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Anti-inflammatory potential of β-cryptoxanthin against LPS-induced inflammation in mouse Sertoli cells

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ABSTRACT

β-cryptoxanthin (CX), a major carotenoid pigment, can inhibit inflammatory gene expression in mice with nonalcoholic steatohepatitis. In the present study, we examined the anti-inflammatory effects of CX on lipopolysaccharide (LPS)-induced inflammation in mouse primary Sertoli cells and the possible molecular mechanisms behind its effects. The results showed that CX significantly inhibited LPS-induced decreases in cell viability and in the percentage of apoptotic cells. Moreover, CX inhibited the LPS-induced up-regulation of tumor necrosis factor α (TNF-α), interleukin-10 (IL-10), interleukin-6 (IL-6) and interleukin-1β (IL-1β) in Sertoli cells. In addition, CX significantly limited the LPS-induced down-regulation of AR, HSF2, CREB, FSHR, INHBB and ABP in Sertoli cells. Western blot analysis showed that CX significantly suppressed NF-κB (p65) activation as well as MAPK phosphorylation. All the results suggested that CX suppressed inflammation, possibly associated with the NF-κB activation and MAPK of phosphorylation. Thus, CX may possess therapeutic potential against inflammation-related diseases.

1. Introduction

β-cryptoxanthin (3-hydroxy-b-carotene, CX), a major carotenoid pigment, exists in tangerines, pumpkins and persimmons. This pigment acts as a chemopreventive agent against lung cancer and is a strong antioxidant, scavenging both reactive oxygen and nitrogen species [1,2]. Furthermore, in our previous research, CX inhibited the proliferation and migration of stomach cancer cell BGC-823 *via* the retinoic acid β-receptor [3]. Recent studies showed that CX significantly decreased smoking-induced lung squamous metaplasia and inflammatory genes expression in mice [4].

Much clinical evidence suggests that testicular function is decreased by inflammatory and infectious diseases, resulting in a temporary or permanent impairment of fertility in humans [5,6]. Lipopolysaccharide (LPS) is one of the primary modulators of the inflammatory response in host cells. LPS induces the release of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) to adjust the acute phase response [7–9]. Previous studies showed that acute inflammation induced by LPS impairs testicular steroidogenesis [10] and disrupts the whole spermatogenetic process *via* activation of NF-κB [11,12], compromising male reproductive function [13].

Sertoli cells are somatic cells in the seminiferous epithelium and play an important role in spermatogenesis. Furthermore, Sertoli cells create tight junctions that make up the blood-testis barrier, forming a special morphological and chemical microenvironment to provide nutrients and regulatory factors for germ cells [14,15] and to protect seminiferous tubules from auto-antigens and invading pathogens [16,17]. Disruption of the Sertoli cell-germ cell association by cytotoxic agents and diseases often leads to a breakdown of spermatogenesis and to infertility [18]. Sertoli cells are used as targets for inflammatory mediators [19] and are a well-established model for testing toxicity in the male reproductive system [20]. Sertoli cells in seven-day-old mouse pups are immature and in a fully differentiated state that determines the number of germ cells produced in adult testis. Although existing findings demonstrate that physiological Sertoli cells retain neonatal characteristics during the fetal and early postnatal periods [21], they can protect germ cells from the noxious effects of inflammation, including inflammation triggered by LPS or IL-1α [22,23]. Furthermore, Sertoli cells from about one-week-old mouse pups, as a neonatal

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model, were used to determine whether LPS and proinflammatory cytokines could influence early postnatal Sertoli cell development *in vitro* [24].

It has increasingly been recognized that vitamin A (retinol) plays an indispensable role in male reproductive function. It was reported to enhance testosterone production [25], to maintain the Sertoli cell tight junctions that contribute to the blood-testis barrier [26] and to regulate spermatogenesis by promoting spermatogonia differentiation [27,28]. In particular, retinoic acid (RA), the active metabolite of vitamin A, regulates spermatogonial maturation and the entry of germ cells into meiotic prophase in the postnatal testes. Furthermore, recent studies reported that RA signaling regulates the transition of germ cells from Aa1 to A1 or A1 to A2 spermatogonia in neonatal testes [20,29]. Nevertheless, no study has reported whether CX can reverse LPS-induced injury in a neonatal model. Thus, we chose the neonatal stage as a model to investigate whether CX can reverse LPS-induced injury and whether the MAPK signaling pathway is involved in the LPS-induced inflammatory response in Sertoli cells.

2. Materials and methods

2.1. Chemicals and reagents

CX, DMSO and LPS were obtained from Sigma–Aldrich (St. Louis, MO, USA). LPS was dissolved in sterile water and CX was dissolved in DMSO. DMEM-F12 and fetal bovine serum (FBS) were supplied by Gibco BRL (Invitrogen Corporation, Carlsbad, CA, USA). Penicillin and streptomycin were purchased from Life Technology (Gaithersburg, MD, USA). All other reagents were of analytical grade. Anti-phospho-ERK (4370), anti-phospho-P38 (4511), and anti-phospho-p65 (3033) were obtained from Cell Signaling Technology (Beverley, MA, USA). Anti-p65 (sc8008) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-P38 (ab32142) was obtained from Abcam Ltd. (Cambridge, MA, USA). Anti-ERK (bs1112) was obtained from Bioworld Technology (Minneapolis, MN, USA).

