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Involvement of prostatic interstitial cells of Cajal in inflammatory cytokines-elicited catecholamines production: Implications for the pathophysiology of chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS)

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ABSTRACT

In a previous work using guinea pig prostate, we have identified a novel interstitial cells of Cajal (ICCs) which possess close contacts between sympathetic nerve bundles and smooth muscle cells. The ability of prostatic ICCs in mediating excitatory neural inputs was therefore studied using isolated murine prostate ICCs by collagenase digestion combined with FACS method. RT-PCR and Western blotting analyses revealed that prostatic ICCs under a quiescent state expressed abundantly the rate-limiting enzymes essential for catecholamine synthesis. Moreover, distinct proinflammatory cytokines (*e.g.* IL-1β, IL-8, ICAM-1 and TNF-α) could significantly stimulate the expression levels of the rate-limiting enzymes of catecholamine production in prostate ICCs. Mechanistically, the above-mentioned stimulatory effects of proinflammatory cytokines appeared to be mediated via activation of NF-κB, HIF-1α and HDACs signaling pathways. Considering that prostatic catecholamine overactivity serves as an essential etiology of pelvic pain by indirectly stimulating the smooth muscle cell proliferation, or by directly causing muscular spasm, our results collectively suggest that targeting the NF-κB, HIF-1α and HDACs pathways in prostate ICCs be considered as a new strategy for treatment of chronic pelvic pain syndrome (CPPS) induced by chronic prostatitis (CP). Overall, the current study should shed novel light on the biology of this unique prostate ICCs.

1. Introduction

Prostatitis is one of the most common urinary disease in the male population. There is a 50% chance a man will develop prostatitis throughout his life. On the basis of classification of National Institutes of Health consensus, prostatitis is categorized into four types, including acute bacterial prostatitis (category I), chronic bacterial prostatitis (category II), chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS; category III) and asymptomatic inflammatory prostatitis (category IV). CP/CPPS is the most common type of prostatitis accounting for ~90% of clinical cases [1]. CP/CPPS is characterized by pain or discomfort in the abdomen, pelvis and genitals, as well as irritative and obstructive urinary problems, so its social impact is quite significant [1]. Experimental evidence obtained from clinical studies and animal work suggest that the development of pelvic pain appears to be tightly associated with the prostate catecholamines production. Prostatic catecholamine overactivity stimulates the smooth muscle cell proliferation via activating α1-adrenoceptor (α1AR)/MAPK/RTK cascade, or directly results in muscular spasm. Both smooth muscle cell proliferation and muscular spasm are well known to cause pelvic pain [2]. Histologically, the prostate catecholamines production has been observed to be originated from stromal cells [3], but what types of stromal cells are the main sources of prostatic catecholamine synthesis remains to be a matter of serious investigation.

Compelling morphological data have documented that interstitial cells of Cajal (ICCs) are involved in many enteric motor neurotransmission pathways in nervous and gastrointestinal systems. These unique cells are interposed between enteric neurons

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and smooth muscle cells, and play a major role in mediating cholinergic excitatory inputs between two cell types [4]. Previously, we have identified a similar ICCs in guinea pig prostate. Like the gastrointestinal and neuronal ICCs, prostate ICCs express abundantly c-kit, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH). Histochemical analysis reveals that there are many close points of contact existing among ICCs, sympathetic nerve bundles and smooth muscle cells. From a functional standpoint, norepinephrine (NE) could evoke a notable single inward current in isolated prostate ICCs via activation of α1AR. Thus, prostate ICCs, by incorporating nerve bundles and smooth muscle cells to form an intrinsic network, would facilitate a role in excitatory neurotransmission [5]. Nevertheless, the exact functions of prostate ICCs under certain pathophysiological conditions remain to be further defined.

In the present study we have explored the function relationship between chronic inflammation and ICCs in the murine prostate. Using isolated prostatic ICCs, we have tested the importance of these cells as an critical converging site where chronic prostatitis positively regulates smooth muscle cells proliferation via augmentation of catecholamine production. We have also found that proinflammatory cytokines potentiates the expression levels of rate-limiting enzymes essential for catecholamine synthesis in ICCs through cooperation with distinct signaling pathways. Overall, our systematic analysis should pave the way for a better understanding of the functions of prostate ICCs.

2. Materials and methods

2.1. Animals

Male C57BL/6 J mice at the age of 10 weeks, obtained from the Laboratory Animal Center of Fourth Military Medical University, were maintained in accordance with the "*Guide for the Care and Use of Laboratory Animals*" from the National Institutes of Health. Mice were fed *ad libitum*, and housed under a constant 12 h light:12 h darkness cycle (lights on at 08:00 h) and controlled conditions of temperature (22 ± 1 °C). Mice were euthanized by CO_2 asphyxiation. All animal work was approved by the Animal Care and Use Committee of our university.

