Proteasome activator REGγ promotes inflammation in Leydig cells via IkBε signaling

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Received September 15, 2018; Accepted February 18, 2019

DOI: 10.3892/ijmm.2019.4115

Abstract. The development of testicular inflammation affects the normal male reproductive function. The proteasome activator complex subunit 3 (REGγ) has been suggested to regulate experimental colitis. However, to the best of our knowledge, a potential association between REGγ and testicular inflammation has not been demonstrated. The present study successfully established inflammatory models in C57 mice, primary Leydig cells and the TM3 cell line. It was observed that the absence of REGγ conveyed a significantly protective effect toward testosterone secretion in Leydig cells. REGγ deficiency significantly decreased the expression levels of phosphorylated transcription factor p65 and inflammatory factors in testis tissues, primary Leydig cells and the TM3 cell line. Inflammation also upregulated the expression levels of REGγ. Furthermore, the degradation of the nuclear factor light-chain-enhancer of activated B cells (NF-κB) inhibitor ε (IkBε) signaling pathway regulated REGγ and NF-κB expression. Double knockdown of REGγ and IkBε restored the response in wild-type cells to LPS-induced inflammation. In summary, these results demonstrated that REGγ regulates NF-κB activity by specifically degrading IkBε to regulate inflammation in testicular Leydig cells.

Introduction

Various lifestyle and environmental factors have been associated with an increase in the incidence of male-specific diseases (1), including orchitis, which is characterised by the inflammation of one or both testicles due to local or systemic infection, or non-infectious factors, with infective orchitis being more common (2). The mammalian testis is composed of spermatogenic tubules that primarily consist of spermatogenic epithelium and are separated by stromal layers. The spermatogenic epithelium consists of Sertoli sustentacular cells and spermaticogenic cells, while the interstitial space between the seminiferous tubules contains capillaries, capillary lymphatic vessels, nerves and various types of cells, including Leydig cells, fibroblasts, giant cells, mast cells, lymphocytes and eosinophils. Leydig cells are primarily responsible for androgen secretion (3,4).

The development of inflammatory diseases is closely associated with the activation of various signaling pathways including the nuclear factor light-chain-enhancer of activated B cells (NF-κB) signaling pathway (5,6), which is closely associated with the development of testicular inflammation (7). Phosphorylated transcription factor p65 (p-p65) is an important marker of inflammation and NF-κB signaling pathway activation (6).

Protein degradation is important for maintaining normal physiological functions and homeostasis (8), as the disruption of this system is a known cause of several diseases. In higher eukaryotes, the majority of proteins (80-90%) are degraded by the proteasome (9,10). The normal function of the proteasome is critical for maintaining relatively stable intracellular activity. The 11S regulatory complex, which includes proteasome activator complex subunit 1, proteasome activator complex subunit 2 and proteasome activator complex subunit 3 (REGγ), has been demonstrated to enhance the proteasomal degradation of substrate proteins and to alter the type and substrate selectivity of enzymes (11). One study has suggested
that inflammatory stimulation significantly increases REGγ expression in mouse colon epithelial cells and human colon cancer epithelial cells (12). Conversely, intestinal inflammation was decreased in a REGγ-deficient mouse model of inflammatory bowel disease (12). However, the association between REGγ and testicular inflammation remains unclear.

Therefore, the present study aimed to determine the role of REGγ in testicular inflammation using mouse and cell models. It was observed that the knockdown of REGγ resulted in a decreased level of inflammation in the animal and the cell models. Furthermore, REGγ may regulate the NF-xB signaling pathway via NF-xB inhibitor (IkB) ε (IkBe). These data indicate that REGγ may serve as a potential target in the treatment of testis inflammation.

Materials and methods

Experimental mice. The 6-week-old C57BL/6 wild-type (WT) (REGγ+/+) and REGγ-deficient (REGγ−/−) male mice were obtained from the Minhang Laboratory Animal Center of East China Normal University (Shanghai, China) and housed under specific pathogen-free conditions at 21±2˚C and 55±10% humidity under a 12 h light/dark cycle, and fed normal chow with ad libitum access to water. The C57BL/6 REGγ+ mice were originally acquired from Dr John J. Monaco (University of Cincinnati College of Medicine, Cincinnati, OH, USA) (11-12). A total of 36 REGγ+ mice and 24 REGγ−/− mice were used for the current study.

