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·*Original Article*·

Involvement of nuclear factor-kappa B on corticosterone-induced rat Leydig cell apoptosis

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Abstract

Aim: To investigate the activation of nuclear factor-kappa B (NF-kappa B) and its function in glucocorticoid-induced Leydig cell apoptosis. **Methods:** The Leydig cells were isolated from male Sprague-Dawley rats (90 days of age) and were incubated with corticosterone (CORT, glucocorticoid in rat) for 6 h, 12 h and 24 h, respectively. The P65 subunit of NF-kappa B (NF-kappa B/P65) in nuclei and the inhibitor of NF-kappa B (Ikappa B) in cytoplasm were analyzed by Western-blotting. The Leydig cells were treated with anti-Fas antibody for 3 h followed by Western blotting to assay the changes of NF-kappa B/P65 in nuclei and in cytoplasm. The role of NF-kappa B in CORT-induced Leydig cell apoptosis was evaluated by observing the effects of NF-kappa B/P65 overexpression and inhibiting activation of NF-kappa B by 100 μmol/L Pyrrolidine dithiocarbamate (PDTC) on this apoptosis. **Results:** The treatment of Leydig cells with CORT increased the levels of NF-kappa B/P65 in nuclei and decreased the levels of Ikappa B in cytoplasm. Following the Leydig cells were treated with anti-Fas antibody, the levels of NF-kappaB/P65 was increased in nuclei and decreased in cytoplasm. The CORT-induced Leydig cell apoptosis was inhibited by overexpressed NF-kappaB/P65 and was enhanced by incubation with PDTC. **Conclusion:** NF-kappa B is activated by increased FasL/Fas in CORT-induced Leydig cell apoptosis. NF-kappa B may play an anti-apoptotic role in this apoptosis. *(Asian J Androl 2006 Nov; 8: 693–702)*

Keywords: rat Leydig cell; NF-kappa B; apoptosis; corticosterone

1 Introduction

The ubiquitously expressed transcription factor nuclear factor-kappa B (NF-kappa B) plays a critical role in regulating inducible gene expression in immunity, cell apoptosis and inflammation [1–3]. Classical NF-kappa B is a heterodimer composed of the p50 and p65/RelA subunits, which exists in the cytoplasm in an inactive complex bound by the inhibitor of NF-kappa B (Ikappa B) proteins. Generally, the activation of NF-kappa B involves the degradation of Ikappa B. This permits NF-kappa B to translocate to the nucleus, where it activates gene transcription. NF-kappa B is involved in the regulation of cell survival [2]. Although NF-kappa B activation provides a survival-promoting signal in a majority of systems, it has been found to cause cell death in some cases [4]. In the past decade, many experiments have shown that NF-kappa B is involved in glucocorticoid-induced apoptosis and it is known that there is mutual transcriptional antagonism between glucocorticoid recep-

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tor (GR) and NF-kappa B [5].

Leydig cells in the testis secrete the steroid hormone testosterone, which is needed for fertility, libido, strength and vitality in adult men. Luteinizing hormone secreted by pituitary is the main factor that stimulates testosterone biosynthesis in Leydig cells. Recent studies have found that psychological and physiological stresses typically result in a suppression of Leydig cell steroidogenesis through sharp elevations in circulating glucocorticoid levels, and it is well known that glucocorticoid inhibits expression and activities of several enzymes in testosterone biosynthesis [6].

Our recent studies have found that excessive exposure to corticosterone (CORT, glucocorticoid in rat) initiates rat Leydig cell apoptosis, which is mediated by FasL/Fas and caspase-3 [7–8]. Although the GR mediates glucocorticoid-induced suppression of steroidogenesis in Leydig cells [9], it is unknown whether CORT-induced Leydig cell apoptosis is mediated by GR or whether NF-kappa B is involved in CORT-induced Leydig cell apoptosis. Whether this transcription factor antagonizes the potential GR-mediated apoptosis has not been investigated. The aim of the present study is to investigate activation of NF-kappa B and its function in CORT-induced Leydig cell apoptosis.

2 Materials and methods

2.1 Animals

Male Sprague–Dawley rats (90 days of age) were purchased from the Animal Centre of the Chinese Academy of Sciences of China (Shanghai, China). The animals were killed by CO_2 asphyxiation for isolation of Leydig cells.

2.2 Chemicals and reagents

Corticosterone (C2505), pyrrolidine dithiocarbamate (P8765), bovine lipoprotein (L3626), Percoll (P1644), DMEM-Ham's F12 (D2906), protease K (P2308) and RNase A (R6513) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Rabbit polyclonal anti-P65 antibody (sc-7151), anti-Fas antibody (M-20, sc-716), anti-Lamin B antibody and anti-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Ikappa B α antibody (06-494) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (170-6515) and Gel shift assay systems was obtained from Bio-Rad (Hercules, CA, USA).

