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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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2.8. Statistical analysis

Statistical comparisons and analyses between 2 groups were performed by 2-tailed, unpaired *Student's t*-test, with the aid of SPSS 15.0. Data are presented as mean ± S.E.M. and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purification of prostatic ICCs using FACS

In an attempt to study the potential role of ICCs at the *in vitro* level, we firstly isolated ICCs from murine prostate tissues using collagenase digestion combined with FACS method [11]. As shown in Fig. 1A, this combined isolation significantly increased the proportion of cells in the ICCs fraction. The ICCs isolated by our enzymatic digestion-FACS method exhibited classical spindle-shape morphology typical of neuronal interstitial cells of Cajal (*black arrow* in the left panel of Fig. 1B). The identity and purity of the ICCs was then confirmed by two methods. Immunofluorescent staining confirmed that the isolated ICCs were c-Kit-positive (*white arrow* in the right panel of Fig. 1B). Consistently, RT-PCR analyses using primer sets specific to macrophages, smooth muscle, mast cells and ICCs marker genes demonstrated an exclusive expression of c-Kit in isolated ICCs (Fig. 1C). These data collectively reflect our success in isolating and purifying the ICCs from murine prostate tissues.

3.2. Prostatic ICCs under a quiescent state express the rate-limiting enzymes essential for catecholamine synthesis

In humans, catecholamines (including dopamine, norepinephrine and epinephrine) are derived from the amino acid L-phenylalanine. L-Phenylalanine is converted into L-tyrosine by the TH, with molecular oxygen (O_2) and tetrahydrobiopterin as cofactors. L-Tyrosine is converted into L-DOPA by TH with tetrahydrobiopterin, O_2, and ferrous iron (Fe^{2+}) as cofactors. L-DOPA is converted into dopamine by the enzyme aromatic L-amino acid decarboxylase (AADC), with pyridoxal phosphate as the cofactor. Dopamine is

