The role of the α7nAChR-mediated cholinergic anti-inflammatory pathway in Hirschsprung associated enterocolitis

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Abstract

Background To investigate the role and the underlying mechanism of the α7nAChR-mediated cholinergic anti-inflammatory pathway in the pathogenesis of Hirschsprung(HSCR) associated enterocolitis（HAEC）.

Methods Experimental group:twenty-one-day-old Ednrb-/- mice were selected (n=10), with comparable-age wild type(Ednrb+/+) mice controls (n=10). Intestinal samples were collected. The experimental colons were divided into narrow and dilated segments according to morphology changes. The control colons were divided into distal and proximal segments.Colon HE staining was used to judge HAEC.Acetylcholine levels in colon was measured using enzyme-linked immunosorbent assays. Detected phosphorylated Jak2 (p-Jak2), Jak2, phosphorylated Stat3 (p-Stat3), Stat3, phosphorylated IκBα (p-IκBα) and IκBα were studied by Western blotting; mRNA levels of Jak2, Stat3, and IκBα were detected by RT-qPCR.

Results Colon HE staining indicated that HAEC mainly occured in the dilated segments of HSCR mice (Ednrb-/- mice) (EDNRB-P).Acetylcholine content in EDNRB-P was significantly lower than that in the narrow segments (EDNRB-D) ($P<0.05$). Western blotting showed that the Jak2, p-Jak2, Stat3 and p-Stat3 levels in EDNRB-D were significantly higher than those in EDNRB-P ($P<0.05$). The p-IκBα and IκBα levels in EDNRB-P were significantly higher than those in EDNRB-D($P<0.05$). The mRNA levels of Jak2 and Stat3 in EDNRB-D were higher than those in EDNRB-P, but the IκBα mRNA level was significantly lower than that in EDNRB-P ($P<0.05$).

Conclusions During HAEC, the inflammation in the dilated segment was more severe ,while in the narrow segment there was no obvious inflammatory reaction and the content of acetylcholine was higher, which was associated with the α7nAChR-mediated cholinergic anti-inflammatory pathway.

Background

Hirschsprung(HSCR) associated enterocolitis (HAEC) is the most common complication of HSCR and an important cause of mortality in children. The pathogenesis of HAEC is not clear, and is thought to be related to dilation of the proximal colon intestine, destruction of mucosal barrier, bacterial translocation and infection, mucous protein changes, decreased number of neuroendocrine cells in the mucosa, and changes of gene expression [1]. Macrophages are innate immune cells, accounting for approximately 10–20% of all monocytes. Macrophages reside in the intestinal tract and constitute the largest macrophage pool in the human body[2]. Under normal circumstances, intestinal resident macrophages maintain weak inflammatory reactivity and strong phagocytic function. However, when the intestinal microenvironment is changed, macrophages undergo a series of changes and participate in a variety of immune inflammatory reactions, thus affecting the occurrence and development of HAEC[3, 4].The cholinergic anti-inflammatory pathway (CAP) [5]plays a very important role in controlling the inflammatory response, during which is mediated by activation of α7nAChR on the surface of macrophages. However, the underlying mechanism is still unclear. This study aims to investigate the role

and the underlying mechanism of the α7nAChR-mediated CAP in the inflammatory response during HAEC.

Methods

Materials

SPF-class Ednrb knockout(Ednrb-/+) mice (B6:129 background) were donated by Nationwide Children's Hospital of Ohio State University and bred in the Animal Experimental Center of Tongji Medical College of Huazhong University of Science and Technology [batch number of production license: SCXK (Hubei) 2014–0004]. The acetylcholine kit, GAPDH, histone H3, HRP-labeled goat anti-rabbit, HRP-labeled donkey anti-goat, HRP-labeled goat anti-mouse, HRP-labeled goat anti-rat, phosphorylated protease inhibitor, primers and RNA extracts were all purchased from Wuhan Servicebio Company. RevertAid First Strand cDNA Synthesis Kit, FastStart Universal SYBR Green Master (Rox) and HyPure TMMolecular Biology Grade Water were purchased from Thermo, Roche and HyClone, respectively.

