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·*Original Article*·

NFAT2 is implicated in corticosterone-induced rat Leydig cell apoptosis

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Abstract

Aim: To investigate the activation of the nuclear factor of activated T cells (NFAT) and its function in the corticosterone (CORT)-induced apoptosis of rat Leydig cells. **Methods:** NFAT in rat Leydig cells was detected by Western blotting and immunohistochemical staining. Cyclosporin A (CsA) was used to evaluate potential involvement of NFAT in the CORT-induced apoptosis of Leydig cells. Intracellular Ca^{2+} was monitored in CORT-treated Leydig cells using Fluo-3/AM. After the Leydig cells were incubated with either CORT or CORT plus CsA for 12 h, the levels of NFAT2 in the nuclei and in the cytoplasm were measured by semi-quantitative Western blotting. The role of NFAT2 in CORT-induced Leydig cell apoptosis was further evaluated by observing the effects of NFAT2 overexpression and the inhibition of NFAT2 activation by CsA on FasL expression and apoptosis. **Results:** We found that NFAT2 was the predominant isoform in Leydig cells. CsA blocked the CORT-induced apoptosis of the Leydig cells. The intracellular Ca^{2+} level in the Leydig cells was significantly increased after the CORT treatment. The CORT increased the level of NFAT2 in the nuclei and decreased its level in the cytoplasm. CsA blocked the CORT-induced nuclear translocation of NFAT2 in the Leydig cells. Both CORT-induced apoptosis and FasL expression in the rat Leydig cells were enhanced by the overexpression of NFAT2 and antagonized by CsA. **Conclusion:** NFAT2 was activated in CORT-induced Leydig cell apoptosis. The effects of NFAT2 overexpression and the inhibition of NFAT2 activation suggest that NFAT2 may potentially play a pro-apoptotic role in CORT-induced Leydig cell apoptosis through the up-regulation of FasL. *(Asian J Androl 2007 Sep; 9: 623–633)*

Keywords: nuclear factor of activated T cells; corticosterone; Leydig cell; apoptosis

1 Introduction

Leydig cells are a preeminent source of the testosterone needed for fertility, libido, strength and vitality in the adult male [1]. The primary regulator of testosterone biosynthesis is luteinizing hormone, which maintains testosterone production in Leydig cells [2]. However, testosterone levels decline during stress, when glucocorti-

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coid levels are increased. The declines in testosterone production are due in part to a direct effect of increased corticosterone (CORT, the endogenous glucocorticoid in rat) on Leydig cells [3]. Our recent studies have demonstrated that exposures to high concentrations of CORT, such as the 100 nm levels that are typically achieved during stress, induced the apoptotic death of rat Leydig cells, mediated by FasL/Fas and caspase-3 [4].

Ca^{2+} has been implicated as a mediator of glucocorticoid-induced cell apoptosis for a number of years. There are significantly increased intracellular Ca^{2+} levels in lymphoid cells undergoing corticosteroid-induced apoptosis [5]. Various Ca^{2+} mediated apoptotic routes share the activation of the Ca^{2+}/calmodulin-dependent calcineurin (CaN) [6].

The nuclear factor of activated T cells (NFAT), which are the substrate for CaN, represent a family of Ca^{2+} dependent transcription factors. Four isoforms, NFAT1, NFAT2, NFAT3 and NFAT4, have been identified. The activities of NFAT proteins are tightly regulated by the Ca^{2+}/calmodulin-dependent CaN, which can be inhibited by cyclosporine A (CsA). CaN controls the translocation of NFAT proteins from the cytoplasm to the nucleus of activated cells [7].

NFAT, which are expressed in most immune system cells, play a pivotal role in the transcription of cytokine genes and other genes critical for the immune response [8]. Although originally thought to be largely restricted to cells of the immune system, NFAT has been shown to play a role in other cell types [9, 10]. In non-immune cells, NFAT has been shown to regulate heart valve development and control the differentiation of skeletal myocytes [11, 12]. It has also been suggested that NFAT plays a role in long-term memory in neurons [13].

It has been demonstrated that NFAT was implicated in glucocorticoid-induced thymocyte apoptosis and other several types of cell apoptosis [14, 15], but it was unknown whether NFAT was involved in glucocorticoid-induced Leydig cells apoptosis mediated by Fas/FasL. The aim of the present study was to investigate the activation of NFAT 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 center of the Academy of Science of China (Shanghai, China). The animals were killed using CO_2. All animal procedures were carried out in accordance with standards set by the Rockefeller University Animal Care and Use Committee (Protocol number 91200).