Cell culture and expression constructs. Primary Leydig cells were collected from mouse testes. TM3 cells were purchased from the Cell Bank of Type Culture Collection Chinese Academy of Sciences (Shanghai, China; cat. no. GNM24). The TM3 cell line is a mouse epithelial Leydig cell line. Primary Leydig cells and the TM3 cell line were grown in Dulbecco’s modified Eagle’s medium/F-12 nutrient mixture (dMEM/F-12) supplemented with 5% fetal bovine serum, 2.5% horse bovine serum (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), L-glutamine (150 mg/l), NaHCO3 (1.5 g/l), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were cultured in a 37˚C incubator with 5% CO2 and 95% air.

Animal and cell inflammation models. Based on preliminary experiments, 12 REGγ+ mice were selected and 6 of them were injected intraperitoneally with LPS (20 mg/kg body weight) dissolved in double distilled (dd) water for 6 h. As a control, 6 mice were injected intraperitoneally with equal volumes of dd water for 6 h.

The optimal conditions for creating an inflammatory cell model were explored. Concentration (0, 1, 5 or 10 mg/ml) and time gradients (0, 10, 20, 40 or 60 min) were established to determine the optimal model conditions. TM3 cells were treated for 10 min with LPS at concentrations of 0, 1, 5 or 10 mg/ml. The optimal concentration of LPS was then selected to treat the cells for different times (0, 10, 20, 40 or 60 min) in order to select the optimal treatment time.

Primary Leydig cells. Upon LPS treatment, mice were sacrificed by CO2 anesthesia (SMQ-II; Tianhuan Technology, Co., Ltd., Shanghai, China; final CO2 concentration, 80-100%). The displacement rates were between 10-30% of the chamber volume per minute (cv/min), and death was confirmed by observation of loss of respiration, heart beat and reflexes. Then, cervical dislocation was performed as a secondary physical method of sacrifice.

The testes were then collected from the mice in a sterile hood. Sterile tweezers were used to remove the testicular capsule. The tissue was digested in 0.25% collagenase at 37˚C for 10 min (1 ml per testis). Next, the tissue suspension was filtered with a 40 μm filter. Following centrifugation at 200 x g and 4˚C for 5 min, the cell suspension was placed in culture dishes and cultured in an incubator for 4 h. The adherent cells that were attached to the dishes were considered to be primary Leydig cells. The purity of the Leydig cells, as assessed by immunocytochemical staining for 3β-hydroxysteroid dehydrogenase.

For immunocytochemical staining, after the cells adhered to the slide, the glass slips were washed three times with 1X PBS, fixed with 4% paraformaldehyde at room temperature for 20 min and again washed three times with PBS. Then the glass slips were put in 0.5% Triton X-100 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 5 min and washed with PBS. Then, the glass slips were washed in 3% H2O2 at room temperature for 15 min, washed with PBS and blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) at room temperature for 1 h. The glass slips were then treated with anti-3β-hydroxysteroid dehydrogenase primary antibodies (cat. no. sc-515120; 1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C, washed with PBS, treated with horseradish peroxidase-conjugated secondary antibodies (cat. no. K5007; 1:200; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) at room temperature for 1 h and again washed with PBS. Next, the glass slips were washed with PBS, stained with 0.5 ug/ml DAPI for 3 min at room temperature and washed with PBS. Then, the glass slips were observed by fluorescence microscopy at a magnification of x600. In each field of vision, the total number of cells and positive cell were counted, positive cells accounted for >90% of total cells.

Knockdown of REGγ. Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) was used to knock down REGγ and IkBe expression in TM3 cells using small hairpin (sh)RNA: shREGγ and shIkBe. TM3 control (shN) and REGγ-knockdown (shR) cells were generated by retroviral shRNA plasmids specific for REGγ or control vectors, respectively, from OriGene Technologies, Inc. (Rockville, MD, USA). The plasmids were provided by Shanghai GenePharma Co., Ltd. (Shanghai, China). A total of 1 μg/μl plshRNAIkBe or plshNAREGγ vectors were incubated with TM3 cells for 12 h to obtain shN or shR cells, respectively. Then, the expression levels of REGγ in the shN and shR groups were detected by western blot analysis.

