Roscovitine protects murine Leydig cells from lipopolysaccharide-induced inflammation

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Abstract. Roscovitine is a cyclin-dependent kinase inhibitor, which has been previously investigated for its anticancer effects. It has also been confirmed that roscovitine can downregulate the expression of myeloid cell leukemia-1 protein to inhibit inflammation. In the present study, roscovitine was used to treat inflammation in lipopolysaccharide (LPS)-induced model mice. At the cellular level, Leydig cells isolated from mouse testis were assessed for inflammatory factors. It was revealed that roscovitine successfully reduced inflammation-associated injury induced by LPS pretreatment. At the molecular level, roscovitine was found to exert this effect through promotion of adenosine monophosphate-activated protein kinase phosphorylation. To the best of our knowledge, the present study was the first to suggest that roscovitine has a protective role in Leydig cells through its anti-inflammatory action.

Introduction

As novel cancer-targeting drugs, cyclin-dependent kinase (CDK) inhibitors are highly anticipated (1). Over the past decade, numerous studies have implicated roscovitine as an anticancer drug with promising therapeutic properties (2). Roscovitine was found to inhibit cell division control protein 2, cdk2 or cdk5 kinase activities (3,4), and was shown to directly inhibit nuclear factor-κb activation in cancer cell lines (5).

Additional studies have demonstrated further properties of cyclin-dependent kinase inhibitors (6) or roscovitine (7); for instance, roscovitine downregulated the expression of myeloid cell leukemia (Mcl)-1 protein, which is likely involved in inflammatory processes (7). Other studies have shown that upregulation of adenosine monophosphate-activated protein kinase (AMPK) activation also inhibited the expression of Mcl-1 (8-10). AMPK is an important regulator of energy homeostasis in cells. In recent years, studies have demonstrated that AMPK has an important role in modulating inflammation in inflammatory pulmonary diseases, including asthma, pulmonary infectious diseases and pulmonary fibrosis (11-14). These results suggested that roscovitine may have anti-inflammatory effects; while it has remained elusive whether it also inhibits inflammation in murine Leydig cells.

Leydig cells are distributed in the loose connective tissue between seminiferous tubules. The main function of Leydig cells is the synthesis and secretion of androgen, which has important roles in male reproductive function (15). Inflammation can affect the normal function of Leydig cells (16). Lipopolysaccharide (LPS) was used to establish a mouse model of inflammation (17) and the testes were collected to investigate the effects of roscovitine on murine testis inflammation.

Materials and methods

Animals and ethics statement. A total of 16, 2-month-old male C57BL/6 mice (four groups, n=4 each group, weight range from 20-25 g) were obtained from the Shanghai Laboratory Animal Company (Shanghai, China). The mice were housed at $21\pm2°C$ with $55\pm10\%$ humidity under a 12-h light/dark cycle. Chow and water were available *ad libitum*. All animal procedures were reviewed and approved by the Animal Care Committee of Tongji University (Shanghai, China).

Experimental groups. Mice were divided into four groups (n=4 each): Control group, case group, LPS group and dimethylsulfoxide (DMSO) group. In the case group, each mouse was injected i.p. with roscovitine (S1153; Selleck Chemicals, Houston, TX, USA) at 5 mg/kg dissolved in DMSO (Sigma-Aldrich, Merck Millipore, Darmstadt, Germany), and LPS (Sigma-Aldrich, Merck Millipore) at 3 mg/kg dissolved in saline (0.9% NaCl). Animals in the LPS group received LPS only (5 mg/kg). Animals in the DMSO and control groups only received equivalent volumes of DMSO and saline, respectively. After 12 h, mice were sacrificed by carbon dioxide anesthesia (SMQ-II; Tianhuan Technology, Co., Ltd., Shanghai, China) with a final concentration of 80-100% CO_2

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in a cage 730x560x600 mm in size. Mortality was confirmed following 5 min of observation. Venous blood was collected from the caudal vein and the testis was removed for further examination.

Behavior and weight of mice. The behavior of the mice was observed and recorded prior to and after drug treatment, and the mice were weighed using an electronic balance at the same time-points.

Observation of histological changes. Mouse testis were fixed at room temperature by soaking overnight in 2% paraformaldehyde and transferred by gradient ethanol (from 50 to 100%) and embedded into paraffin. Sections were sliced to 5 μm and stained with hematoxylin and eosin. Tissue sections were also stained immunohistochemically to observe specific expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) in testis tissue after the indicated treatments. The dilution ratio of Anti-HSD3B1 antibody (catalogue no. ab55268; Abcam, Cambridge, UK) was 1:1,000. Histology of testicular tissues was observed by light microscopy (Eclipse E100, Nikon, Tokyo, Japan).

