured in 24-well plates with DMEM/F12 medium. The tubules were divided into three treatment groups: Insulin-Transferrin-Selenite (ITS) (treated with ITS and 0.1% BSA), LH (treated with ITS, 0.1% BSA and 1 ng/ml LH) and NGF (treated with ITS, 0.1% BSA, 10 ng/ml NGF, 100 ng/ml NGF and 200 ng/ml NGF). For the cell proliferation assays, the treated tissues were incubated for 24 h, and cell proliferation was measured using Click-iT EdU HCS assays. To measure cell differentiation, the ITS and LH groups were incubated with the medium described for 21 days. The NGF group was incubated for 3 days and then treated with DMEM/F12-ITS media containing ITS, 0.1% BSA and 1 ng/ml LH for up to 21 days.

2.5. Isolation of Leydig cells and treatment

Leydig cells were isolated as previously described [23]. Leydig cells were typically enriched more than 90% and showed intense staining. PLCs and ILCs were cultured for 24 h in DMEM/F12 medium with 1 mg/ml cholesterol-rich lipids (Sigma, C7305) alone, with a stimulating dose of LH (1 ng/ml), or with NGF (100 ng/ml) for 24 h.

2.6. RIA for testosterone

The culture media from the seminiferous tubules were collected on day 21 and stored at −20 °C prior to analysis. Testosterone concentrations in the media were measured using I^{125}-based RIA.

2.7. Real-time quantitative RT-PCR

Total RNA was extracted using RNeasy Plus Mini Kits (Qiagen, 74134). Total RNA was used as the template for cDNA synthesis primed with random hexamers (Bio-Rad, 170-8890). The reaction mixture was incubated at 42 °C for 30 min followed by 5 min at 85 °C. All PCRs were performed using Bio-Rad SsoAdvanced SYBR Green (172-5261). The Bio-Rad CFX connect Real-Time system (Bio-Rad Laboratories, California, USA) and Bio-Rad CFX Manager Software (version 2.0) were used to collect the PCR data. The primers were described previously [24,25] and were synthesized by Beijing Genomic Institue (BGI). The RNA levels of each gene were normalized to *Rps16*.

2.8. Western blot analysis

Western blot analysis was conducted as described previously [22]. Tissue proteins were isolated from the seminiferous tubules on day 21. The membranes were incubated with anti-StAR antibody (Santa Cruz, sc-25806, 1:1000), anti-Hsd3b1 antibody (abcam, ab150384) or anti-β-actin antibody (abcam, ab8227). The western blots were repeated three times using samples from three independent experiments. The protein levels were quantified using Image J software from BioRad and normalized to β-actin.

3. Results

3.1. Up-regulation of NGF mRNA during LC regeneration

Changes in serum testosterone levels indicated that ALCs were depleted, followed by LC regeneration. To investigate testicular NGF changes during this process, we measured NGF mRNA levels by real-time PCR. The results showed a sharp rise at day 7 (Fig. 1B). NGF mRNA expression returned to the baseline level on day 28 and remained at this level on days 42 and 63.

3.2. NGF induced SLCs to proliferate

We examined whether NGF induced SLCs to proliferate. In a culture model of seminiferous tubules, we examined the numbers of proliferating SLCs with EdU labeling. As shown in Fig. 2, using the Click-iT EdU cell proliferation assay, spindle-shaped, EdU-labeled cells were observed on the surfaces of the tubules during 24 h in culture. NGF induced marked cell proliferation, especially at 200 ng/ml. The NGF-stimulated groups showed significantly enhanced SLC proliferation, but the effect on cell proliferation varied significantly among the different NGF-stimulated groups. NGF