2.3 Isolation of Leydig cells

Adult Leydig cells were isolated from 90-day-old rats according to the procedure of Sriraman *et al.* [10, 11], which is a modification of the procedure described by Klinefelter *et al.* [12]. The decapsulated testis was subjected to collagenase digestion in a 50-mL plastic tube containing 10 mL medium with collagenase (600 units) and DNase (750 units). The tubes were placed in a shaking water bath with constant agitation (50 times/min) at 34ºC for 15–20 min until the seminiferous tubules were separated. The enzyme action was terminated by adding excess medium. The tubules were allowed to settle by gravity and the medium, consisting of interstitial cells, was aspirated and filtered through a 100-μm nylon mesh. The filtrate was centrifuged at 250 × *g* for 10 min at 25ºC, which yielded a crude interstitial pellet. The pellet obtained was suspended in 35 mL 55% isotonic Percoll with 750 units DNase in Oakridge tubes. The tubes were centrifuged at 20 000 × *g* for 1 h at 4ºC. Percoll fractions corresponding to densities of 1.070–1.090 g/mL were collected and the cells present in this fraction were pelleted by centrifugation at 250 × *g* for 10 min at 25ºC after diluting with 3–4 volumes of medium. The purities of isolated cell fractions were evaluated by histochemical staining for 3β-hydroxysteroid dehydrogenase activity, with 0.4 nmol/L etiocholanolone as the steroid substrate [13]. The mean purity of Leydig cells was 85%.

2.4 Cell culture and treatments

Cell culture was conducted as previously described [14]. Briefly, freshly isolated Leydig cells were seeded in 50 mm Petri dishes (BD Falcon USA), at a density of 4×10^6 cells per flask, and cultured for 24 h in phenol red-free DMEM/Ham's F12 medium supplemented with 1 mg/mL bovine lipoprotein in an incubator gassed with 5% O_2, 5% CO_2, at 34ºC [14]. In the first line of the experiment, we treated cultured Leydig cells with CORT, RU486 and CORT plus RU486 for 12 h, respectively, followed by flow cytometric analysis for apoptotic frequencies of Leydig cells. In the second line of the experiments, the cultured Leydig cells were treated with 100 nmol/L CORT for 6 h, 12 h and 24 h *in vitro* respectively, then cells were collected with trypsin and the expression of NF-kappa B/P65 in nucleus and Ikappa Bα in cytoplasm in Leydig cells were analyzed by Western blotting. In the third line of the experiments, anti-

Fas antibody (analogue of FasL) was added to the cultures at a concentration of 5 μg/mL. Following the treatment for 6 and 12 h *in vitro*, genomic DNA was extracted and analyzed by agarose gel electrophoresis to detect the "DNA ladder", a characteristic biochemical feature of apoptosis. After the ability of the anti-Fas antibody to induce apoptosis was substantiated, isolated Leydig cells were incubated with 5 μg/mL anti-Fas antibody for 3 h and the levels of NF-kappa B/P65 in nuclei and in cytoplasm were measured by Western blotting. In the fourth line of the experiments, Leydig cells were incubated with pyrrolidine dithiocarbamate (PDTC) for 2 h and then treated with CORT for another 12 h. Other groups of Leydig cells were treated with vehicle (DMSO), PDTC and CORT for 12 h. Flow cytometric analysis for determining apoptotic frequencies of Leydig cells was performed at the end of treatments.

2.5 Preparation of cytoplasmic and nuclear extracts

The cytoplasmic and nuclear protein extracts were prepared according to the protocol of Schreiber *et al.* [15] with some modifications. Briefly, cultured cells were collected and washed twice with cold phosphate-buffered saline, lysed in 400 μL of cold buffer A (HEPES-NaOH 10 mmol/L pH 7.8, KCl 15 mmol/L, $MgCl_2$ 1 mmol/L, EDTA 0.1mmol/L, phenylmethanesulphonylfluoride [PMSF] 1 mmol/L, dithiothreitol (DTT) 1 mmol/L, leupeptin 1 mg/L). After 15 min incubation on ice, 0.1% NP-40 was added to the homogenates and the tubes were vigorously rocked for 1 min. Then the homogenates were centrifuged (10 000 × *g*, 20 s) in a microcentrifuge at room temperature. The supernatant fluid (cytoplasmic extracts) was collected and stored in aliquots at –70ºC. The nuclear pellet was washed once with cold buffer A, then suspended in 100 μL of cold buffer B (HEPES-NaOH 20 mmol/L pH 7.9, NaCl 420 mmol/L, $MgCl_2$ 1.5 mmol/L, EDTA 0.2 mmol/L, glycerol 25%, PMSF 0.5 mmol/L, DTT 0.5 mmol/L, leupeptin 1 mg/L), and incubated on ice for 30 min with rocking at maximum speed of 3 500 rpm between times. The solution was clarified by centrifugation at 12 000 × *g* for 4 min at –4ºC, and the supernatant fluid (nuclear extract) was stored in aliquots at –70ºC. The protein concentration was determined using a kit (Bio-Rad, Hercules, CA, USA) with bovine serum as a standard.

2.6 Western blot analysis

Nuclear or cytoplasmic extracts (20 μg total protein/lane) were separated by 12% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes using a wet blotting apparatus. Membranes were blocked (1 h, room temperature) in 5% non-fat dried milk plus Tris-buffered saline (TBS) plus 0.05% Tween-20 (blocking buffer), incubated with primary antibody (1 h, room temperature) in blocking buffer, and washed five times with TBS/0.05% Tween-20 before incubation (1 h, room temperature) with secondary HRP-conjugated antibody in blocking buffer. After successive washes, the membranes were developed with an enhanced chemiluminescence kit (ECL, Amersham, Arlington Heights, IL, USA). Anti-P65 Ab and anti-Ikappa Bα Ab were applied at a dilution of 1:500. HRP-conjugated IgG was applied at a dilution of 1:2 000. β-actin and Lamin B served as loading control of cytoplasmic and nuclear extracts, respectively. Semi-quantitative analysis of immunoreactivity was measured by GeneGenius system (SYNGENE, Cambridge, UK), and the results were expressed as absorbance ratio.