Hscr Animal Model

Animal protocols were approved by the Institutional Animal Care and Use Committee at Tongji Medical College of Huazhong University of Science and Technology.After gene identification of Ednrb gene knockout mice, heterozygous mice were screened out. Heterozygous mice were interbred to obtain homozygous mice. The 21-day-old homozygous(Ednrb-/-) mice were used as HSCR model mice,and wild type(Ednrb+/+) mice with the same age were used as the control group including 10 mice regardless of gender in each group. The Ednrb-/- mouse model is an established model for studying the pathogenesis of HAEC[6]. At 3 weeks, all mice presented with a small distal colon, obvious dilated proximal colon, and a large amount of dry feces (Figure-1). Moreover, the weight of HSCR mice was significantly lower than that of wild-type mice.

Intestinal Pathological Tissues Were Obtained

At the time of euthanization using isoflurane anesthesia in a desiccator jar for all mice,colon specimens were collected and washed with PBS. The experimental group was divided into narrow and dilated segments according to intestinal morphology. The colon specimens of the control group were divided into distal and proximal segments. Each specimen was divided into 4 parts for the Hematoxylin eosin(HE) staining,acetylcholine assay, Western blots and RT-qPCR.

He Staining And Hscr Interpretation

Specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with HE. Morphological changes of intestinal tissues were observed under light microscope.The extent of inflammatory damage in the intestinal tissue was assessed using the criteria described by Teitelbaum[7] (Table-1).If the pathological score was higher than or equal to grade III, it was judged as HAEC and used for the experiment.

Acetylcholine Assay

Acetylcholine was determined by enzyme-linked immunosorbent assays (Synergy, HI) using a commercial available kit (Wuhan Servicebio Company). The measurement was performed according to the instructions.

Western Blotting

Protein expression levels of p-Jak2, Jak2, p-Stat3, Stat3, p-IκBα and IκBα in intestinal wall tissues were detected by Western blots. Total proteins were extracted with RIPA lysate containing 1% PMSF. The protein concentration was determined by the BCA method. Protein samples were denatured and electrophoresed by SDS-PAGE and then transferred to PVDF membranes. The PVDF membrane was sealed with 5% skim milk powder for 1 h and then incubated overnight with the first diluted antibody at 4℃ (TBST with dissolved 5% skim milk, phosphorylated protein with TBST with dissolved 5% BSA). The secondary antibody was diluted 3000 times with TBST, incubated at room temperature for 30 min, and then washed with TBST in a decolorizing shaker 3 times at room temperature for 5 min each time. In the dark room, ECLA and ECLB reagents were mixed in the centrifuge tube at a medium volume, the mixed ECL solution was added for the full reaction and then, the samples were exposed, developed, fixed and analyzed. β-actin was used as an internal reference. The density ratio of target protein to β-actin was used as the relative content of the target protein and was compared between groups.

The Mrna Levels Of Jak2, Stat3 And Iκbα

The mRNA levels of Jak2, Stat3 and IκBα were detected by RT-qPCR. Total RNA was extracted by TRIzol reagent, and RNA concentration and purity were determined by Nanodrop 2000. RNA template was reverse transcribed into cDNA using RevertAi M-MuLV reverse transcriptase. Quantitative PCR was used to detect the mRNA expression. The results were processed by the ΔΔCT method: A = CT (target gene, sample to be tested)-CT (internal standard gene, sample to be tested), B = CT (target gene, control sample)-CT (internal standard gene, control sample), K = AB, expression multiple = 2^{-K}. GAPDH: sense primer 5'-CCTCGTCCCGTAGACAAAATG-3', antisense 5'-TGAGGTCAATGAAGGGGTCGT-3'; JAK2: sense primer 5'-TGGAGTGGCTAAGCAGTTGGC-3', antisense 5'-TCAGGGGCTTATCTCCTCCAC-3'; Stat3: sense primer 5'-CTGGTGTGAACTACTCAGGGTGT-3', antisense 5'-GGGCTTTGTGCTTAGGATGG-3'; IκBα:sense primer 5'-CCGCACAGCCATGTTTCAG-3', antisense 5'-CATGGAGTCCAGGCCGCTGTCGTG-3'. PCR reaction

conditions: predenaturation 95 °C, 10 min; cycle (40 times) 95 °C, 15 s → 60 °C, 60 s; melting curve 60 °C → 95 °C, 0.3 °C per 15 s.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software. The measurement data are expressed as $\bar{x}$ ±S. The t-test was used for statistical analysis between groups. P<0.05 was considered statistically significant.