Western blot analysis. Following treatment, Leydig and TM3 cells were lysed in 1X loading buffer (5X loading buffer: 250 mM Tris-HCl pH 6.8, 10% SDS, 10% bromphenol blue, 50% glycerin and 5% β-5 Mercaptoethanol) for total protein collection. Protein concentrations were determined using the bichononic acid method. The buffer, including all the extracted proteins, was then heated at 100°C for 30 min. Protein samples (50 μg/lane) were subsequently separated using SDS-PAGE.
on 11% gels and transferred onto nitrocellulose membranes. Upon blocking with 7% BSA dissolve in PBS at room temperature for 1 h, the membranes were incubated with primary antibodies (1:1,000) at 4°C for 12 h and then with IRDye® 800CW-conjugated donkey anti-rabbit secondary antibodies (cat. no. 925-32213; 1:5,000; LI-COR Biosciences, Lincoln, NE, USA). β-actin (1:5,000) was used as a loading control. Finally, the membranes were scanned using an Odyssey imaging system (LI-COR Biosciences). Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used for the densitometric analysis. The following antibodies were employed: Anti-IkBε (cat. no. sc-7155; Santa Cruz Biotechnology, Inc.), anti-IkBα (cat. no. 4812), anti-IkBβ (cat. no. 94101; both Cell Signaling Technology, Inc., Danvers, MA, USA), anti-β-actin (cat. no. a2228; Sigma-Aldrich; Merck KGaA) and anti-p-p65 (cat. no. ab16502; Abcam, Cambridge, UK).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using TRizol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and reverse transcribed into complementary DNA using the Mx3005P qPCR system (Agilent Technologies, Inc.) under the following thermocycling conditions: Denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55-58°C for 30 sec, 72°C for 30 sec and 70°C for 1 min, and a final step at 4°C for 10 min. Each PCR mixture contained 10 µl SYBR-Green Premix Ex Taq polymerase (Takara Biotechnology Co., Ltd., Dalian, China). For each sample, the mRNA expression levels of the genes of interest were normalized to those of GAPDH. The 2−ΔΔCq method was used for quantification (13). The primers used for the qPCR were as follows: Tumor necrosis factor-α (TNF-α) forward (F), 5'-GACGGACACTGGCACAGAGAC-3' and reverse (R), 5'-TCGACAACAGGAAATGGA-3'; interleukin (IL)-6 F, 5'-CCACGGCTTTCCTACTCTC-3' and IL-6 R, 5'-CTG TTGGAGTGTGATCTCTGTG-3'; IL-1β F, 5'-GATGATACAC TGCTGGTGTGGTGTA-3' and R, 5'-GTGGTTCTACCTCGGA GCCGTCTAG-3'; and GAPDH F, 5'-AGGTCGGTGAACG GATTGG-3' and R, 5'-GGGGTCTTGTGATGGCAACA-3'.

Immunohistochemistry. Tissues were fixed with Bouin's solution (Sigma-Aldrich; Merck KGaA) overnight at room temperature. The tissues were transferred to 70% ethanol for 1 h and then with IR dye® antibodies (1:1,000) at 4°C for 12 h and observed under a light microscope (Eclipse E100; Nikon Corporation, Tokyo, Japan) to evaluate histological changes. The following antibodies were employed: Anti-IkBε (cat. no. sc-7155; Santa Cruz Biotechnology, Inc.) and anti-p-p65 (cat. no. ab16502; Abcam). The slides were observed by a fluorescence microscope at a magnification of x200.

Detection of cytokines. Venous blood was collected from the tail vein of mice, coagulated for 2 h at room temperature and then centrifuged at 1,800 x g for 15 min at 4°C (Centrifuge 5408R; Eppendorf, Hamburg, Germany). The upper serum layer was collected to determine the testosterone levels using the F-TESTO ELISA kit (cat. no. JLI0895; Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's protocol.

Flow cytometry. Leydig and TM3 cells were stained with fluorescein isothiocyanate and propidium iodide, and then analysed with a BD® LSR II flow cytometer (all BD Biosciences, San Jose, CA, USA) using FlowJo software (version 10; FlowJo LLC, Ashland, OR, USA).

Cycloheximide treatment. TM3 shN and shR cells were seeded into a six-well plate at a density of 1x10⁶ cells/well. Cells were then treated with 50 µg/ml cycloheximide (Sigma-Aldrich; Merck KGaA) for 0, 20, 40 and 60 min. For this treatment, cells were culture in DMEM/F-12 supplemented with 5% fetal bovine serum, 2.5% horse bovine serum, L-glutamine (150 mg/l), NaHCO₃ (1.5 g/l), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C with 5% CO₂.