2.7 DNA extraction and agarose electrophoresis

Recent studies [16, 17] show that in some types of cells, either FasL or anti-Fas antibody that can induce apoptosis is able to activate NF-kappa B after binding to Fas through a signal pathway different from that of apoptosis triggered by FasL-Fas. We considered that increased levels of Fas and FasL in CORT-induced rat Leydig cell apoptosis might also activate NF-kappa B. The anti-Fas antibody was used to simulate Fas after establishing the fact that this antibody can induce Leydig cells apoptosis. Induction of apoptosis with anti-Fas Ab was examined by DNA fragmentation. DNA was extracted and analyzed by agarose gel electrophoresis as described by Wilson *et al.* [18]. Briefly, aliquots of 1×10^6 Leydig cells were collected 6 and 12 h after treatment with anti-Fas antibody. The samples were centrifuged at 500 × *g* for 5 min at 4ºC, and the supernatant was discarded and the pellet was resuspended in 20 μmol/L of lysis buffer (50 mmol/L Tris–HCl, pH 8.0, containing 10 mmol/L EDTA, 0.5% sodium lauryl sarcosinate and 0.5 μg/mL proteinase K) and incubated for 1 h at 50ºC. RNase A (10 μL, 0.5 mg/mL) was added and incubated for an additional 1 h at 50ºC. Low melting temperature agarose (10 μL, 1%) was added to the sample and 40 μL of each sample was placed into wells of 2% agarose gel (containing 10 μg/mL ethidium bromide), which was electrophoresed at 40 V for 2 h; DNA bands were visualized

by ultraviolet fluorescence.

2.8 Construction of nuclear factor-kappa B/P65 expression vectors

Total RNA was extracted from thymus tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Using avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany), 3 μg of total RNA were reverse transcribed. The coding sequence of NF-kappa B/P65 (1653 bp) was amplified using the polymerase chain reaction (PCR) primers 5'-CCGCTCGAGATGGACGATCTGTTTC-3' (forward) and 5'-CTAGTCTAGATTAGGAGCTGATCTGACTCAGAAGAGC-3' (reverse). PCR was performed for 34 cycles at 94ºC for 40 s; 60ºC for 40 s and 72ºC for 120 s. The fragments of the coding sequences of NF-kappa B/P65 were gel-excised using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After digestion with XbaI and XhoI, the purified PCR products were ligated to pcDNA3.0 vector (Invitrogen, Carlsbad, CA, USA) using T4 DNA ligase (Promega, Madison, WI, USA) to yield the full-length NF-kappa B expression plasmids.

2.9 Overexpression of nuclear factor-kappa B/P65 protein in Leydig cells

The Leydig cells were isolated and cultured in 24 well culture plates (1×10^6/well) in fresh medium for 24 h. Then, 1.0 μg of recombinant plasmids purified with the QIAQEN Plasmid Midi Purification kit (QIAGEN, Hilden, Germany) was transiently transfected into the Leydig cells using LIPOFECTAMINE 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. After another 24 h of culture, the expression of P65 protein was identified by Western blotting. The Leydig cells transfected with pcDNA3.0 vector served as control.

2.10 Effect of nuclear factor-kappa B/P65 overexpression on corticosterone-induced rat Leydig cell apoptosis

The transfected cells were cultured for 24 h. Then, after 100 nmol/L CORT (Sigma, St. Louis, MO, USA) was added to the medium, the cells were continually incubated for 12 h followed by analysis of apoptosis. The cells transfected with naked pcDNA3.0 and without vector served as controls. The apoptotic frequency of transfected Leydig cells and controls was assessed using Annexin-V/PI (BD Biosciences, San Jose, CA, USA) following the manufacturer's specifications. Binding of fluorescein-conjugated Annexin-V and propidium iodide in CORT-treated Leydig cells was analyzed by flow-cytometry (FACScan; Becton Dickinson, San Jose, CA, USA).

2.11 Statistics

Each experimental design was repeated for three times. The data were analyzed by pairwise multiple comparisons procedures with general linear model least squares means using the Bonferroni adjustment to identify significant differences between treatment and control [19]. Differences were regarded as significant at $P < 0.05$.

3 Results

3.1 Suppression of corticosterone-induced rat Leydig cell apoptosis by RU486

To observe whether CORT-induced rat Leydig cell apoptosis is a GR-mediated process, isolated Leydig cells were treated with CORT and CORT plus RU486 for 12 h. The effect of RU486 on CORT-induced Leydig cell apoptosis was analyzed by FACScan (Figure 1A-D). The results showed that RU486 blocked the CORT-induced Leydig cell apoptosis (Figure 1E).