Results

HE staining results

In the EDNRB-D(narrow segments of Ednrb-/-), the ganglion cells in the intermuscular and submucosal plexus were absent, and the nerve fibres was significantly thickened and morphologically disordered.The number and the morphology of ganglion cells in the EDNRB-P(dilated segments of Ednrb-/-)were generally normal.In the control group, normal ganglion cells were found in the plexus of each intestinal segment, with large and round nuclei,rich and dark cytoplasm,and normal structure and quantity of the plexus(Figure-2).The pathological score of intestinal tissue inflammation damage showed that the wild type mice in the WT-D (distal segments of wild-type) and WT-P (proximal segments of wild-type) were evaluated as grade 0 ,and the EDNRB-D had grade 0, whereas the EDNRB-P had grade III or higher,which indicated that HAEC mainly occured in the EDNRB-P(Figure-2).

Acetylcholine Content Of The Colon

There was no significant difference in the acetylcholine content between the WT-D and WT-P groups ($P > 0.05$). The acetylcholine content in the EDNRB-P group was significantly lower than that in the EDNRB-D group ($P < 0.05$) (Figure-3).

The Expression Level Of Pathway Proteins In Colon

There was no significant difference in the expression levels of p-Jak2, Jak2, p-Stat3, Stat3, p-IκBα, and IκBα between the WT-D and WT-P groups ($p > 0.05$). The protein levels of p-Jak2, Jak2, p-Stat3 and Stat3 in the EDNRB-D group were significantly higher than those in the EDNRB-P group ($p < 0.05$). The protein levels of p-IκBα and IκBα in the EDNRB-P were significantly higher than those in the EDNRB-D ($p < 0.05$) (Figure-4).

The mRNA expression levels of Jak2, Stat3 and IκBα in colon

There was no significant difference in the expression levels of Jak2, Stat3 and IκBα between the WT-D and WT-P groups ($P > 0.05$). The level of IκBα mRNA in the EDNRB-P group was significantly higher than that in the EDNRB-D group ($P < 0.05$). The mRNA levels of Jak2 and Stat3 in the EDNRB-D group were significantly higher than those in the EDNRB-P group ($P < 0.05$) (Figure-5).

Discussion

HSCR is a congenital disorder of the intestinal nervous system, which is characterized by the absence of muscular and submucosal ganglion cells in the distal colon due to the impeded migrations of the neural crest cells, leading to intestinal motility dysfunction and functional obstruction[8, 9]. HAEC is one of the most serious complications of HSCR. HAEC is associated with a variety of risk factors, including family history, concomitant trisomy 21, Bardet-Biedl syndrome, long-segment HSCR, delayed diagnosis of HSCR, and repeated HAEC[10]. The pathogenesis of HAEC is still unclear. It was found that mechanical obstruction, mucosal immunodeficiency, abnormal mucin, and infection were associated with HAEC. Among them, immune cells and cytokines play important roles in the development of HAEC. The gastrointestinal mucosa contains numerous macrophages. These resident macrophages participate in the regulation of inflammatory responses to bacteria and antigens, protect the mucosa from damage by pathogenic microorganisms, and remove dead cells and debris. Therefore, intestinal macrophages are the first line of defense against microbes in the innate immune system to maintain the intestinal microenvironment and normal physiological functions[4, 11].

In HSCR, pathogenic bacteria have the opportunity to enter the lamina propria through the mucosal barrier of injury, activate resident macrophages, and recruit mononuclear cells in the blood to release high levels of cytokines to activate the adaptive immune response, further inducing tissue damage. Macrophages play an important role in inflammatory responses, and their activation is mainly initiated by intestinal lipopolysaccharide (LPS). LPS activates the MAPK and NF-κB signaling pathway by binding to TLR4, which results in the production of cytokines interleukin-6 (IL-6) and TNF-α[12]. NF-κB is a major transcription factor for the expression of numerous cytokines and inflammatory mediators, which plays a key role in the regulation of the expression of many inflammatory factors and growth factors. Phosphorylation of IκBα is an important marker of NF-κB activation. In our study, we found that the expression levels of p-IκBα and IκBα proteins and IκBα mRNA in the dilated segment were higher than those in the narrow segment, which suggested that the inflammation of HSCR mice mainly occurred in the dilated segment.This result was consistent with HE staining.