Serum testosterone levels. Venous blood was extracted from the mice and blood was allowed to coagulate for 12 h at room temperature, followed by centrifugation for 10 min at 4°C at 1006.2 x *g* (Centrifuge 5408R; Eppendorf, Hamburg, Germany). The upper serum layer was assessed using an ELISA kit (cat. no. YC30087; Yuanchuang Bio-Chemical Co., Ltd., Shanghai, China) to quantify testosterone levels according to the manufacturer's instructions.

Cell culture and reagents. Mouse testes were collected to extract primary Leydig cells for RNA and protein isolation. Furthermore, Leydig cells were isolated and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Merck-Millipore) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (18).

RNA analysis by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA from cells was extracted using TRIzol (Life Technologies, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA samples were reverse-transcribed into complementary DNA using the Mx3005P qPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). The PCR reaction was completed as follows: 94°C for 5 min followed by 94°C for 30 sec, 55-58°C for 30 sec, 72°C for 30 sec-1 min and 70°C for 10 min at 4°C for 30 cycles. PCR reaction mixtures contained 10 ml SYBR Green Premix Ex Taq (Takara Bio Inc., Dalian, China). Relative gene expression levels were normalized to GAPDH mRNA levels in each sample. Melting curve analysis for each primer set revealed only one peak for each product. The Applied Biosystems 7900HT fast real-time system (Thermo Fisher Scientific, Inc.) was used. Primer sequences are listed in Table I (Takara, Dalian, China) (19) and quantification was completed using the 2(-Delta Delta C(T)) method (20).

Western blot analysis. After the indicated treatments, Leydig cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4,

150 mM NaCl, 5 mM EDTA, 1 mM phenylmethane sulfonyl fluoride and 1% Triton X-100). Samples were fractionated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked with 10% nonfat milk and incubated at room temperature with primary antibodies for 2 h, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Li-cor Biosciences, Lincoln).

NE, USA; catalogue no. 926-32211, 926-68021) at a dilution of 1:10,000 at room temperature for 1 h. Images were obtained using Odyssey (LI-COR Biosciences, Lincoln, NE, USA). Membranes were then stripped and re-probed with GAPDH antibody to ensure equal protein loading. TNF-α (D2D4, 1:1,000) and AMPK (catalogue no. 23A3, 1:1,000) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Interleukin (IL)-1β antibody (catalogue no. CC36131) at a dilution of 1:1,000 was purchased from Bioworld Technology (Nanjing, China). Odyssey infrared imaging system (version 3.0.21; LI-COR Biosciences, Lincoln, NE, USA) was used for densitometry.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Statistical differences were analyzed with Student's *t*-test or one-way analysis of variance if there were more than two groups. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Behavior and body weight of mice. The behavior of mice in the LPS group showed obvious changes at 12 h after injection of LPS. They showed less activity and eating, and their body weight was reduced (Fig. 1). In the DMSO and case groups, only minor weight reductions were observed, while no reduction was present in the control group (Fig. 1).

Serum testosterone levels. Venous blood was collected from the mice and serum testosterone levels were detected using an ELISA kit. Testosterone levels were significantly reduced in the LPS group compared to that in the control, with only a minor reduction in the case group and no reduction in the DMSO group (Fig. 2).

Histology of mouse testes. Hematoxylin and eosin staining revealed that the structure of the testis was intact in the control animals (Fig. 3A), with a large number of Leydig cells localized to the gaps between convoluted seminiferous tubules. In the LPS group, the structure of the testis was altered and fewer Leydig cells were present, while the case group treated with roscovitine showed partial rescue of the changes induced by LPS (Fig. 3B and C). There were no obvious morphological changes in the DMSO group (Fig. 3D). In addition, the expression of 3βHSD was observed by immunohistochemical staining (Fig. 4), revealing a marked decrease in the LPS group compared to that in the other groups.

Roscovitine inhibits the expression of proinflammatory cytokines. As roscovitine was observed to exert protective

Table I. Primer sequences used for PCR.