3.2 Activation of nuclear factor-kappa B in corticosterone-induced rat Leydig cell apoptosis

Whether the activation of NF-kappa B is present in CORT-induced rat Leydig cell apoptosis was determined through observing the change in expression levels of NF-kappa B/P65 in nuclei and Ikappa B in cytoplasm (Figures 2, 3). A 60-kDa band corresponding to NF-kappa B/P65 in the nuclear extracts of control Leydig cells was observed (Figure 2A). An increase in the intensity of this band was seen after 6 h of exposure to 100 nmol/L CORT *in vitro*, with further increase at 12 h and 24 h (Figure 2B). A 35-kDa band corresponding to Ikappa B in cytoplasm was detected (Figure 3A). The intensity of this band was reduced following CORT treatment with 6 h and there was a significant decrease at 12 h and 24 h (Figure 3B).

3.3 DNA ladder electrophoresis

The DNA extracted from isolated Leydig cells treated with 5 μg/mL anti-Fas antibody for 6 h and 12 h was subjected to agarose gel electrophoresis. The charac-

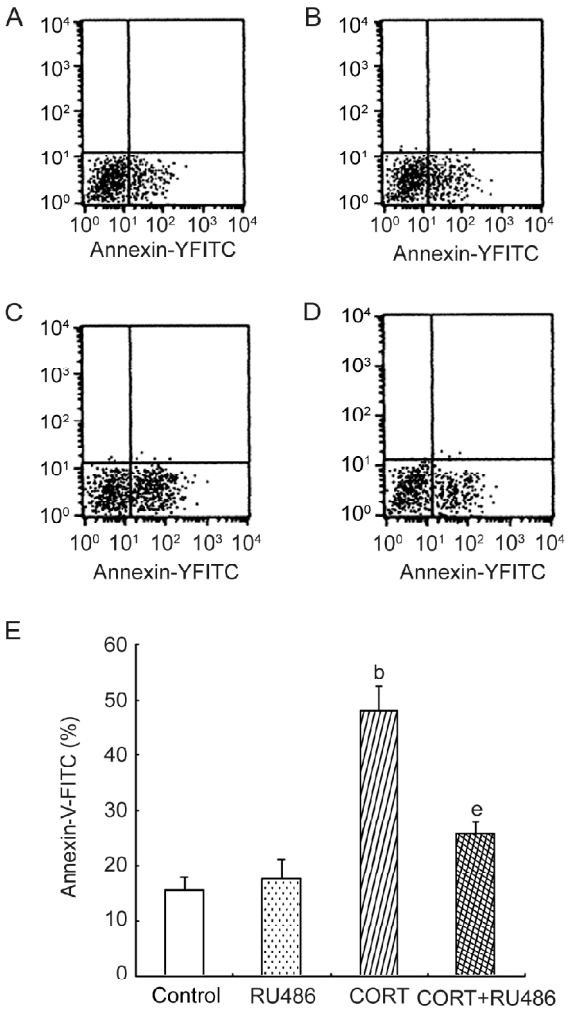

Figure 1. Effect of RU486 on corticosterone (CORT)-induced Leydig cell apoptosis. (A)-(D): isolated cells were incubated with 100 nmol/L RU486 (B), 100 nmol/L CORT (C) and 100 nmol/L RU486 plus 100 nmol/L CORT (D), respectively. The Leydig cells treated with vehicle (DMSO) served as control. After 12 h incubation, Leydig cells were analyzed by FACS with Annexin-V/PI double staining. Cells in the right lower quadrant were designated apoptotic (PI-negative/annexin V-FITC-positive), cells in left lower quadrant were designated living (PI-negative/annexin V-FITC-negative), cells in right upper quadrant were designated dead (PI-positive/annexin V-FITC-positive), and cells in left upper quadrant were designated damaged (PI-positive/annexin V-FITC-negative). (E): the Leydig cells treated with CORT showed increased frequencies of apoptotic labeling in the righ lower quadrant compared with control, $^{b}P < 0.05$, compared with the control. The Leydig cells treated with RU486 plus CORT showed decreased frequencies of apoptotic labeling in the righ lower quadrant compared with the cells treated with CORT alone. $^{e}P < 0.05$, compared with the control.

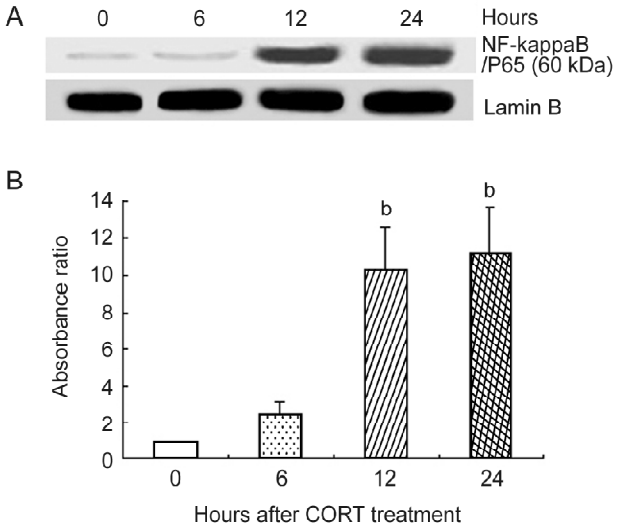

Figure 2. (A): Western blotting analysis of nuclear extracts of Leydig cells treated with corticosterone (CORT). Immunoblotting of the P65 subunit of NF-kappa (BNF-kappaB/P65) in nuclear extracts of Leydig cells from control and with 6-24 h CORT treatment. (B): Quantification of the resulting NF-kappaB/P65 band intensities. $^{b}P < 0.05$, compared with the control (0 h).