Statistical analysis. All data analyses were conducted using the Student's t-test or one-way analysis of variance followed by a Fisher's Least Significant Difference post-hoc test for multiple comparisons with SPSS v12.0 software (SPSS, Inc., Chicago, IL, USA). Values are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

Validation of inflammation models. Testes were collected from C57BL/6 WT mice upon LPS treatment for hematoxylin and eosin (H&E) staining and western blot analysis. The results of the western blot analysis indicated that the p-p65 levels were significantly increased in the inflammatory group (Fig. 1A). H&E staining revealed that the testicular structure of the inflammatory group was damaged compared with the control group (Fig. 1B). These results confirmed the successful establishment of a mouse model of testicular inflammation. The western blot analysis results of primary Leydig cells demonstrated that the p-p65 expression levels in the Leydig cells in the inflammation group were increased compared with those in the control group (Fig. 1C). For the TM3 cell line, the inflammation was most pronounced in cells treated with LPS at a concentration of 5 mg/ml (Fig. 1D) for 10 min (Fig. 1E).

Inflammation affects the function of Leydig cells. In addition, the ELISA results demonstrated that the serum testosterone levels in the inflammatory group were decreased compared with the control group (Fig. 1F). Leydig cells are the
primary cells that secrete testosterone, which indicated that the function of these cells was impaired.

**REGγ deficiency decreases LPS-induced inflammation in vivo.** Control and inflammatory groups were established in 6-week-old C57 WT male mice and REGγ−/− mice. Then, testes from these mice were collected for western blot analysis, qPCR assays and H&E staining. The results confirmed by H&E staining (Fig. 2A). The results of qPCR analyses demonstrated that the expression levels of TNF-α, IL-1β and IL-6 were significantly increased in the C57 WT mice compared with the REGγ−/− mice following LPS treatment (Fig. 2C).

**REGγ deficiency decreases LPS-induced inflammation in vitro.** Primary Leydig cells were extracted from C57 WT and REGγ−/− mice testes and divided into LPS− or LPS+ groups. Then, western blot analysis and qPCR assays were performed. The results confirmed by H&E staining (Fig. 2A). The qPCR results indicated that the expression levels of TNF-α were significantly increased in REGγ−/− mice compared with the WT group (Fig. 2B).

Western blot analysis confirmed that REGγ was successfully knocked down (Fig. 2C). shN and shR cells were treated with LPS (5 mg/ml) for 0, 10, 20, 40 and 60 min. The western blot analysis results revealed that the expression level of p-p65 in the shN groups was increased compared with in the shR groups, with increasing incubation time (Fig. 3D).

**REGγ deficiency promotes apoptosis.** Flow cytometry was used to detect levels of cell apoptosis. Briefly, shN and shR cells were treated with 5 mg/ml LPS for 10 min. The results indicated that the number of apoptotic cells was significantly increased in the shN cells compared with the shR cells (Fig. 3E).

**Reciprocal regulation of REGγ and NF-κB in vivo and in vitro.** Inflammation models were established in two groups of REGγ+/+ and REGγ−/− mice. The testes of these mice were collected, sliced and stained with an anti-REGγ antibody. The immunohistochemical staining results revealed no REGγ expression in REGγ−/− mice testes, whereas REGγ was highly expressed in REGγ+/+ mouse testis (Fig. 4A). The expression level of p-p65 was markedly decreased in the REGγ−/− mice compared with the REGγ+/+ mice following LPS treatment (Fig. 4B). LPS treatment increased the expression level of REGγ in Leydig cells of REGγ+/+ mice (Fig. 4C). In addition, LPS treatment upregulated the expression levels of REGγ in TM3 WT cells compared with the control group (Fig. 4D).

**REGγ promotes NF-κB activity by degrading IκBe.** To explore the mechanism behind the association between REGγ and
NF-κβ in Leydig cells, several upstream signaling pathways were identified from previous studies (5‑7,12). Of these, the present study focused on the IkB family proteins. Western blot analysis of IkBα, IkBβ and IkBε were performed in shN and
The results demonstrated significant differences in IκBε expression levels between shN and shR cells (Fig. 5A). The results of the immunohistochemical staining also revealed that IκBε expression levels were increased in the testicular tissues of REGγ-/- mice compared with REGγ+/+ mice (Fig. 5B). Based on these results, cycloheximide degradation analyses were conducted. The results revealed that IκBε degradation was increased in shN cells compared with in shR cells treated for the same time interval. These results demonstrated that the degradation of IκBε increased with increased expression of REGγ (Fig. 5C).

REGγ/IκBε double knockdown (dKD) restores inflammation levels. shRNA was used to knock down the REGγ and IκBε genes in the TM3 cell line. shN, shR (IκBε knockdown) and dKD (REGγ and IκBε knockdown) cells were treated with 5 mg/ml LPS for 10 min. The western blot analysis results demonstrated that the expression levels of REGγ and IκBε were significantly decreased in the dKD cells compared with the shN cells (Fig. 5D). In addition, p-p65 was highly expressed in dKD cells, which was similar to the expression levels observed in WT cells (Fig. 5D).