2.2. Isolation and purification of prostatic ICCs

The prostatic ICCs were isolated according to our previous work [5]. Briefly, after dissection under a stereo microscope (Leica Biosystems, Beijing, China), murine prostate tissues were cut into ~1 mm^3 pieces and subjected to collagenase digestion (1 mg/ml, Sigma-Aldrich, Beijing, China) in Ca^{2+}-free Hanks' solution at 37 °C for 30 min. After three times of gentle washes, the tissues were cultured in DMEM medium containing 50 ng/ml of SCF (Sigma-Aldrich) at 37 °C for 24 h. Subsequently, the ICCs attached to the culture flasks were collected and labeled with anti-c-Kit antibody (Thermo Fisher Scientific, Shanghai, China) that was conjugated with the fluorescein isothiocyanate (FITC) using the Pierce™ FITC Antibody Labeling Kit (Thermo Fisher Scientific). Final fluorescence-activated cell sorting (FACS) was carried out using the BD LSR II System (BD Biosciences, Hong Kong, China).

2.3. ICCs treatment

Recombinant murine proinflammatory cytokines were purchased from R&D Systems (Minneapolis, MN, USA). To study the potential modulation of ICCs by inflammation, ICCs were incubated with 20 ng/ml IFN-γ and IL-17, in the presence or absence of the anti-inflammatory reagent hyaluronic acid (HA) hylan G-F 20 (G-F 20, Sigma-Aldrich), for 48 h [6], followed by determination of catecholamine concentrations as described below. To determine what types of proinflammatory cytokines can exert stimulatory effects on catecholamine production, ICCs were treated with different proinflammatory cytokines including 10 pg/ml of IL-1β, IL-8, ICAM-1 and IL-1α, 100 ng/ml of TNF-α, 20 ng/ml of IL-6, and 100 ng/ml of RANTES for 48 h, followed by RT-qPCR analysis. To investigate the signal transduction pathways involved in inflammation-dependent catecholamine induction, ICCs were treated with 10 pg/ml IL-1β alone or with 10 pg/ml IL-1β + different pathway inhibitors (Selleck, Shanghai, China) as indicated for 48 h. After 48 h of incubation, control (0.2% DMSO), stimulated (10 pg/ml IL-1β) and treated (different pathway inhibitors) ICCs were collected and subjected to RT-qPCR analysis as described below.

2.4. Determination of catecholamine concentrations

ICCs were incubated with 20 ng/ml IFN-γ and IL-17 (Sigma-Aldrich), in the presence or absence of the anti-inflammatory reagent G-F 20 (250 μg/ml), for 48 h. Subsequently, catecholamine levels in extracellular medium from ICCs cultures were quantified using the QuickDetect™ Catecholamine (CA) (Mouse) ELISA Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

2.5. Immunofluorescence

Freshly isolated ICCs were fixed using 4% paraformaldehyde (PFA, BOSTER, Wuhan, China) at room temperature (RT) for 15 min, followed by incubation with Blocking Solutions for Immunofluorescence (Vector Labs, Shanghai, China) at RT for 30 min. Cells were then treated with anti-c-Kit antibody (Thermo Fisher Scientific) at 4 °C overnight. After a thorough rinse, the immunoreactions were finally revealed by incubating cells with anti-rat FITC 488−conjugated IgG (Thermo Fisher Scientific) at RT for 60 min. The immunofluorescent staining was evaluated by a Zeiss 510 microscope.

2.6. RT-qPCR

Total RNA was isolated from ICCs or prostate tissues using a RNeasy mini kit (Qiagen, Shanghai, China), as per the manufacturer's instructions. After a routine DNase treatment (Promega, Madison, WI, USA), RNA samples were subjected to reverse transcriptase (RT) using Superscript III (Rnase H-Reverse Transcriptase; Thermo Fisher Scientific). Subsequent PCR was set up according to Promega's protocol [7,8]. The primers used were listed in Supplementary Table 1. PCR products were then quantified by SYBR green intercalation on a Two Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The relative abundance of each target transcript was quantified using the comparative △△Ct method, with *Gapdh* as an internal control [9].

2.7. Western blotting

Western blotting was carried out as described elsewhere [10]. ICCs were lysed using ReadyPrep™ Protein Extraction Kit (Bio-Rad) according to the manufacturer's instructions. An equal amount of total cell lysates were subjected to SDS-PAGE, followed by Western blotting analysis using anti-tyrosine hydroxylase (TH) antibody (Abcam, Shanghai, China), anti-dopamine-β-hydroxylase (DBH) antibody (Thermo Fisher Scientific), anti-dopa decarboxylase (AADC) antibody (Sigma-Aldrich) and anti-phenylethanolamine *N*-methyltransferase (PNMT) antibody (Abcam).

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