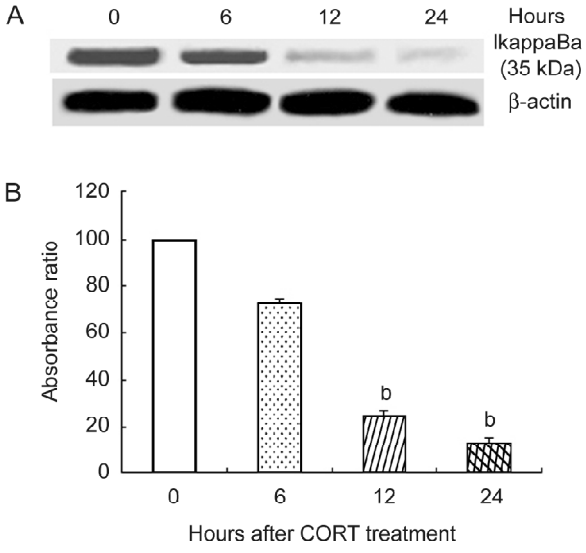

Figure 3. (A): Western blotting analysis of cytoplasmic extracts of Leydig cells treated with corticosterone (CORT). Immunoblotting of Ikappa B α in cytoplasmic proteins of Leydig cells from control and with 6–24 h CORT treatment. (B): Quantification of the resulting Ikappa B α band intensities. $^{b}P < 0.05$, compared with the control (0 h).

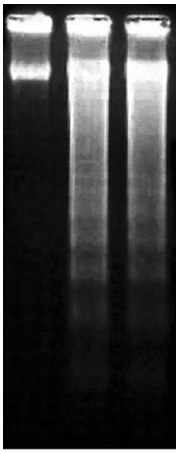

Figure 4. Agarose gel electrophoresis of genomic DNA of cultured Leydig cells from control (lane 1) and treated with 5 μg/mL anti-Fas Ab for 6 h (lane 2) and 12 h (lane 3).

teristic apoptotic DNA ladder representing the cleavage of DNA into multimers of 200 bp could be seen in all experimental sample lanes (Figure 4).

3.4 Activation of NF-kappa B with anti-Fas antibody

To evaluate the manner of NF-kappa B activation in CORT-induced Leydig cell apoptosis, we compared the levels of NF-kappa B/P65 in cytoplasmic extracts and nuclear extracts from Leydig cells treated with anti-Fas antibody, analogue of FasL, for 3 h with the corresponding controls by Western blotting (Figure 5A). There was a significant decrease in cytoplasm (Figure 5B) and a significant increase in the levels of NF-kappa B/P65 in nuclei (Figure 5C).

3.5 Inhibition of corticosterone-induced rat Leydig cell apoptosis by overexpression of NF-kappa B/P65

It was found that NF-kappa B/P65 is involved in CORT-induced rat Leydig cell apoptosis. In other words, there is an activation of NF-kappa B/P65 in this process. Although NF-kappa B/P65 has been shown to inhibit apoptosis in most kinds of cells, it is not clear whether NF-kappa B/P65 can protect rat Leydig cells from CORT-induced apoptosis. Western blot analysis showed that Leydig cells transiently transfected with NF-kappa B expression vector possessed higher expression level of NF-kappa B than control cells that were transfected with pcDNA 3.0 (data not shown). After treatment with CORT for 12 h, the apoptotic frequencies of cells transfected