2.2 Chemicals and reagents

Corticosterone (C2505), Percoll (P1644), DMEM-Ham's F12 (D2906), protease K (P2308), Dimethyl Sulfoxide (DMSO, D-2650), CsA (C1832) and RNase A (R6513) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, U.S.A). Goat polyclonal anti-NFAT (sc-1049), donkey anti-goat immunoglobulin G-fluorescein isothiocyanate (IgG-FITC; sc-2024), anti-Lamin B and anti-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (170–6515) and Gel shift assay systems were obtained from Bio-Rad Co. (Hercules, CA, USA).

2.3 Isolation of Leydig cells

Adult Leydig cells were isolated from the 90-day-old rats according to the procedure of Sriraman *et al.* [16], which is a modification of the procedure described by Klinefelter *et al.* [17]. The decapsulated testes were 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 and constantly agitated (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 down by centrifugation at 250 × *g* for 10 min at 25ºC after diluting it with 3–4 volumes of the medium. The purities of the isolated cell fractions were evaluated by histochemical staining for 3β-hydroxysteroid dehydrogenase activity, with 0.4 nm etiocholanolone as the steroid substrate [18]. The enrichment of the Leydig cells was up to a purity of 85% on average.

2.4 Cell culture and treatments

The cell culture was conducted as previously described [19]. Briefly, freshly isolated Leydig cells were seeded in 50-mm Petri dishes (BD Falcon, Franklin Lakes, NJ, USA), at a density of 4×10^6 cells per dish, 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. In the first experiment, the cultured Leydig cells were collected with trypsin and the expression of NFAT2 in the Leydig cells was analyzed by Western blotting. In the second experiment, we treated the cultures of Leydig cells with either 100 nmon/L CORT or CORT plus 100 ng/mL Cyclosporin A (CsA) for 12 h. When CsA was used at a concentration of 100 ng/mL, it blocked the CORT-induced Leydig cells apoptosis without the ability to initiate the apoptosis of the Leydig cells alone. Analysis by flow-cytometry (FACS, Becton Dickinson, Franklin Lakes, NJ, USA) for the apoptotic frequencies of the Leydig cells and the DNA ladder, a characteristic biochemical feature of apoptosis, were performed. In the third experiment, intracellular Ca^{2+} was monitored in CORT-treated Leydig cells using the Ca^{2+}-sensitive dye, Fluo-3/AM. In the fourth experiment, the cultured Leydig cells were treated with CORT or CORT plus CsA as above, then the cells were collected with trypsin and the cytoplasmic and nuclear expression of NFAT2 in the Leydig cells were analyzed by Western-blotting. In the fifth experiment, the Leydig cells were transfected with pcDNA3.1 or pcDNA3.1-NFAT2 vector and then treated with a vehicle (DMSO), CORT or CORT plus CsA for 12 h, respectively. Analyses for apoptotic frequencies of Leydig cells and detection for FasL expression in the Leydig cells were performed at the end of the treatments.

2.5 Detection of NFAT2 expression by Western blotting

The expression of NFAT2 protein was detected by Western blotting. After being prepared with a radioimmunoprecipitation assay (RIPA) lysis buffer (Cat. No. 20–188, Upstate Co. Charlottesville, VA, USA) and phenyl-methane-sulphonylfluoride (PMSF, Cat. No. P7626, Sigma Co., St. Louis, MO, USA) at a dilution of 1:100, total protein was separated by 8% SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose membranes using a wet blotting apparatus. The membranes were blocked (for 1 h at room temperature) in 5% non-fat dried milk plus Tris-buffered saline plus 0.05% Tween-20 (blocking buffer), incubated with primary antibody (for 1 h at room temperature) in a blocking buffer and washed three times with Tris-buffered saline 0.05% Tween-20 before incubation (for 1 h at room temperature) with a secondary HRP-conjugated antibody in the blocking buffer. After successive washes, the membranes were developed with an enhanced chemi-luminescence kit (ECL, Amersham, Arlington Heights, IL, USA). Anti-NFAT2 antibody and HRP-conjugated IgG were applied at a dilution of 1:1 000 or 1:2 000. β-actin and Lamin B served as a loading control of the cytoplasmic and nuclear extracts, respectively. The semi-quantitative analysis of immunoreactivity was measured by GeneGenius system (Syngene, Cambridge, UK) and the results were expressed as optical density.