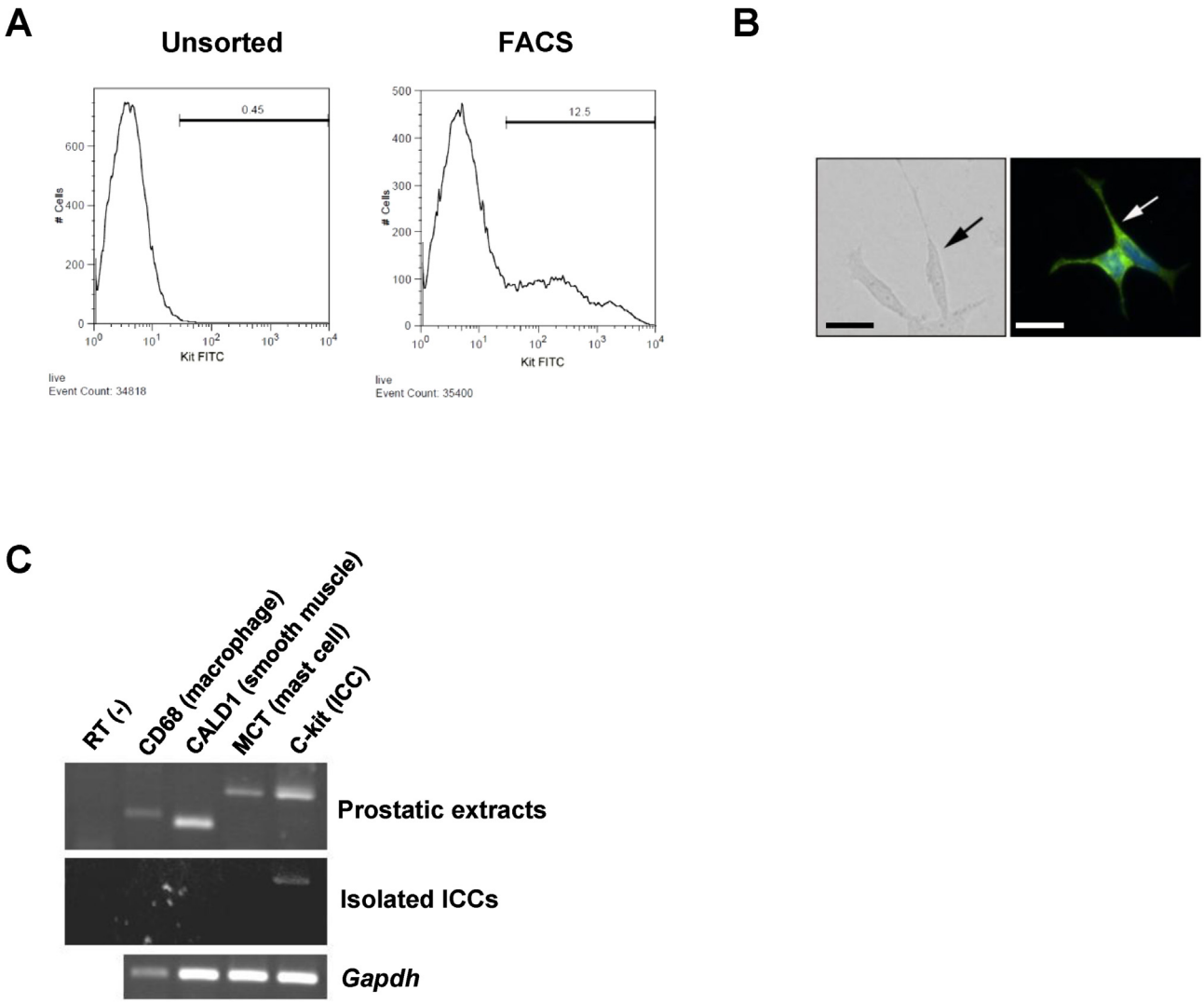

Fig. 1. *Isolation and purification of prostatic interstitial cells of Cajal (ICCs).* (A) Representative FACS analyses of c-kit expression in isolated prostatic ICCs. (B) Morphological analysis of isolated prostatic ICCs: *left panel*, cultures of ICCs isolated from mouse prostate exhibited the classical spindle-shape morphology typical of neuronal ICCs; right panel, immunofluorescence images shows a cytoplasm and membrane expression of the ICCs-specific marker c-kit in isolated prostatic ICCs, further confirming the cell identity. (C) Purity of isolated ICCs assessed by RT-PCR analyses using primer sets specific to macrophages, smooth muscle, mast cells and ICCs marker genes. Amplification of target genes in prostatic extracts was used as the positive control.

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converted into norepinephrine by the enzyme dopamine β-hydroxylase (DBH), with O_2 and L-ascorbic acid as cofactors. Norepinephrine is converted into epinephrine by the enzyme phenylethanolamine *N*-methyltransferase (PNMT) with S-adenosyl-L-methionine as the cofactor (Fig. 2A) [12]. By using RT-PCR and Western blotting analyses, we confirmed that the unstimulated prostate ICCs expressed the rate-limiting enzymes (namely TH, AADC, DBH and PNMT) essential for catecholamine synthesis, at both transcriptional (Fig. 2B) and translational (Fig. 2C) levels.

3.3. *In vitro chronic inflammation stimulates catecholamine synthesis in cultured prostate ICCs*

Catecholamine overactivities caused by excessive chronic inflammation induce muscular spasm and smooth muscle cell proliferation, which is an important etiology of chronic pelvic pain syndrome. However, very little is known about what types of interstitial cells are the main sources of catecholamine production in prostate [2]. We addressed this point by investigating the potential role of prostate ICCs in catecholamine overproduction triggered by proinflammatory cytokines. For establishing *in vitro* chronic inflammation model, ICCs were grown in the presence of IFN-γ+IL-17 (20 ng/mL for each) for 48 h [6], followed by ELISA analysis of catecholamine levels in extracellular medium (Fig. 3A). The catecholamine production of ICCs was significantly stimulated by ~37.19 fold after IFN-γ+IL-17 co-treatment (Fig. 3B). In line with this observation, the expression levels of four rate-limiting enzymes along the catecholamine synthesis, namely TH, AADC, DBH and PNMT, were all notably increased upon proinflammatory stimulation (Fig. 3C). Of note, the induction of catecholamine production and expression levels of four rate-limiting enzymes by proinflammatory cytokines were both substantially abolished by treatment with the strong anti-inflammatory reagent G-F 20 [6]. Thus, IFN-γ+IL-17-mediated inflammatory response had a significant impact on ICCs function. Next, we further characterized what types of proinflammatory cytokines can exert stimulatory effects on catecholamine production in ICCs. We incubated ICCs with different proinflammatory cytokines for 48 h. Expression levels of TH and DBH, were used as the read-outs because they are sensitive indicators of catecholamine synthesis following proinflammatory stimulations [13]. IL-1β, IL-8, ICAM-1 and TNF-α were the four cytokines capable of inducing significant levels of TH and DBH expressions. The other cytokines (IL-1α, IL-6 and RANTES) tested failed to induce expression levels of TH and DBH (Fig. 3D and E). Thus, enhancement of catecholamine production in ICCs appeared to be intrinsic characteristics of distinct proinflammatory cytokines.