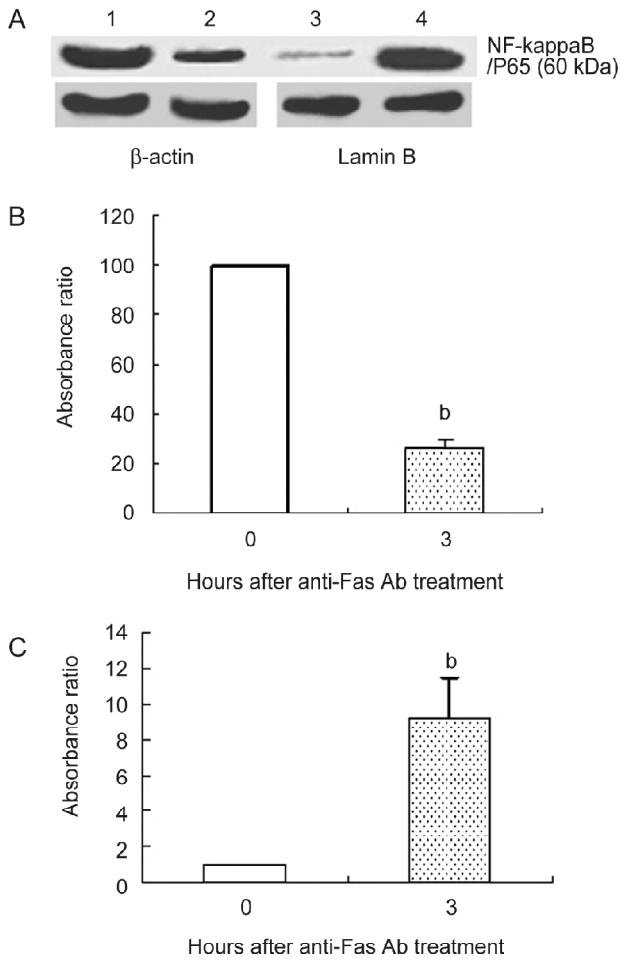

Figure 5. Western blotting analysis of cytoplasmic and nuclear extracts of Leydig cells treated with corticosterone (CORT). (A): Immunoblotting of NF-kappa B/P65 in cytoplasmic and nuclear extracts of Leydig cells from control and with 3 h anti-Fas Ab incubation. Lane 1, NF-kappa B/P65 in cytoplasmic extracts of controls; Lane 2, NF-kappa B/P65 in cytoplamic extracts of Leydig cells treated with anti-Fas Ab; Lane 3, NF-kappa B/P65 in nuclear extracts of controls; Lane 4, NF-kappa B/P65 in nuclear extracts of Leydig cells treated with anti-Fas Ab. (B)–(C): Quantification of the resulting NF-kappa B/P65 band intensities. (B) is corresponding to the level of NF-kappa B/P65 in cytoplasmic extracts; (C) is corresponding to the level of NF-kappa B/P65 in nuclear extracts. $^{b}P < 0.05$, compared with the control (0 h).

with NF-kappa B expression vector and controls was analyzed by FACScan (Figure 6A-C). It is shown that transfected cells that overexpress NF-kappa B/P65 are less susceptible to CORT-induced apoptosis than the controls (Figure 6D). These results suggest that NF-kappa B exerts antiapoptotic activity in CORT-induced rat Leydig cell apoptosis.

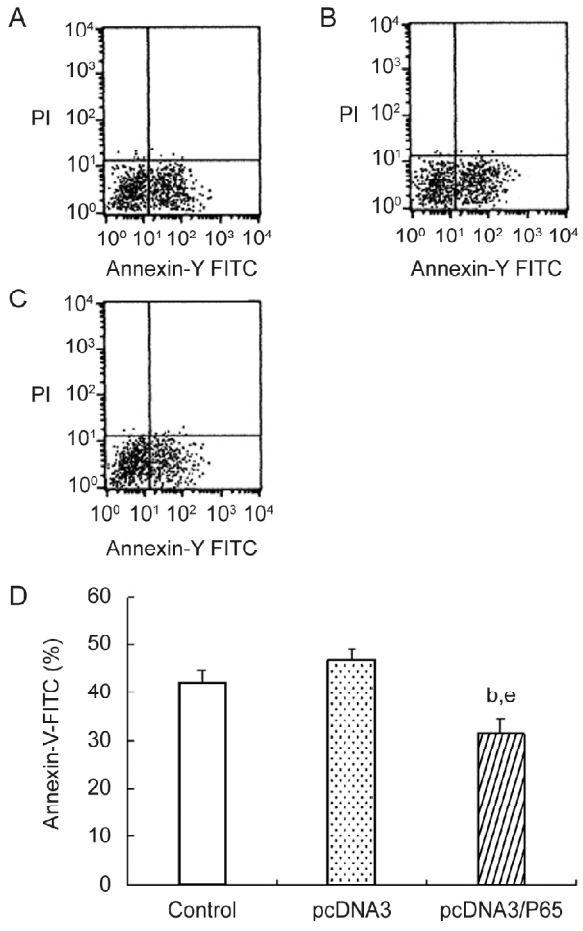

Figure 6. FACS analysis of annexin V-FITC labeling of apoptotic Leydig cells treated with corticosterone (CORT) *in vitro*. (A)–(C): Leydig cells were treated with 100 nmol/L CORT following 24 h transfection, and the frequency of apoptotic Leydig cells labeling with Annexin-V were analyzed by FACS. The Leydig cells of experimental group were transfected with the P65 subunit of NF-kappa (BNF-kappa B/P65) expression vectors (C), The cells transfected with naked pcDNA3.0 (B) and without vectors (A) served as controls. Cells in the right lower quadrant were designated apoptotic (PI-negative/annexin V-FITC-positive), cells in left lower quadrant were designated living (PI-negative/annexin V-FITC-negative), cells in right upper quadrant were designated dead (PI-positive/annexin V-FITC-positive), and cells in left upper quadrant were designated damaged (PI-positive/annexin V-FITC-negative). (D): Leydig cells transfected with NF-kappa B/P65 expression vectors showed decreased frequencies of apoptotic labeling in the right lower quadrant compared with the two controls, $^{b}P < 0.05$, compared with the control; $^{e}P < 0.05$, compared with pcDNA3.