2.6 Immunohistochemical staining

NFAT immunohistochemistry was performed on the paraffin-embedded testis sections that were deparaffinized and rehydrated in graded concentrations of xylene and ethanol. The slides were washed (for 2 × 10 min) in phosphate-buffered saline (PBS), buffer A (PBS containing 0.1% Triton X-100; for 2 × 5 min), and buffer B (PBS containing 0.1% Triton X-100, 2% BSA; for 2 × 5 min). The sections were incubated overnight at 4ºC in buffer B containing anti-NFAT antibody at a dilution of 1: 200 or PBS, washed in buffer B (2 × 5 min), and incubated for 2 h at room temperature in FITC-conjugated anti-goat IgG. Following another rinse in buffer B (for 2 × 5 min) and PBS (for 2 ×10 min), the sections were dehydrated stepwise through xylene and ethanol. Randomly chosen interstitial spaces, defined as the space bounded by at least three seminiferous tubule profiles, were evaluated. All measurements were performed blind on an image analysis workstation (Zeiss LSM-510 META, Postfach, Germany).

2.7 Apoptosis assay

The annexin-V and DNA ladder assay were used to assess apoptosis. For the annexin-V assay, the Leydig cells were incubated with 10 μL propidium iodide (PI) and 5 μL FITC-annexin-V (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 15 min. Then, the Leydig cells were analyzed on FACS. Annexin-V binds to those cells that express phosphotidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of cells with a compromised cell membrane. This allows for the discrimination between live cells (unstained with either fluorochrome) from apoptotic cells

(stained only with annexin-V) and necrotic cells (stained with both annexin-V and PI). To detect damage to the nuclear chromatin, DNA was extracted and analyzed by agarose gel electrophoresis, as described by Wilson *et al*. [20]. Briefly, aliquots of 1×10^6 Leydig cells were collected 12 h after treatment with CORT or CORT plus CsA treatment. The samples were centrifuged at 500 × *g* for 5 min at 4ºC and then 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 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. The DNA bands were visualized by UV fluorescence.

2.8 Assays of intracellular Ca^{2+} level in CORT-treated Leydig cells

The Leydig cells were divided the two groups, one treated with the vehicle (DMSO) that served as the control, the other one treated with 100 nm CORT. The intracellular Ca^{2+} was monitored using the Ca^{2+}-sensitive dye, Fluo-3/AM. The intracellular Ca^{2+} were labeled with Fluo-3/AM at a concentration of 5 μmol/L for 30 min at 37ºC, then abandoned the medium with Fluo-3/AM, washed with medium twice. The dye-loading glass coverslips were transferred to an observation chamber mounted on the stage of an inverse microscope equipped with laser confocal scanning microscopy (LCSM, Zeiss KS 400, Postfach, Germany). The level of intracellular Ca^{2+} at the resting stage was measured first. Following this, 100 nm CORT was added to the observation chamber, and the change in the Ca^{2+} concentration was monitored. Confocal fluorescence images of 512 × 512 pixels were recorded every 5 s. Ca^{2+} labeling with fluo-3/AM was excited at 488 nm krypton/argon laser. The emitted light was detected at 505–530 nm. The numerical aperture of the objective was 1.0 and that of the ocular was 3.0.

2.9 Preparation and analysis of cytoplasmic and nuclear extracts

The cytoplasmic and nuclear protein extracts were prepared according to the protocol of Schreiber *et al*. [21] with some modifications. Briefly, after culture, the cells were collected and washed twice with cold PBS, 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 0.1 mmol/L, 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* for 20 s) in a micro centrifuge 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, being rocked at maximum speed between several 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. Nuclear or cytoplasmic extracts (20 μg total proteins) were analyzed by Western blotting as described above.

2.10 Construction of NFAT2 expression vectors

The vector pblue-NFAT2 was generously provided by Prof. Anjana Rao (Center for Blood Research and Department of Pathology, Harvard Medical School, Boston, MA, USA). After digestion with ScaI and EcoRI, the purified NFAT2 products were ligated to pcDNA3.1 vector (Invitrogen, Grand Island, NY, USA) using T4 DNA ligase (Promega, Madison, WI, USA) to yield the pcDNA3.1-NFAT2 expression vector.

2.11 Transient overexpression of NFAT2 protein in Leydig cells

The Leydig cells were isolated and cultured in 24-well culture plates (1×10^6/well) in a fresh medium for 12 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, Grand Island, NY, USA) according to the supplier's protocol. After another 24 h of culture, the expression of NFAT2 protein was identified by Western blotting. The Leydig cells transfected with pcDNA3.1 vector served as the control.

2.12 Effect of NFAT2 overexpression on CORT-induced Leydig cell apoptosis

The transfected cells were cultured for 24 h. Then, after 100 nm CORT or 100 nm CORT plus CsA was added to the medium, the cells were continually incubated for 12 h followed by an analysis of the apoptosis. The cells transfected with the empty expression vector pcDNA3.1 served as the control. The apoptotic frequency of the transfected Leydig cells and the controls was assessed using annexin-V/PI (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's specifications. The binding of the fluorescein-conjugated Annexin-V and PI in CORT-treated Leydig cells was analyzed by flow cytometry.