Gene	Primer (5'-3')
Hsd17b3	F: ATTTTACCAGAGAAGACATCT
	R: GGGGTCAGCACCTGAATAATG
Cyp17a1	F: CCAGGACCCAAGTGTGTTCT
	R: CCTGATACGAAGCACTTCTCG
Cyp11a1	F: AGGTGTAGCTCAGGACTTCA
	R: AGGAGGCTATAAAGGACACC
Srd5a1	F: CACATCCTGCGGAATCTGA
	R: TGCTGCCTCGCTCTGGT
Cox2	F: TTCAACACACTCTATCACTGGC
	R: AGAAGCGTTTGCGGTACTCAT
iNOS	F: GTTCTCAGCCCAACAATACAAGA
	R: GTGGACGGGTCGATGTCAC
GAPDH	F: AGGTCGGTGTGAACGGATTTG
	R: TGTAGACCATGTAGTTGAGGTCA

F, forward sequence; R, reverse sequence; hsd, hydroxysteroid dehydrogenase; CYP, cytochrome P450; Srd5a1, steroid 5 alpha-reductase 1; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase.
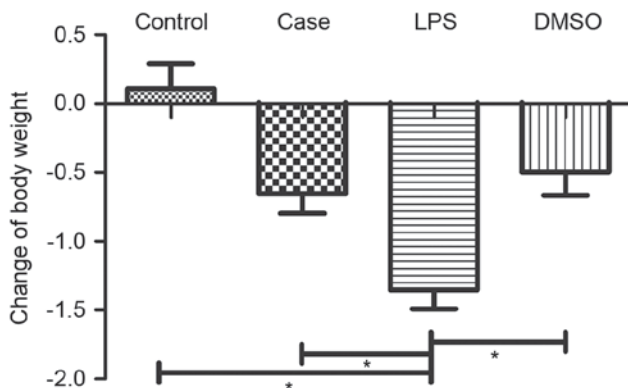

Figure 1. Change in body weight of the mice after drug treatment. Mice were weighed prior to and 12 h after the respective treatments. The mean weight change (g) in the LPS group was significantly greater compared to that in the other three groups. *P<0.05. Groups: Control, injected with saline; case, injected with roscovitine (5 mg/kg) dissolved in DMSO followed by injection of 5 mg/kg LPS in saline; LPS, injected with LPS only; DMSO, injected with DMSO only; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide.

effects on Leydig cells, it was hypothesized that roscovitine inhibits inflammatory and proinflammatory signaling pathways. To examine this hypothesis, western blot and RT-qPCR analyses were performed to detect inflammatory proteins and mRNAs in Leydig cells from mice of the various treatment groups. TNF-α and IL-1β were significantly reduced in the case group compared to the LPS group (Fig. 5A and B).

Roscovitine inhibits inflammation by upregulating AMPK. The western blot analysis results showed an obvious upregulation of AMPK levels in the case group compared to that in the other groups. Compared with the control group, a slight upregulation of AMPK expression was observed in the LPS group as well as a downregulation in the DMSO group (Fig. 5C).
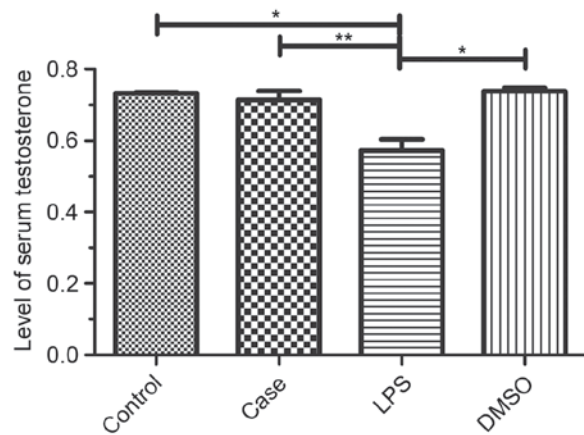

Figure 2. Serum testosterone levels. After 12 h of treatment, testosterone levels in venous blood were examined by ELISA. Serum testosterone was significantly lower in the LPS group compared to the other three groups. *P<0.05; **P<0.01. Groups: Control, injected with saline; case, injected with roscovitine (5 mg/kg) dissolved in DMSO followed by injection of 5 mg/kg LPS in saline; LPS, injected with LPS only; DMSO, injected with DMSO only; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide.

Roscovitine inhibit the pro-inflammatory gene. The protective effect of roscovitine in the case group was further confirmed by the inhibition of the pro-inflammatory genes cyclooxygenase (COX) -2 and inducible nitric oxide synthase (iNOS; Fig. 6A and B).

Roscovitine rescues LPS-induced inhibition of testosterone synthesis gene transcription. RT-qPCR was used to assess the effect of roscovitine on the transcription of the key reproduction-associated genes Hsd17b3, cytochrome P450 (Cyp) 17a1, Cyp11a1 and steroid 5 alpha-reductase 1 (Srd5a1). As shown in Fig. 7, LPS treatment significantly reduced the mRNA levels of each of these genes, while roscovitine partly attenuated these decreases, which was significant for Hsd17b3 and Cyp17a1 (Fig. 7A and B), but not for Cyp11a1 and Srd5a1 (Fig. 7C and D). There was a significant change in Srd5a1 expression in the DMSO group compared with that in the control group (Fig. 7C).