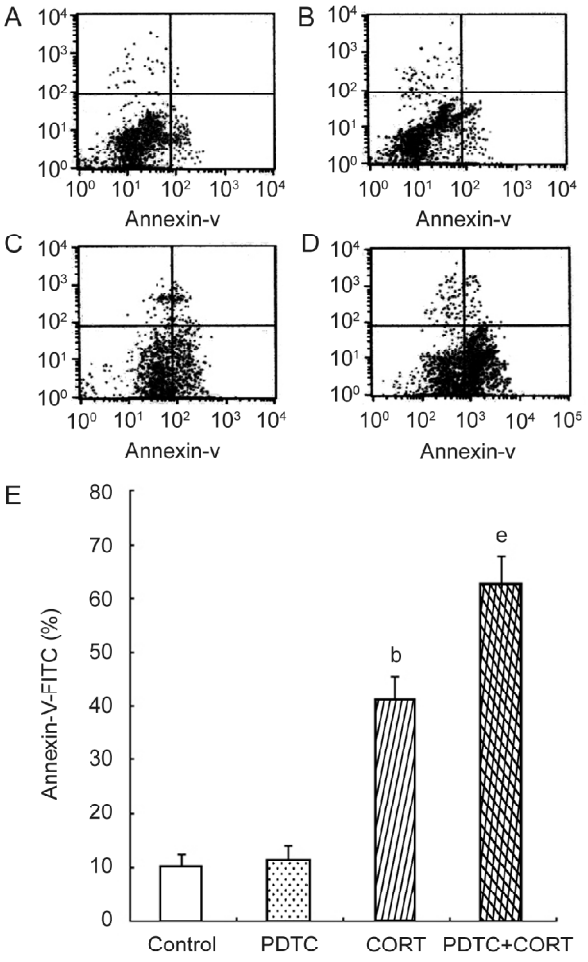

Figure 7. Effect of inhibition of NF-kappaB by Pyrrolidine dithiocarbamate (PDTC) on corticosterone (CORT)-induced Leydig cell apoptosis. (A)–(D): one group of Leydig cells were preincubated with 100 μmol/L PDTC for 2 h before 100 nmol/L CORT treatment for 12 h (D). Other groups of Leydig cells were treated with vehicle (DMSO) (A), 100 μmol/L PDTC (B), and 100 nmol/L CORT (C) respectively for 12 h. At the end of treatments, Leydig cells were analyzed by FACS with Annexin-V/PI double staining. Cells in the right lower quadrant were designated apoptotic (PI-negative/annexin V-FITC-positive), cells in left lower quadrant were designated living (PI-negative/annexin V-FITC-negative), cells in right upper quadrant were designated dead (PI-positive/annexin V-FITC-positive), and cells in left upper quadrant were designated damaged (PI-positive/annexin V-FITC-negative). (E): The Leydig cells treated with CORT showed increased frequencies of apoptotic labeling in the righ lower quadrant compared with control, $^{b}P < 0.05$, compared with the control. The Leydig cells treated with PDTC plus CORT showed increased frequencies of apoptotic labeling in the right lower quadrant compared with the cells treated with CORT alone, $^{e}P < 0.05$, compared with CORT.

3.6 Enhancement of corticosterone-induced Leydig cell apoptosis by inhibition of NF-kappa B activation

To further evaluate the function of NF-kappa B, the

effect of inhibition of NF-kappa B by PDTC, a well known inhibitor of NF-kappa B, on CORT-induced Leydig cell apoptosis was observed with FACScan (Figure 7A-D). It was shown that the apoptotic frequency of cells treated with PDTC plus CORT was higher than that of cells treated with CORT alone (Figure 7E). This result further indicates that NF-kappa B could protect Leydig cells against CORT-induced apoptosis.

4 Discussion

Recent studies reveal that the transcription factor NF-kappa B is a potential regulator of apoptosis [2]. Given that the antagonism between NF-kappa B and GR was identified in a variety of cell types, much interest has been devoted to the role of NF-kappa B in GR-mediated apoptosis and its activation manner [5]. Our previous work has established that high stress levels of CORT could induce rat Leydig cells apoptosis through activation of Fas/FasL, and subsequent caspase family [8]. However, it is unclear whether NF-kappa B is involved in CORT-induced Leydig cell apoptosis. The experiments reported herein are the first to demonstrate that NF-kappa B plays an anti-apoptotic role in CORT-induced Leydig cell apoptosis, and is activated by Fas/FasL signaling pathway.

Owing to the considerable role of NF-kappa B in GR-mediated apoptosis, our present work tested the involvement of NF-kappa B in this process following establishment of GR mediation in CORT-induced Leydig cell apoptosis. It is well known that CORT-induced inhibition of the testosterone biosynthesis in rat Leydig cells is a GR-mediated process. Blockade of GR by the receptor antagonist RU486 alleviates CORT-mediated suppression of testosterone production in Leydig cells [9]. It is very possible that CORT-induced Leydig cell apoptosis is also a GR-mediated process, but it has not yet been experimentally established. RU486, antagonist of GR, was used to treat isolated rat Leydig cells together with high stress levels of CORT, and it was found that RU486 decrease apoptotic frequency of Leydig cells compared to treatment with CORT alone. This result suggests that CORT-induced Leydig cell apoptosis is a GR-mediated process.