2.13 Effect of NFAT2 overexpression on FasL expression in CORT-induced Leydig cell apoptosis

The transfected cells were cultured for 24 h. Then, 100 nm of CORT or 100 nm CORT plus CsA was added to the medium and the cells were further incubated for 12 h followed by Western blot analyses. These analyses were performed as described in section 2.5 above, but the primary antibody, anti-FasL antibody, was applied at a dilution of 1:500.

2.14 Statistical analysis

Each experimental design was done in triplicate. The data were analyzed by pairwise multiple comparisons procedures with a general linear model least squares means via Bonferroni adjustment to identify significant differences between the treatment and the control (SAS Institute, 2000). Differences were regarded as significant at $P < 0.05$.

3 Results

3.1 Expression of NFAT2 in Leydig cells

To verify whether NFAT was involved in the CORT-induced apoptosis of the Leydig cells, the levels of NFAT2 protein in the Leydig cells were assayed by Western blotting. Using an antibody against NFAT2, Western blotting analysis detected a 100 kDa band, which was similar to the band in T cells (Figure 1). NFAT2 fluorescence was identified in Leydig cells by immunohistochemical staining. (Figure 2). These data suggest that NFAT2 was present in rat Leydig cells.

3.2 Suppression of CORT-induced Leydig cell apoptosis by CsA

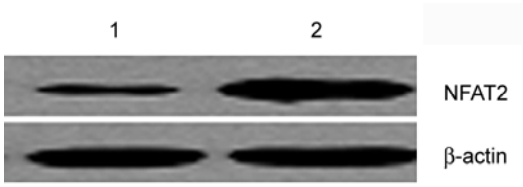

Figure 1. Expression of nuclear factor of activated T cells (NFAT) protein in Leydig cells by Western blotting. Lane 1, the lysates extracted from Leydig cells; Lane 2, the lysates extracted from T cells as a positive control. Beta-actin was used as a control to monitor the protein quality. The proteins were resolved by SDS-PAGE and immunoblotted with anti-NFAT2. NFAT2 protein in Leydig cells run as high molecular mass bands, which is similar to that in T cells. The experiments were repeated at least twice with identical results.

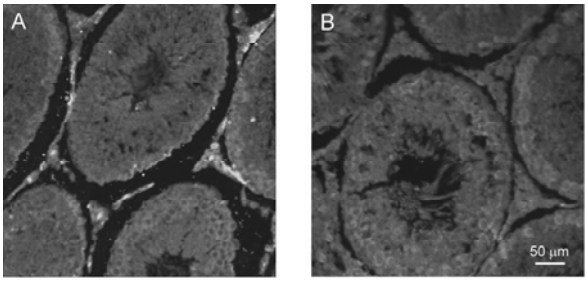

Figure 2. Immunohistochemical localization of nuclear factor of activated T cells (NFAT) in rat testes. Asterisks indicate Leydig cells that were positively stained for NFAT. (A): NFAT2 expression in Leydig cells. (B): NFAT2 immunostainings were abolished by incubating with Phosphate-buffered saline (PBS) buffer instead of anti-NFAT antibody. Scale bar = 50 μm.

CsA inhibits CaN, thus preventing the dephosphorylation of NFAT and its translocation to the nucleus. We selected CsA as an inhibitor to evaluate whether NFAT is potentially implicated in CORT-induced Leydig cell apoptosis. Isolated Leydig cells were divided into three groups, which were treated with vehicle, 100 nm CORT and CORT plus CsA for 12 h, respectively. The effect of CsA on CORT-induced Leydig cell apoptosis was analyzed by FACS (Figure 3A). The results showed that CsA blocked the CORT-induced Leydig cell apoptosis (Figure 3B). In addition, DNA prepared from isolated Leydig cells cultured for 12 h in the presence of vehicle, 100 nm CORT, and CORT plus CsA, was subjected to agarose gel electrophoresis. The characteristic apoptotic