3.4. *Catecholamine synthesis induction by proinflammatory cytokines involves NF-κB, HIF-1α and HDACs pathways*

To gain mechanistic insights into the regulation of catecholamine production in ICCs by proinflammatory stimulation, we

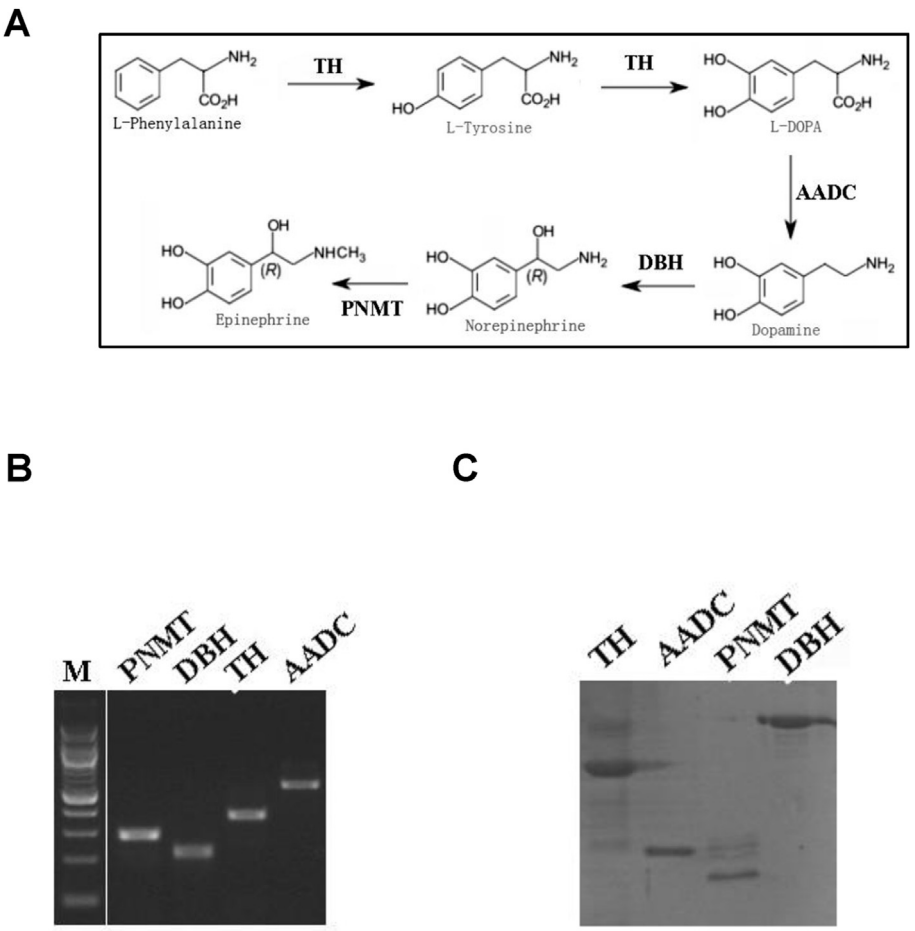

Fig. 2. *Prostatic ICCs express the rate-limiting enzymes essential for catecholamine synthesis.* (A) Schematic presentation depicting the main metabolic pathways important for catecholamine production. (B) RT-PCR analysis of the rate-limiting enzymes essential for catecholamine synthesis in isolated prostatic ICCs. (C) Western blotting analysis of the rate-limiting enzymes essential for catecholamine synthesis in isolated prostatic ICCs.