AMPK inhibitor reverses the effect of roscovitine. Leydig cells were induced with TNF-α and pretreated with or without AMPK inhibitor (compound C) for 1 h. Subsequently western blot and RT-qPCR were used to detect changes of the inflammatory factor COX-2. The results revealed that AMPK inhibitor reversed the inhibitory effect of roscovitine (Fig. 8).

Discussion

Inflammation in the male reproductive system has been considered to be one of the causes of male infertility (21-23). Infection of the male reproductive system can affect the growth of the testis through direct damage to testicular sperm tissue or lead to the obstruction of sperm delivery ducts (24). Leydig cells are important for the maintenance of male reproductive function. Hong *et al* (15) found that inflammation can inhibit the function of Leydig cells. Thus, it is important to further study inflammation and anti-inflammatory mechanisms. To explore possible treatments, the present study established an experimental inflammation model in mice employing LPS
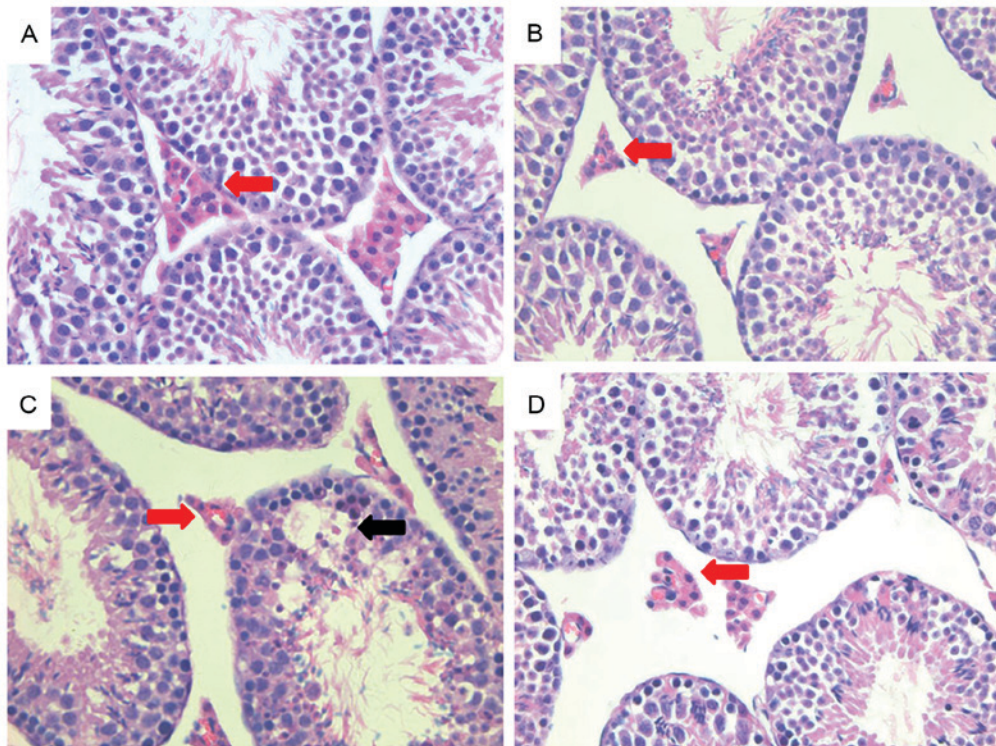
Figure 3. Interstitial morphology of mouse testes. Testicular tissue from each group was paraffin-embedded and stained with hematoxylin and eosin. (A) The intact structure of testicular tissue was clearly evident in the control group, with large numbers of Leydig cells present. (B) Tissue morphology appeared similar to that in the control tissue after treatment roscovitine and subsequently with LPS. (C) In testicular tissue from mice treated with LPS only, few Leydig cells were detected and tissue morphology was altered (black arrow). (D) After treatment with DMSO vehicle only, the structure was similar to that in the control group. Magnification x200. Leydig cells are indicated by red arrows. LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide.
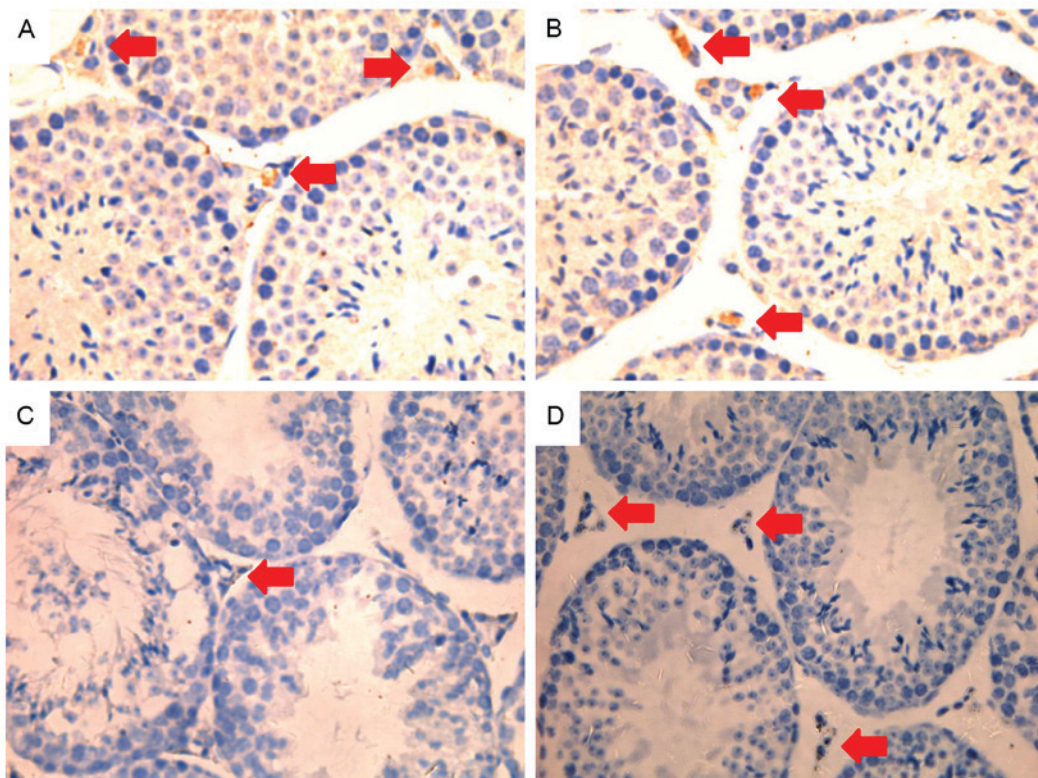