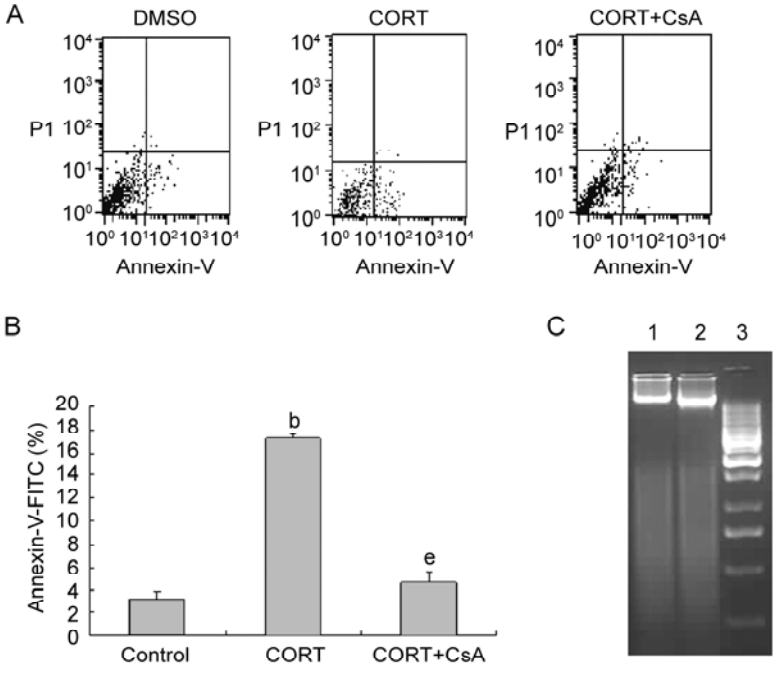

Figure 3. Effect of Cyclosporin A (CsA) on corticosterone (CORT)-induced Leydig cell apoptosis. (A): Isolated cells were incubated with either 100 nm CORT or 100 ng/mL CsA plus 100 nm CORT. The Leydig cells treated with a vehicle (Dimethyl Sulfoxide, DMSO) served as control. After 12-h incubation, the Leydig cells were analyzed by FACS with annexin-V/PI double staining. The cells in the right lower quadrant were designated apoptotic (propidium iodide (PI)-negative/annexin V-fluorescein isothiocyanate-positive), the cells in the left lower quadrant were designated alive (PI-negative/annexin V-FITC-negative), the cells in right upper quadrant were designated dead (PI-positive/annexin V-FITC-positive), and the cells in left upper quadrant were designated damaged (PI-positive/annexin V-FITC-negative). (B): The Leydig cells treated with CORT showed increased frequencies of apoptotic labeling compared with the control. $^{b}P < 0.05$, compared with the control. The Leydig cells treated with CORT plus CsA showed decreased frequencies of apoptotic labeling compared with the cells treated with CORT alone. $^{e}P < 0.05$, compared with CORT. (C): The agarose gel electrophoresis of the DNA extracted from the Leydig cells. Lane 1 shows the DNA extracted from the vehicle-treated Leydig cells for 12 h; lane 2 shows the Leydig cells after a 12-h exposure to CORT plus CsA; lane 3 shows the Leydig cells after a 12-h exposure to CORT.

DNA ladder representing the cleavage of DNA into multimers of 200 bp could be seen in the experimental sample lane 3 (Figure 3C) except for the controls treated with the vehicle (lane 1) and the group treated with CORT plus CsA (lane 2). These results indicate that CsA blocks CORT-induced Leydig cell apoptosis. Since CsA also regulates NFAT intracellular localization, these data suggest that NFAT could be involved in CORT-induced Leydig cell apoptosis.

3.3 Measurement of intracellular Ca^{2+} concentration in CORT-treated Leydig cells

To measure changes in the intracellular Ca^{2+} concentration caused by CORT treatment, the levels of Ca^{2+} labeled with Fluo-3/AM were determined by LCSM. The results showed that 100 nm CORT treatment results in a sharp increase of Ca^{2+} levels (about five-fold) in cultured Leydig cells (Figure 4).

3.4 Activation of NFAT2 in CORT-induced Leydig cell apoptosis

Whether the activation of NFAT2 occurs in CORT-induced Leydig cells apoptosis remains unknown. This was determined through observing the change in expression levels of NFAT2 in the nuclei and cytoplasm (Figure 5), A stronger band corresponding to NFAT2 was observed in the cytoplasmic extracts of the control Leydig cells (Figure 5A, lane 1), the cytoplasmic extracts of the Leydig cells treated with 100 nm CORT plus CsA for 12 h (Figure 5A, lane 3) and the nuclear extracts of the Leydig cells treated with 100 nm CORT for 12 h (Figure 5A, lane 5). A decrease in the intensity of this band was seen

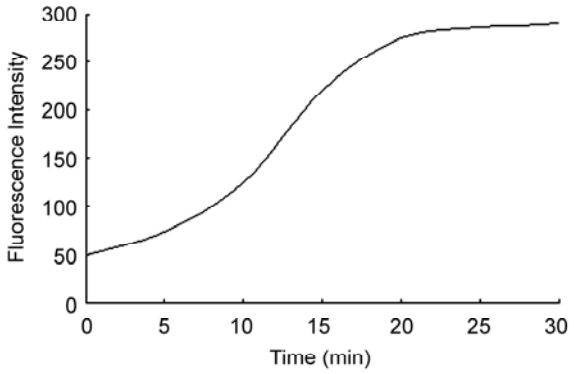

Figure 4. Measurement of the intracellular Ca^{2+} concentration in Leydig cells treated with corticosterone (CORT). Intracellular Ca^{2+} labeling with Fluo-3/AM was measured by laser confocal scanning microscopy. Before the Leydig cells were treated, the fluorescence intensity was steady. When treated with 100-nm CORT for 20 min, the fluorescence intensity significantly increased to more than five-fold that of the untreated Leydig cells.