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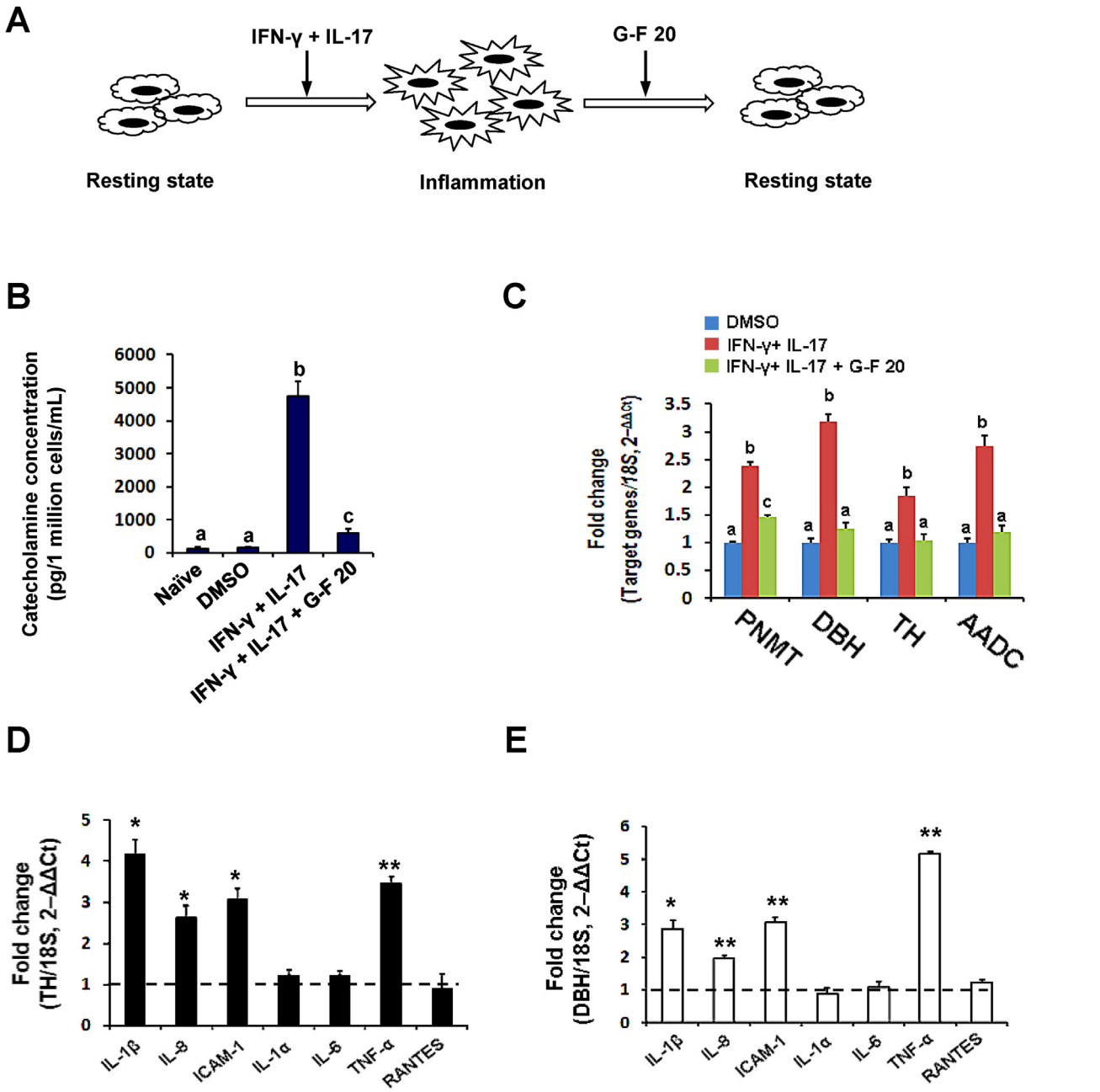