Figure 4. Expression of 3β-HSD in Leydig cells. 3β-HSD expression in testicular tissue obtained from mice in each group 12 h after each treatment was examined by immunohistochemistry. (A) Expression of 3β-HSD was observed in Leydig cells from control mice. (B) In mice treated with roscovitine and subsequently with LPS, 3β-HSD expression did not appear to differ from that in the control in extent or intensity. (C) Staining was clearly diminished in animals treated with LPS alone. (D) After treatment with DMSO vehicle only, 3β-HSD expression was similar to that in the control group. Red arrows indicate cells positive for 3β-HSD. Magnification x200. LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; 3β-HSD, 3β-hydroxysteroid dehydrogenase.
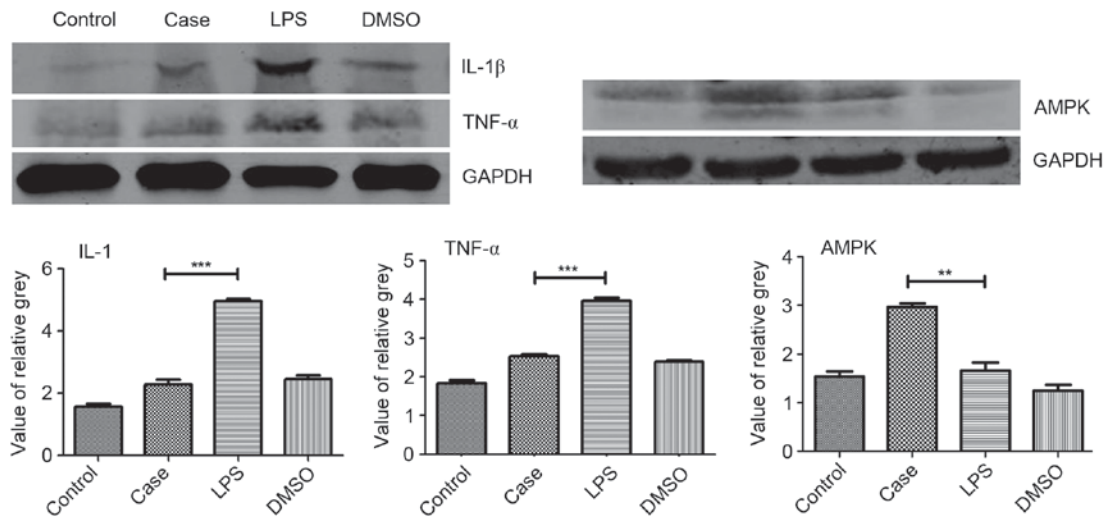
Figure 5. Protein expression of IL-1β, TNF-α and AMPK. Protein levels of IL-1β, TNF-α and AMPK were determined by western blot analysis of testicular tissue from each group 12 h after each treatment. Roscovitine was found to significantly increase the expression of AMPK compared with that in the other three groups. Furthermore, IL-1β and TNF-α expression in the case group were significantly downregulated compared with that in the LPS group. **$P<0.01$; ***$P<0.001$. Groups: Control, injected with saline; case, injected with roscovitine (5 mg/kg) dissolved in DMSO followed by injection of 5 mg/kg LPS in saline; LPS, injected with LPS only; DMSO, injected with DMSO only; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; TNF, tumor necrosis factor; IL, interleukin; AMPK, adenosine monophosphate-activated protein kinase.
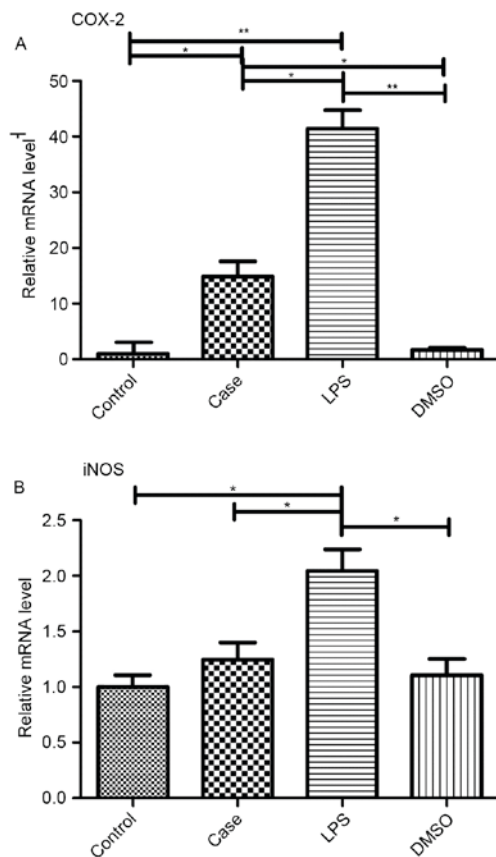