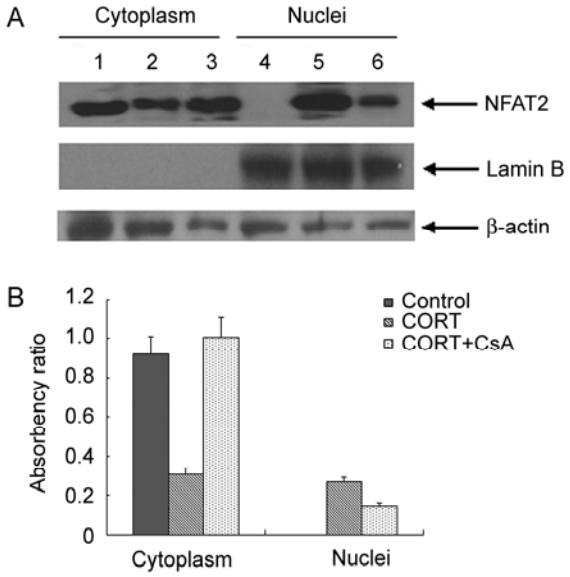

Figure 5. Analysis of nuclear factor of activated T cells (NFAT) protein in the cytoplasm and nuclei by Western blotting. (A): The nuclear and cytoplasmic proteins were extracted and subjected to Western blotting for the detection of the NFAT2 protein. Lamin B was used as a nuclear protein control. Immunoblotting of NFAT2 in cytoplasmic and nuclear proteins of Leydig cells from the control, with a 12-h corticosterone (CORT) treatment and with a 12-h CORT plus Cyclosporin A (CsA) treatment. NFAT2 was present in the cytoplasm of the Leydig cells without CORT treatment, while it increased in the nucleus of CORT-treated Leydig cells. This suggested that the treatment with CORT increased the level of nuclei NFAT2 in the Leydig cells. The quantification of the resulting NFAT2 band intensities is shown in (B). Values are mean ± SD.

in the cytoplasmic extracts of the Leydig cells treated with 100 nm CORT for 12 h (Figure 5A, lane 2) and the nuclear extracts of the Leydig cells treated with 100 nm CORT plus CsA for 12 h (Figure 5A, lane 6). The results indicate that the activation of NFAT occurs in CORT-induced Leydig cell apoptosis, i.e., that NFAT2 exists in the cytoplasm of resting Leydig cells and that it translocates into the nucleus upon CORT treatment. This translocation can be inhibited by CsA.

3.5 Enhancement of CORT-induced Leydig cell apoptosis by overexpression of NFAT2

To further study the implication of NFAT in CORT-induced Leydig cell apoptosis, an NFAT2 expression vector was transfected into isolated Leydig cells. Western blotting was then performed to detect NFAT2 in cell lysates 12 h later. As expected, Leydig cells transiently transfected with the NFAT2 expression vector expressed higher levels of NFAT2 than the control cells (Figure 6A). After a 12-h CORT treatment, the apoptotic frequencies of cells transfected with NFAT2 expression vector and controls were analyzed by FACS (Figure 6B). It was shown that the transfected cells overexpressing NFAT2 were more susceptible to CORT-induced apoptosis than the control (Figure 6C). Furthermore, the addition of CsA alleviated CORT-induced Leydig cell apoptosis (Figure 6). These results further support a role for NFAT2 in CORT-induced Leydig cell apoptosis.

3.6 Up-regulation of FasL by NFAT2 in CORT-induced Leydig cell apoptosis

Our previous study showed that FasL is a pro-apoptotic regulator in CORT-induced Leydig cell apoptosis [4]. To assess whether NFAT2 enhancement of apoptotic frequency involves FasL up-regulation in CORT-treated Leydig cells, the expression levels of FasL in Leydig cells transfected with NFAT2 expression vector and controls were analyzed by Western blotting. After being treated with CORT for 12 h, the transfected cells which overexpress NFAT2 possessed a higher expression level of FasL than the control cells. The addition of CsA alleviated the CORT-induced up-regulation of FasL expression (Figure 7). Taken together, these results suggest that NFAT2 may potentially play a pro-apoptotic role in CORT-induced Leydig cell apoptosis through the up-regulation of FasL.