Fig. 3. *Potentiation of catecholamine synthesis by distinct proinflammatory stimulations in prostatic ICCs.* (A) Schematic illustration of a previously validated *in vitro* chronic prostate inflammation model. (B) ICCs were incubated with IFN-γ+IL-17 (20 ng/mL for each), in the presence or absence of G-F 20 (250 μg/mL), for 48 h, followed by ELISA analysis of catecholamine levels in extracellular medium. Different superscript letters denote groups that are statistically different ($P < 0.05$). (C) ICCs were incubated with IFN-γ+IL-17 (20 ng/mL for each), in the presence or absence of G-F 20 (250 μg/mL), for 48 h, followed by RT-qPCR analysis of the expression of the rate-limiting enzymes essential for catecholamine synthesis. Different superscript letters denote groups that are statistically different ($P < 0.05$). 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 of TH (D) and DBH (E) levels. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared to the values in cells treated with 0.2% DMSO.

stimulated ICCs with 10 pg/ml IL-1β in the presence of different pathway inhibitors including SC75741 (NF-κB, 0.2 μM), Ascorbate (HIF-1α, 5 μM), TSA (HDACs, 1.5 nM), Wortmannin (PI3K/AKT, 3 nM),WZ4003 (AMPK, 20 nM) and Go6976 (PKC, 8 nM). Concomitant incubation with SC75741, Ascorbate and TSA significantly reduced the TH and DBH induction elicited by IL-1β (Fig. 4A and B), suggesting that augmentation of catecholamine synthesis by proinflammatory stimulation may be selectively dependent on the activation of NF-κB, HIF-1α and HDACs pathways.

4. Discussion

In the present communication, we attempted to evaluate the potential paracrine cytokine regulation in the prostatic ICCs. To achieve this, we firstly isolated ICCs from murine prostate tissues using collagenase digestion combined with FACS method. Subsequent immunofluorescent staining and RT-PCR analyses confirm that this combined isolation significantly increased the proportion of cells in the ICCs fraction (Fig. 1). To be noted, the purity of the isolated ICCs must be verified using RT-PCR analysis of markers for

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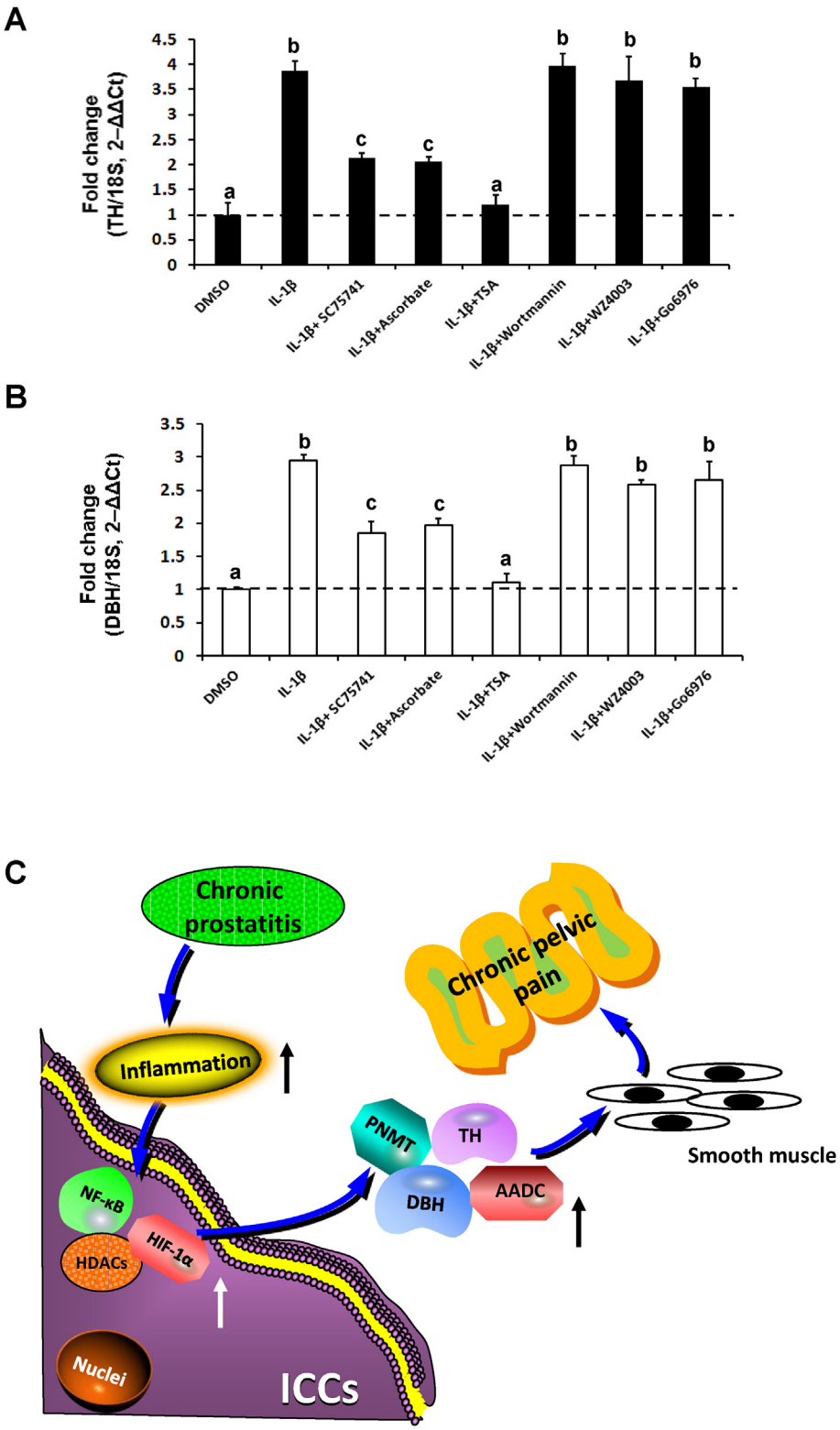