Figure 6. Transcriptional regulation of COX-2 and iNOS in Leydig cells. Gene expression of (A) COX-2 and (B) iNOS was examined by reverse-transcription quantitative polymerase chain reaction analysis 12 h after the indicated treatments. LPS alone significantly increased the expression of COX-2 and iNOS, which was inhibited by roscovitine, while COX-2 mRNA levels were still higher than those in the control and DMSO groups. *$P<0.05$; **$P<0.01$. Groups: Control, injected with saline; case, injected with roscovitine (5 mg/kg) dissolved in DMSO followed by injection of 5 mg/kg LPS in saline; LPS, injected with LPS only; DMSO, injected with DMSO only; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase.

as the most commonly used reagent for producing inflammation (17). The present study was using this mode following the successful experience of a previous study (19).

As a CDK inhibitor, roscovitine is a compound that competes for adenosine triphosphate binding sites on CDK (25). It has been demonstrated that roscovitine has significant anti-tumor effects (26-28) and can downregulate Mcl-1 expression (7). Other studies have confirmed that upregulation of AMPK activation inhibits the expression of Mcl-1 (8-10). In the present study, it was observed that roscovitine protected mice and their testes from inflammatory injury. Compared with the LPS group, mice in the case group were more active and had less weight reduction following drug treatment. Moreover, LPS induced damage in mouse testes and reduced 3β-HSD expression, which was attenuated by roscovitine. 3β-HSD is a key enzyme regulating dihydrotestosterone generation and is a good marker for Leydig cells (29-31).

Serum testosterone levels were then determined in the experimental groups, revealing LPS significantly decreased testosterone levels ($P<0.05$), with no significant change in the other three groups. Xue *et al* (32) previously reported that inflammation induced by LPS affects the levels of androgen in the microenvironment. It was therefore speculated that roscovitine may have a protective effect on androgen production in the testis.