4 Discussion

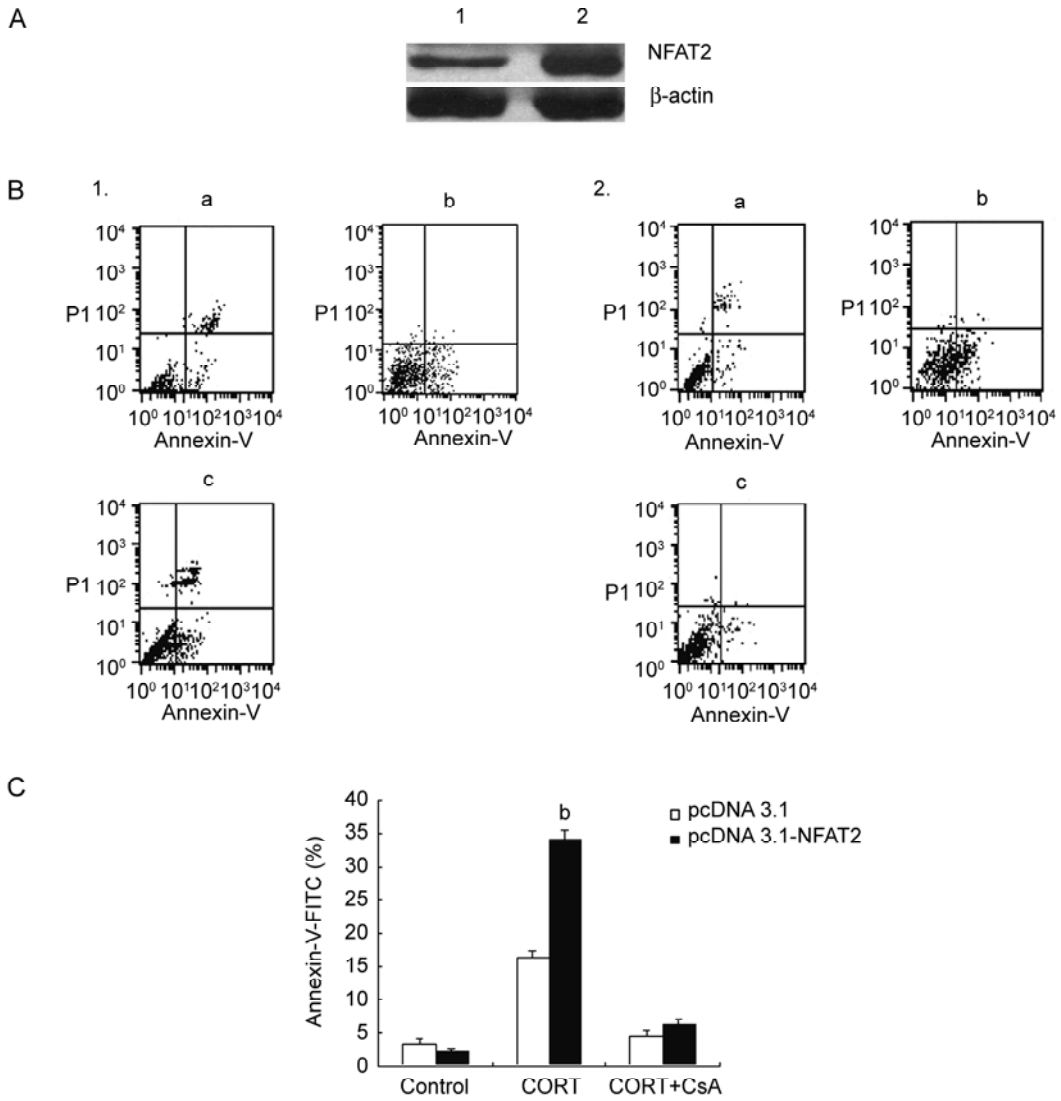

Figure 6. Analysis of apoptotic frequencies of Leydig cells with nuclear factor of activated T cells (NFAT) overexpression. (A): Western blot shows the overexpression of NFAT2 protein in the Leydig cells. Lane 1, the NFAT2 protein level in Leydig cells transfected with pcDNA3.1; Lane 2, the NFAT2 protein level in Leydig cells transfected with pcDNA3.1-NFAT2. β-actin was used as a control to monitor the protein quality. Leydig cells transiently transfected with the NFAT2 expression vector possessed a higher expression level of NFAT2 than the control cells which were transfected with pcDNA 3.1. (B): An annexin-V assay was performed, as described in the materials and methods section. 1. Leydig cells transfected pcDNA3.1 as a control were assayed in the presence of a vehicle (Dimethyl Sulfoxide, DMSO), corticosterone (CORT) and CORT plus Cyclosporin A (CsA); 2. Leydig cells overexpressed NFAT2 were assayed in the presence of vehicle (DMSO), CORT and CORT plus CsA. (C): As shown in Figure 5D, the NFAT2 overexpression of the Leydig cells treated with 100 nm CORT for 12 h showed increased frequencies of apoptotic labeling, compared with Leydig cells without an NFAT2 expression vector. $^{b}P < 0.05$, compared with the Leydig cells without an NFAT2 expression vector.

A high concentration of CORT induces the apoptosis of rat Leydig cells, thus causing a decreased testosterone level [4]. The present study is the first to demonstrate that the activation of NFAT2 is associated with the CORT-induced apoptosis of rat Leydig cells via the up-regulation of FasL. The ubiquitous transcription factor NFAT is regulated primarily at the level of its subcellular localization through the actions of the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase CaN. In resting cells, NFAT family members are normally located in the