Fig. 4. *Catecholamine synthesis induction by proinflammatory cytokines involves NF-κB, HIF-1α and HDACs pathways.* ICCs were stimulated with 10 pg/ml IL-1β in the presence of different pathway inhibitors including SC75741 (NF-κB, 0.2 μM), Ascorbate (HIF-1α, 5 μM), TSA (HDACs, 1.5 nM), Wortmannin (PI3K/AKT, 3 nM),WZ4003 (AMPK, 20 nM) and Go6976 (PKC, 8 nM) for 48 h, followed by RT-qPCR analysis of TH (A) and DBH (B) levels. Different superscript letters denote groups that are statistically different ($P < 0.05$). (C) A working model proposed by the current study.

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potential contaminating cells (*e.g.* macrophages, smooth muscle and mast cells) and cannot be inferred only from the detection of c-kit expression. This is because the actual enrichment values of prostatic ICCs can be remarkably affected by small changes in c-kit mRNA in the unsorted cells. Given that the percentage of prostatic ICCs is relatively low when compared to other contaminating cells, the single use of c-kit mRNA values in isolated ICCs fraction may lead to less reliable measurements [11].

Like the gastrointestinal ICCs which are interposed between enteric neurons and smooth muscle cells, the prostate ICCs express all the rate-limiting enzymes (namely TH, AADC, DBH and PNMT) essential for catecholamine synthesis (Fig. 2), indicating that this kind of ICCs may play a potential role in mediating intramuscular cholinergic neurotransmission [14]. Accumulated data evidence that catecholamine overproduction induced by chronic inflammation cause the constant proliferation of smooth muscle cells in the prostatic stroma, thus leading to muscular spasm and consequent pelvic pain. The catecholamine production has been found to be originated from stromal cells [2]. However, the identities of the key stromal cell types of prostatic catecholamine synthesis under inflammatory conditions remain open, though it is evident that their identifications might provide valuable information for CPPS treatment. Our findings extend these understanding by emphasizing a prevalent stromal involvement, specifically of the ICCs, in the prostatic catecholamine synthesis. Indeed, we have observed that distinct proinflammatory stimulations by IL-1β, IL-8, ICAM-1 and TNF-α could significantly evoke the catecholamine synthesis in prostate ICCs (Fig. 3D and E). In accordance with our results, a very recent study has shown that the expression of IL-8, IL-1β and ICAM-1 is significantly increased in prostatic secretions from patients with CP/CPPS, and these cytokines are therefore suggested to be possible indicators for the clinical diagnosis of CP/CPPS and evaluation of erectile function on patients with CP/CPPS [15]. Similarly, TNF-α and IFN-γ have been both suggested to trigger intraprostatic inflammation and to mediate prostate enlargement under inflammatory conditions [16]. These previous reports and the current study consistently support the notion that the prostatic ICCs may play a major role in receiving proinflammatory inputs from the stromal environment in the murine prostate, and interference with the excessive catecholamine production in prostatic ICCs appears to be attractive therapeutic strategy for CPPS treatment.