Further study using the present inflammation model revealed that roscovitine downregulated the expression of inflammatory factors and proinflammatory genes, including TNF-α, IL-1β, COX-2 and iNOS. Inflammatory cytokines are mainly produced by activated lymphocytes and monocytes and are important in mediating inflammatory responses. Inflammatory cytokines such as IL-1β and TNF-α, secreted by endotoxin and immune complexes, may cause damage via autocrine and paracrine effects. COX-2 is an inducible enzyme similar to ring oxidase. It is highly expressed in inflammatory cells and catalyzes the synthesis of prostaglandin E2 and other
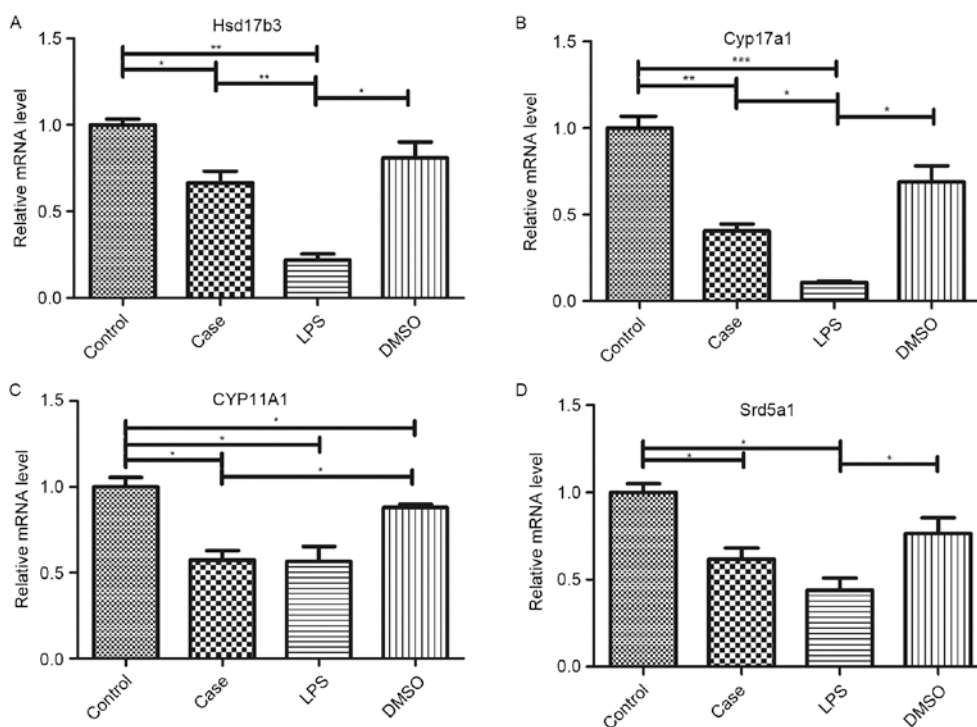
Figure 7. mRNA expression of testosterone synthesis genes. mRNA levels of (A) Hsd17b3, (B) Cyp17a1, (C) Cyp11a1 and (D) Srd5a1 were determined by reverse-transcription quantitative polymerase chain reaction analysis. While all genes were downregulated in the LPS group, this was preserved by co-treatment with roscovitine. The preserving effect was significant for Hsd17b3 and Cyp17a1 but not for Cyp11a1 and Srd5a1. *$P<0.05$; **$P<0.01$; ***$P<0.001$. Groups: Control, injected with saline; case, injected with roscovitine (5 mg/kg) dissolved in DMSO followed by injection of 5 mg/kg LPS in saline; LPS, injected with LPS only; DMSO, injected with DMSO only; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; hsd, hydroxysteroid dehydrogenase; CYP, cytochrome P450; Srd5a1, steroid 5 alpha-reductase 1.
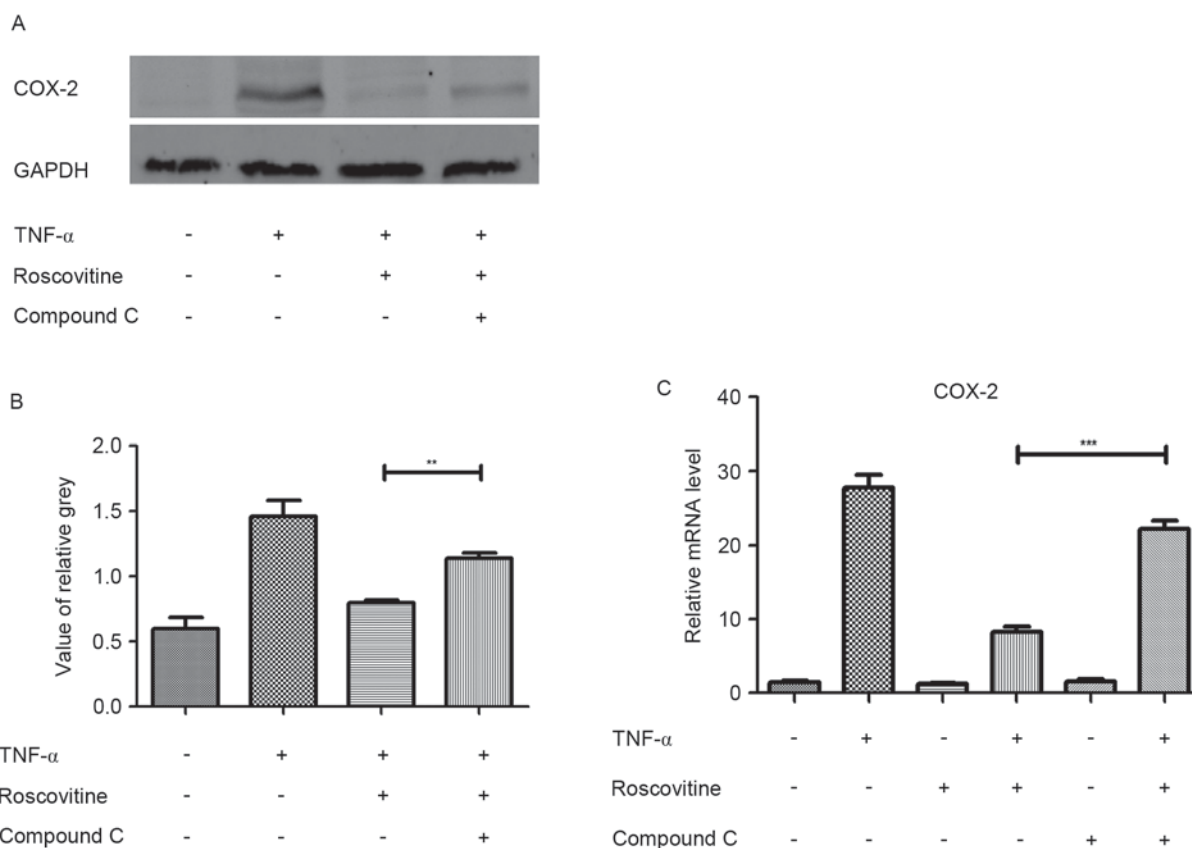