Gene mutations, regulatory sequences, microRNAs and environmental factors can cause disorders of the intestinal nervous system[13]. In human HSCR, acetylcholine transferase was elevated in the non-ganglionic intestine, but neuronal nitric oxide synthase was reduced in the non-ganglionic intestine. These neurotransmitter changes were also confirmed in HSCR animal models[14, 15]. Our study suggested that the acetylcholine content in the colon of HSCR mice varied in the different location of colon. However, contrary to the location of inflammation, the acetylcholine content in the colon of HSCR mice decreased in the dilated segment but increased in the narrow segment. Agrawal RK et al[16]. found

that hyperplastic parasympathetic fibers were distributed in the intestinal wall of the narrow segment of HSCR children,but the cause of parasympathetic fiber hyperplasia was unclear.Parasympathetic nerve fibers can produce a large amount of acetylcholine, which may explain the significant increase in acetylcholine content in the narrow section; however, in the dilated segment, the expansion of the intestinal wall may reduce the distribution density of parasympathetic nerve fibers per unit area, resulting in a relative decrease in acetylcholine content.

The main receptor for acetylcholine on immune cells is α7nAChR, which is expressed in a variety of immune cells[17, 18, 19]. Studies[20]also found that the distributions of intestinal cholinergic nerve fibers were tightly associated with macrophages with positive expression of α7nAChR, which laid a morphological foundation for acetylcholine to inhibit the activation of macrophages through α7nAChR. Acetylcholine was found to alleviate inflammation by inhibiting the expression of CD14, TLR4, icam-1, B7.1 and CD40 in inflammatory cells via binding α7nAChR.The mechanism may be to dampen nuclear translocation of NF-κB, activate the Jak2-stat3 signaling cascade and up-regulate of heme oxygenase-1 and PI3K[21, 22, 23]. In this study, we found the inflammation was more severe in the dilated colon of HSCR mice. The signal pathway proteins Jak2, p-jak2, Stat3, and p-Stat3 and the mRNA expression levels of Jak2 and Stat3 in the narrow colon of HSCR mice were higher than those in the dilated segment. The acetylcholine content in the narrow colon was higher than that in the dilated colon, which may inhibit the occurrence of inflammation by activating the jak2-stat3 signaling pathway through α7nAChR.

Macrophages are the targets in the CAP, which play anti-inflammatory roles mainly by activating α7nAChR on macrophages. After α7nAChR activation, the expressions of TNF-α, IL-1β, IL-6, and high-mobility group box-1 were significantly inhibited. This is the main mechanism of its anti-inflammatory effect[24]. Activation of α7nAChR mainly affects the NF-κB pathway and Jak2-Stat3 pathway[25]. First, it rapidly inhibits the activation of NF-κB inhibitory protein kinase, which prevents IκB from being phosphorylated. Therefore, it maintains the inhibition of NF-κB by IκB and further inhibits the production of proinflammatory factors[26]. Second, activation of α7nAChR phosphorylates Jak2, and subsequently activates the downstream transcription factor Stat3. Moreover, stat3 is a key anti-inflammatory transcription factor, and its anti-inflammatory effect is believed to be related to the anti-inflammatory effect of IL-10 rather than directly inhibit the transcription of pro-inflammatory cytokines[25]. Third, the Jak2-stat3 pathway also interacts with the NF-κB signaling pathway to inhibit the production of inflammatory cytokines[27]. In our study, we found that activation of the two major anti-inflammatory pathways of α7nAChR in the narrow segment of HSCR mice was associated with increased acetylcholine content:firstly, it decreased the expression of p-IκBα and IκBα proteins and IκBα mRNA, thereby inhibiting NF-κB; secondly, it increased the expression levels of p-Jak2, Jak2, p-Stat3 and Stat3 proteins and mRNA of Jak2 and Stat3, thereby inhibiting the occurrence of inflammation.

Conclusion

In summary, the inflammation in the HSCR mouse model mainly occurred in the dilated segment. The higher content of acetylcholine in the narrow segment without obvious inflammation was associated with

the α7nAChR-mediated CAP. This finding may provide new perspective for the treatment of HAEC.

Declarations

Abbreviations

Hematoxylin eosin(HE)

Hirschsprung(HSCR)

Hirschsprung associated enterocolitis(HAEC)

the cholinergic anti-inflammatory pathway(CAP)

HSCR mice(EDNRB-/-)

the narrow segments of HSCR mice(EDNRB-D)

the dilated segments of HSCR mice(EDNRB-P)

lipopolysaccharide(LPS)

wild type mice(WT)

the distal segments of wild-type mice(WT-D)

the proximal segments of wild-type mice(WT-P)

Competing Interest

The authors declare that they have no conflict of interest.