A central question addressed by the current study was how proinflammatory cytokines promotes catecholamine synthesis in prostatic ICCs. Apparently, different signaling pathways including NF-κB, HIF-1α and HDACs were involved. In fact, it has been shown that IL-1β, IL-6, and TNF-α cytokines are able to induce HIF-1α expression in prostate epithelial cells, and this induction is required for the pathogenesis of prostatic hyperplasia [16]. Likewise, compelling data evidence that regulation of fundamental cellular inflammatory responses demands dynamic coordinated participation of key transcription factors (*e.g.* NF-κB and MTA1) and their coregulators (*e.g.* Pol II and HDACs) at the target gene chromatin, and aberrant expression of these transcription factors and coregulators plays a critical role in the development of pathologic phenotypes [17,18]. Thus, our results extend this understanding by identifying prostatic ICCs as another innate immune site where distinct proinflammatory cytokines efficiently elicit the activation of NF-κB, HIF-1α and HDACs pathways. On the other hand, emerging data suggest a potential involvement of NF-κB, HIF-1α and HDACs pathways in the regulation of catecholamine production. For example, NF-κB-dependent activation of Grx1 signaling potentiates dopaminergic loss (decrease of tyrosine hydroxylase activity and reduction of TH-positive striatal axonal terminals) and thus contributes to Parkinson's disease (PD) pathogenesis [19]. Likewise, HIF-1α signaling pathway controlling energy metabolism under nutrient deprivation condition has been shown to play a causative role in regulation of dopaminergic metabolism [20]. Additionally, HDACs in cooperation with the nuclear factor of activated T-cells (NFAT) regulate PMCA2x splicing variant generation, and this cooperation potentiates an excessive Ca^{2+}-dependent secretion of catecholamines in pheochromocytoma cells [21]. These previous studies all suggest that alteration in the expression levels and activities of NF-κB, HIF-1α and HDACs pathways may exert a prominent effect of on the catecholamine synthesis. To be noted, in contrast to the promoting effects of NF-κB activation on catecholamine production observed in the current study, upregulation of NF-κB pathway significantly inhibits catecholamine synthesis through repressing TH expression in the central nervous system [22]. Based on the available data, we conclude that while NF-κB, HIF-1α and HDACs pathways serve as essential mediators integrating proinflammatory stimulations and catecholamine production in prostate ICCs, these mediators may exhibit dual functions on catecholamine synthesis in response to different state of cell contexts. Our study also emphasizes the plausibility of using alterations in NF-κB, HIF-1α and HDACs pathways as sensitive biomarkers for surveillance of proinflammatory response in prostate ICCs.

In summary, we have defined a novel role of prostate ICCs as an critical converging site where chronic prostatitis positively regulates smooth muscle cells proliferation via augmentation of catecholamine production. Regarding, the mechanism responsible for deregulated catecholamine synthesis, we propose that infiltration of the inflammatory cells significantly stimulates the prostate cytokines secretion (*e.g.* IL-1β, IL-8, ICAM-1 and TNF-α), and these proinflammatory cytokines thereby potentiates the expression levels of rate-limiting enzymes essential for catecholamine synthesis through cooperation with NF-κB, HIF-1α and HDACs pathways (Fig. 4C). To this end, we 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 induced by chronic prostatitis. Overall, our study should shed novel light on the biology of prostate ICCs.

Conflicts of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.04.050.

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