Figure 8. Effects AMPK inhibitor on the effect of roscovitine in Leydig cells *in vitro*. After stimulation with and TNF-α, AMPK inhibitor (Compound C) reversed the inhibitory effect of roscovitine and the expression levels of the proinflammatory cytokine COX-2 at (A and B) the protein level and (C) at the mRNA level. *$P<0.05$; **$P<0.01$; ***$P<0.001$. AMPK, adenosine monophosphate-activated protein kinase; TNF, tumor necrosis factor; COX, cyclooxygenase.

products at inflammatory sites to promote an inflammatory reaction resulting in tissue damage. Chen et al (33) found that LPS induced inflammatory reactions in RAW264.7 cells, as well as overexpression of COX-2 and iNOS. Yang et al (34) used LPS to treat microglial cell lines and observed overexpression of COX-2 and iNOS, which was inhibited by application of curcumin.

The pathways via which roscovitine inhibits inflammation were further examined, revealing that the roscovitine-induced overexpression of AMPK was inversely correlated with the production of inflammatory factors. AMPK is a cellular energy regulator. Studies have found that AMPK has a significant inhibitory effect on inflammatory cells, inflammatory factors and inflammation pathways (35-40). Therefore, AMPK and its associated signaling may be important pharmacological targets in the treatment of inflammatory diseases.

The present also investigated the expression of reproduction-associated genes in the experimental groups. Hsd17b3 and Cyp17a1 are key enzymes in testosterone synthesis. Yu et al (40) used dibutylphthalate to inhibit the expression of the Cyp17a1 gene, which reduced testicular rostenedione levels and inhibited testosterone synthesis in Leydig cells. A reduction in testosterone was also detected in male mice treated with LPS, and it was suggested that LPS treatment inhibited the expression of testosterone synthase. The results of the present study, showing diminished mRNA levels of Hsd17b3 and Cyp17a1 after LPS exposure, are consistent with this hypothesis. No significant affect was observed for Cyp11a1 and Srd5a1. However, roscovitine was demonstrated to inhibit these LPS-induced reductions.

In conclusion, to the best of our knowledge, the present study was the first to suggest that roscovitine has a protective role against inflammation in murine Leydig cells. The mechanism for these anti-inflammatory actions is likely to involve upregulation of AMPK. Future studies will further explore the specific roles of AMPK and its upstream and downstream signaling in mediating the development of testicular interstitial cell inflammation and examine the effect of roscovitine on these pathways.

Acknowledgements

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