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Informed consent

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not Applicable

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Author Contribution form

Study conception and design:JF,FC

Data acquisition:FC,LX,XM

Analysis and data interpretation:FC,XW,LX,XC

Drafting of the manuscript:FC

Critical revision:JF

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Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Table

Due to technical limitations, all tables are only available for download from the Supplementary Files section.

Figures

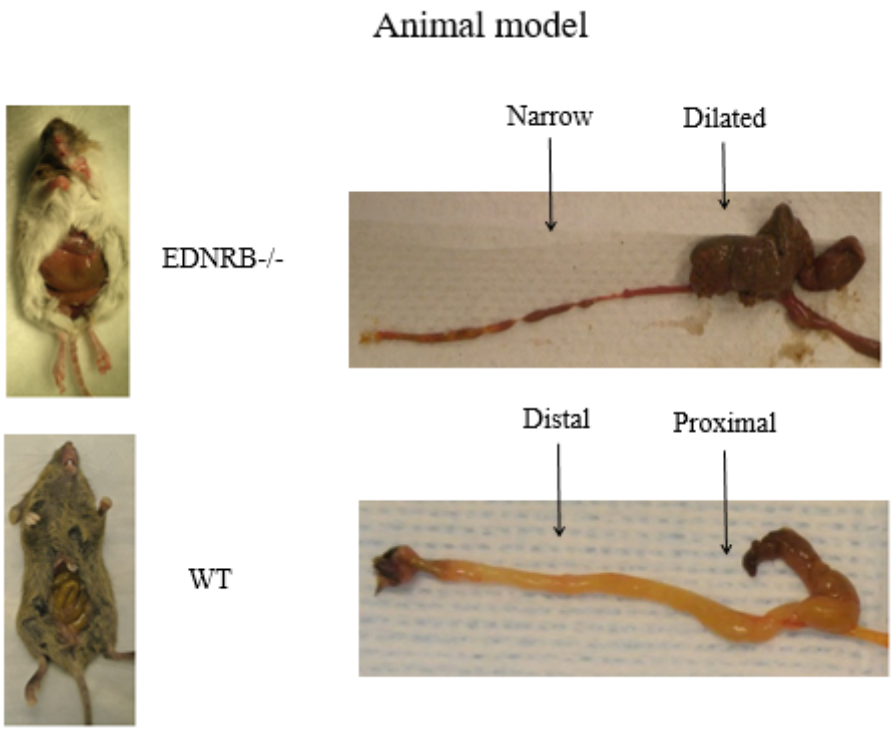

Figure 1

EDNRB-/- mice as HSCR animal model and wild type mice(WT) as control group animal model