**Discussion**

The results of the present study demonstrated that the protease activator REGγ was involved in the development of Leydig cell inflammation in a mouse model of LPS-induced inflammation. These results were additionally validated in primary Leydig cells and the TM3 cell line. Deletion of REGγ increased the accumulation of IκBε and inhibited the activation of the NF-κB signaling pathway, thereby inhibiting inflammation. Furthermore, dKD of REGγ and IκBε produced a successful cell model of inflammation.

With environmental degradation and unhealthy habits, male fertility continues to exhibit a downward trend (14). Numerous factors contribute to male infertility (15-17), including congenital genital abnormalities, genetic and endocrine diseases, reproductive system infection and inflammation, and physical and chemical factors. Inflammation of the male reproductive system is an important pathogenic factor of male infertility (16).

LPS is the primary constituent of the cell wall of gram-negative bacteria and a key cause of infection (18). LPS has been widely used to generate animal models of inflammation to study the effect of inflammation *in vivo*. Injection of LPS produces an inflammatory response and inhibits testicular steroid production and subsequent spermatogenesis, and may also damage the blood-testis barrier, thereby decreasing fertility (19). In a previous study, C57 mice were intraperitoneally injected with LPS to induce an inflammatory response, in order to observe the histopathological changes in the cells (19). In the present study, the serum testosterone levels decreased following LPS treatment, which indicated that inflammation damaged the normal functions of the Leydig cells.

Previous studies have demonstrated the associations between REGγ regulation and various diseases, which have confirmed the important roles of REGγ in the regulation of biological processes (20-23). The majority of these studies on REGγ have focused on cancer, neurological diseases and heart.
disease (20-23), while few have investigated the association between REGγ and inflammation. A previous study revealed that REGγ was involved in the development of inflammatory bowel disease in a mouse model of enteritis (12). Therefore, the present study focused on the role of REGγ in testicular inflammation.

The NF-κB signaling pathway has been recognised as a key signaling pathway in the development of inflammatory-associated diseases (24,25). As IkBe is activated upstream of NF-κB, previous studies explored the association between REGγ and inflammation (26-28). The results of the present study demonstrated the role of the protein kinase-activating factor REGγ in testicular inflammation in animal and cell models.

Testicular tissue damage and NF-κB activation induced a high expression level of REGγ in Leydig cells, and REGγ upregulated the activity of the NF-κB signaling pathway by degrading IkBe, which led to the secretion of proinflammatory cytokines and the persistence of testicular Leydig cell and testis injury, ultimately promoting the development of testicular Leydig cell inflammation. Therefore, the absence of REGγ may promote the accumulation of IkBe, thereby inhibiting the activity of the NF-κB signaling pathway in testicular Leydig cells in mice.

To the best of our knowledge, the present study demonstrated the physiological functions of REGγ and IkBe in testicular inflammation for the first time. REGγ is a specific regulatory factor of IkBe in testicular Leydig cells, which extends awareness of the IkBe regulatory pathway. The testicular tissue of REGγ−/− mice exhibited decreased levels of inflammatory molecules. However, the roles of other testicular tissue cells in this REGγ testicular inflammation model were not investigated. Therefore, the effects of other types of cells in this process cannot be ruled out. The knockdown of IkBe was performed in the present study, but the results were not satisfactory as no significant differences were identified. This is a limitation of the study, and will be a focus in future studies.

Various anti-inflammatory drugs, including glucocorticoids, methotrexate and anti-TNF-α antibodies, completely or partially inhibited the NF-κB signaling pathway (29). However, these anti-inflammatory drugs do not specifically inhibit NF-κB, and the response to these drugs differs among individuals, which highlights the ongoing challenges in proteasome studies. Notably, the results of the present study indicated that REGγ regulates NF-κB activity by specifically degrading IkBe, thereby providing novel molecular targets of the atypical proteasomal pathway. It may be possible to prevent and treat inflammation by attenuating, as opposed to completely inhibiting the NF-κB signaling pathway.

Acknowledgements

Not applicable.

Funding

The present study was partially supported by a grant from the National Natural Science Foundation of China (grant no. 81671446).
Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
LL and YX designed the experiments. TX, HC, SS, GH and BH performed the experiments and TH analyzed the data. TX wrote the manuscript.

Ethics approval and consent to participate
All animal procedures were reviewed and approved by the Animal Care Committee of East China Normal University, which followed the Guide for the Care and Use of Laboratory Animals by the National Research Council.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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