Recent findings [20] have enabled researchers to deepen their understandings of mechanisms of glucocorticoids-induced apoptosis. One hotspot of these findings is the crosstalk between pro-apoptotic and anti-apoptotic signaling. Much work has been devoted to the involvement of NF-kappa B, an apoptosis-related transcription factor, as a result of antagonism between it and GR in transcriptional regulation [5]. In unstimulated cells, NF-kappa B is kept in the cytoplasm through interaction with the inhibitory proteins termed as Ikappa B. When cells are exposed to inducers of NF-kappa B, Ikappa B is phosphorylated at two specific serine residues. This phosphorylation is a signal for ubiquitination and degradation of Ikappa B by the 26S proteasome. Free NF-kappa B dimers are released and translocated to the nucleus, where they activate transcription of target genes. Glucocorticoid can downregulate NF-kappa B-DNA binding before apoptosis occurs in some cases [21, 22]. Increased NF-kappa B DNA binding activity desensitizes CD4(+)CD8(+) double-positive thymocytes to glucocorticoid [23]. These findings suggest that NF-kappa B might be the anti-apoptotic factor in glucocorticoid-mediated apoptosis. Several studies reveal that glucocorticoids repress transactivation of NF-kappa B by upregulating expression of Ikappa B, which sequester NF-kappa B in an inactive cytoplasmic form [5]. Previous studies show that Ikappa B upregulation is predominantly and consistently observed in lymphocytes and monocytes, for instance, in glucocorticoid-sensitive 6TG1.1 human leukemic T cells apoptosis, dexamethasone elicited an increase in the amount of immunoreactive Ikappa Ba, the main member of Ikappa B family, whereas no such mechanism can be retrieved for endothelial or synoviocytes *in vitro* [24–26]. This suggests that different cell types might use alternative pathways to mediate glucocorticoid effects. To investigate the behaviour of NF-kappa B in CORT-induced rat Leydig apoptosis, we firstly examined the effect of CORT on expression of Ikappa B alpha with Western blotting. Surprisingly, the result showed that Ikappa B protein was significantly reduced at 12 h of incubation with CORT rather than increased. We suspected that activation of NF-kappa B (i.e. nuclear translocation) might be present in CORT-induced Leydig cell apoptosis following degradation of Ikappa B. Consequently, the effect of CORT on the level of NF-kappa B expression in nuclei was examined with Western blotting. The level of NF-kappa B expression in nuclei was dramatically increased at 12 h of Leydig cell treatment with CORT in accordance with the timing of Ikappa B degradation. As with our data mentioned above, Slater *et al.* [27] found that nuclear NF-kappa B DNA binding activity in rat immature CD4(+)CD8(+) thy-

mocytes is increased by methylprednisolone, which could induce thymocytes apoptosis. However, the activation manner of NF-kappa B in glucocorticoid-induced apoptosis is unclear.

Fas (APO-1/CD95), a prototype of the death receptors, is a member of the TNF alpha/nerve growth factor receptor superfamily, which directly recruits Fas Associated protein with Death Domain (FADD) and strongly induces apoptosis in a variety of cell types upon its ligation with FasL. However, the function of Fas does not appear to be exclusively restricted to the induction of apoptosis. For instance, Fas-FasL system plays pivotal roles in various aspects of immune regulation and function, such as self-tolerance [28] and cell-mediated cytotoxicity [29]. In the last decade, some studies have shown that ligation of Fas by FasL or agonistic anti-Fas antibody (analog for FasL) could stimulate NF-kappa B activation, irrespective of their sensitivity or resistance to Fas-mediated apoptosis [16, 17]. Our recent study [8] shows that excessive exposure to CORT increases expression of Fas and FasL in Leydig cells, which appears to be noted after 12 h of CORT administration. These increases in expression paralleled the timing of NF-kappa B activation represented above. We hypothesized that NF-kappa B activation is a result of Fas/FasL increase. To substantiate the above hypothesis, isolated Leydig cells were treated with agonistic anti-Fas antibody, which was used to achieve engagement of Fas, and we then examined the potential alteration of levels of NF-kappa B/P65 in nuclei and in cytoplasm with Western blotting. This anti-Fas antibody induced rat primary hepatocytes apoptosis [30]. The ability of the commercial anti-Fas antibody to induce Fas engagement was identified by detecting apoptosis induced by anti-Fas antibody in CORT-treated Leydig cells. After 6 h and 12 h of incubation with anti-Fas antibody, characteristic apoptotic DNA ladders representing the cleavage of DNA into multimers of 200 bp were shown. Western blot analysis revealed that there was an increase in NF-kappa B/P65 in nuclei and a decrease in cytoplasm of Leydig cells after 3 h of incubation with anti-Fas antibody. This result suggests that increased Fas/FasL might be responsible for the NF-kappa B activation in CORT-induced rat Leydig cell apoptosis.

In many types of cells, NF-kappa B activates anti-apoptotic gene whose products block the process of apoptosis [4]. However, NF-kappa B has been shown to serve as both a proapoptotic and antiapoptotic regulatory factor within the same cell type and whether NF-kappa B leads to cell survival or cell death is dependent on the environment [31]. Furthermore, NF-kappa B activation is required for synthetic bile acid derivatives induced apoptosis in human cervical carcinoma cells [32]. What kind of function NF-kappa B exerts in CORT-mediated rat Leydig cells apoptosis is not known. In the present study, overexpression of NF-kappa B/P65 in rat Leydig cells alleviated the CORT-induced apoptosis, although the effect was not dramatic owing to the relatively low efficiency of transient transfection. To further evaluate the function of NF-kappa B in this Leydig cell apoptosis, the effect of PDTC, which can inhibit NF-kappa B activation by blocking signal passage of NF-kappa B activation [33, 34], was observed. CORT-induced Leydig cell apoptosis was enhanced when activation of NF-kappa B was inhibited. These facts suggest that NF-kappa B serves as an anti-apoptotic factor in CORT-induced Leydig cell apoptosis.

CORT-induced Leydig cell apoptosis is a GR-mediated process. Activation of NF-kappa B attributed to FasL/Fas is present in CORT-induced Leydig cell apoptosis. NF-kappa B might potentially play an anti-apopotic role in the Fas-mediated killing.

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