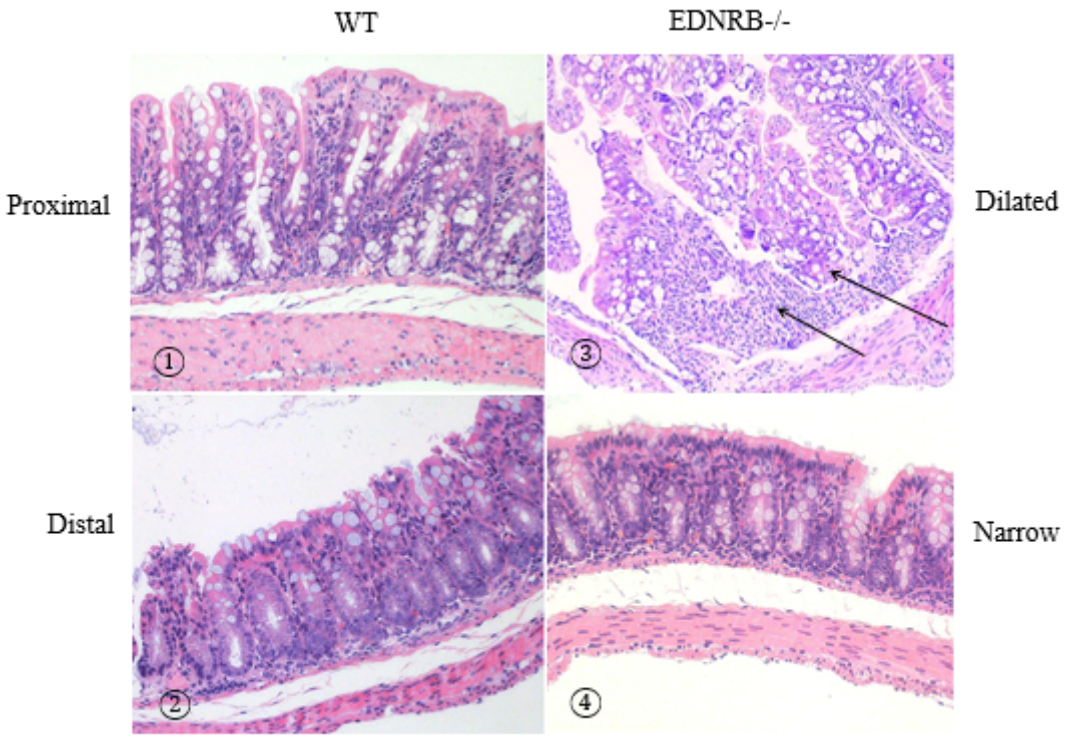

Figure 2

HE staining arrows in ▯ shows multiple crypt abscesses and a large number of inflammatory cell infiltration

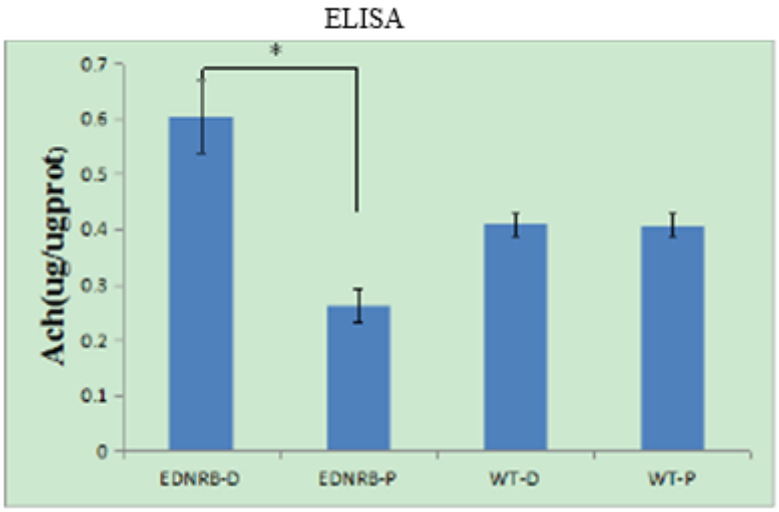

Figure 3

Acetylcholine assay by ELISA:EDNRB-D compare with EDNRB-P,*P<0.05;WT-D compare with WT-P,P>0.05(EDNRB-D:narrow segments of EDNRB-/-, EDNRB-P:dilated segments of EDNRB-/-,WT-D: distal segments of wild-type,WT-P:proximal segments of wild-type.)