2.2. Isolation and purification of Sertoli cells

Primary Sertoli cells were obtained from immature (7-day-old) male KM mice (Hubei Provincial Center for Disease Control and Prevention, Wuhan, China) as previously described [30] with minor modifications. Briefly, the mice testes were removed, minced and rinsed five times in phosphate-buffered saline (PBS); then, the samples were digested with 0.25% trypsin at 37 °C for 5 min before they were digested with DNAse I and Collagenase IV for 5 min in HBSS. Cells were collected by centrifugation after terminating digestion with DMEM-F12 and then washed twice with PBS. Finally, the dispersed cells were suspended with DMEM-F12 containing 0.1% FBS, sodium bicarbonate (2.2 mg/ml), penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml) and incubated with the same medium for 48 h at 37 °C in a 5% CO_2 atmosphere. After the Sertoli cells were attached to the bottom of culture flasks, the unattached germ cells and debris were discarded [31]. The purified Sertoli cells could quickly spread to form a monolayer in new medium containing 10% FBS, penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml).

2.3. Assessment of cell viability

Cell viability was assessed using a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Tokyo, Japan) according to the manufacturer's protocol. Briefly, the cells (5×10^4 per ml) were cultivated at 37 °C in a 96-well microplate in 100 μl culture medium. After culturing for 48 h, the cells were pre-incubated with different concentrations of LPS (50 mM) for 24 h and then stimulated with CX (15 μM) for another 48 h. After CX incubation, the culture medium was removed and fresh culture was added. The cell counting kit-8 reagents were added, and the cells were then incubated in the dark for 4 h. The absorbance was measured on an automated microplate reader (Bio-Rad, Hercules, Tokyo, Japan) at 450 nm.

Table 1
Oligonucleotide sequences and size of primers.

Gene Name	Primer F/R	Product size (bp)
AR	AAAATCCCACATCCTGCTCAA	136
	GGAAAGTCCACGCTCACCA	
FSHR	TCTATTCCCTGCCCAACCAT	227
	AGTCATATCATCAATATCTTGCCTTG	139
HSF2	TCGGAAGATTGTCCAGTTTATTG	
	AGTTGGTTCTTTGACTATGTGCTG	139
CREB	CTGGAGTTGTTATGGCGTCCT	
	ATCTGATTTGTGGCAGTAAAGGTC	233
ABP	ACCCACGCAGAATTCAGTCTC	249
	CAGGCAGAAGGAAGCAGAAGA	
INHBB	GGTCCGCCTGTACTTCTTCGTCTC	183
	TTCTCCACCACATTCCACCTGTCTC	
TNF-α	GTCCCCAAAGGGATGAGAAGTT	125
	GTTTGCTACGACGTGGGCTACA	
IL-10	ATTTGAATTCCCTGGGTGAGAAG	75
	CACAGGGGAGAAATCGATGACA	
IL-6	GAGGATACCACTCCCAACAGACC	141
	AAGTGCATCATCGTTGTTCATACA	
IL-1β	TGTTTTCCTCCTTGCCTCTGAT	106
	GAGTGCTGCCTAATGTCCCCTT	
β-actin	CTGAGAGGGAAATCGTGCGT	208
	CCACAGGATTCCATACCCAAGA	

2.4. Analysis of apoptosis

Primary Sertoli cells were treated with the indicated amount of CX for 72 h, washed in pre-cooled PBS buffer, and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Cell apoptosis was analyzed by flow cytometry using a BD FACSCalibur (Becton Dickinson, San Jose, CA, USA), and the percentage of the cell population at a particular phase was estimated with ModFit LT for Mac V3.0 software. For each measurement, at least 20,000 cells were analyzed.

2.5. RNA extraction and quantitative RT-PCR (q-PCR)

Total RNA was extracted from Sertoli cells using EZNA® Total RNA Kit I (Omega Bio Tek, Norcross, GA, USA), and then RNA was measured using 260/280 UV spectrophotometry. Equal quantities of RNA were reverse transcribed into cDNA using the PrimeScript Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's protocol. The primers were synthesized by Tsingke Biological Technology (Beijing, China) and the primers sequences used are given in Table 1.

PCR reactions were set up with the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Dalian, China) according to the manufacturer's protocol. Q-PCR analysis was performed with a Roche LC480 II Lightcycler (Roche, Mannheim, Germany). PCR was performed with 40 cycles as follows: 95 °C for 1 min, 95 °C for 20 sec (denaturation), 60 °C for 30 s (primer annealing) and 72 °C for 20 s (primer extension). All q-PCR experiments were performed in triplicate, including non-template controls. The levels of mRNA were determined using the $2^{(-\Delta\Delta Ct)}$ method and standardized by comparison to β-actin.

2.6. Western blotting

After treatment, Sertoli cells were washed with pre-cooled PBS and then lysed with radio immune precipitation (RIPA) buffer supplemented with protease and phosphatase inhibitors, scraped