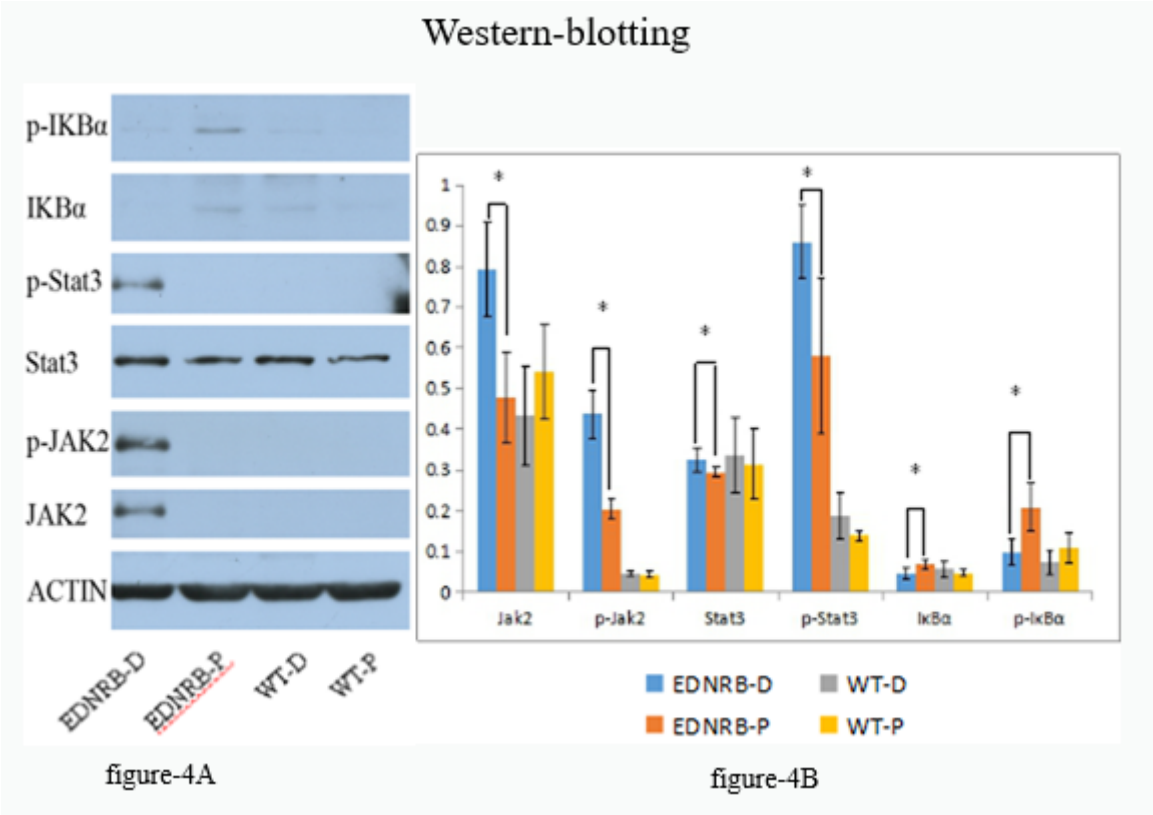

Figure 4

Analysis of expression levels of pathway proteins in EDNRB and WT colon by Western-blotting:EDNRB-D compare with EDNRB-P,*P<0.05;WT-D compare with WT-P,P>0.05 (EDNRB-D: narrow segments of EDNRB-/-, EDNRB-P:dilated segments of EDNRB-/-,WT-D: distal segments of wild-type, WT-P: proximal segments of wild-type)

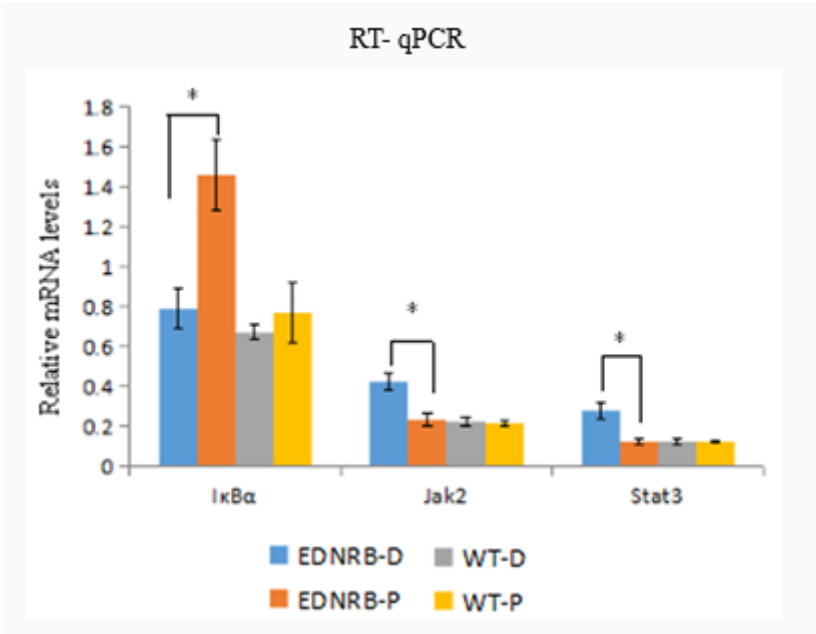

Figure 5

The mRNA levels of Jak2, Stat3 and IκBα by RT- qPCR:EDNRB-D compare with EDNRB-P,*P<0.05;WT-D compare with WT-P,P>0.05(EDNRB-D: narrow segments of EDNRB-/-, EDNRB-P:dilated segments of EDNRB-/-, WT-D: distal segments of wild-type, WT-P: proximal segments of wild-type)

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