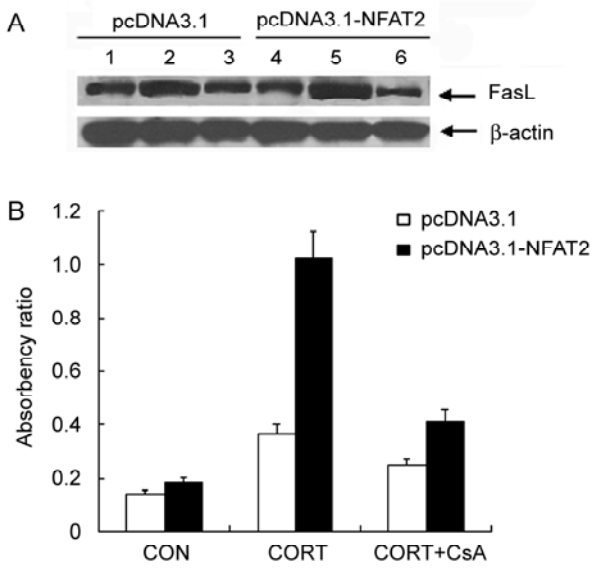

Figure 7. (A): Detection of expression of FasL in overexpressed nuclear factor of activated T cells (NFAT) Leydig cells by Western blotting. The band intensity was normalized to the β-actin product generated using a specific antibody to β-actin. Lane 1, expression of FasL in Leydig cells transfected with pcDNA3.1. Lane 2, expression of FasL in Leydig cells transfected with pcDNA3.1 and treated with corticosterone (CORT) for 12 h. Lane 3, expression of FasL in Leydig cells transfected with pcDNA3.1 and treated with CORT plus Cyclosporin A (CsA) for 12 h. Lane 4, expression of FasL in Leydig cells transfected with pcDNA3.1-NFAT2. Lane 5, expression of FasL in Leydig cells transfected with pcDNA3.1-NFAT2 and treated with CORT for 12 h. Lane 6, expression of FasL in Leydig cells transfected with pcDNA3.1-NFAT2 and treated with CORT plus CsA for 12 h. Quantification of the resulting FasL band intensities is shown in (B). Values are mean ± SD.

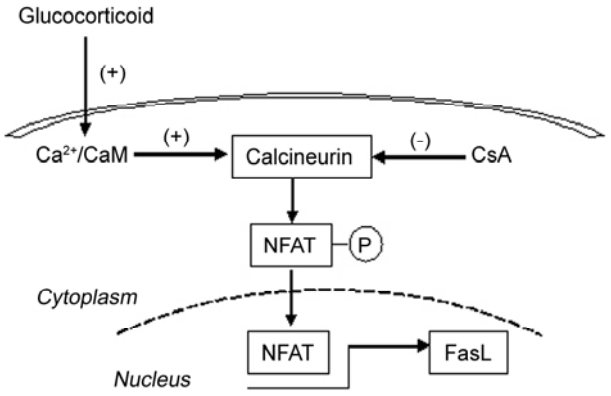

Figure 8. Model for Ca^{2+} Calcineurin nuclear factor of activated T cells (NFAT) signal pathway in corticosterone (CORT)-induced rat Leydig cell apoptosis. CORT-treatment increases intracellular Ca^{2+} concentration in the rat Leydig cell through an unknown, transcription-independent mechanism. This activates calcineurin, which dephosphorylates NFAT2, thus permitting nuclear translocation. Activated NFAT2 induces FasL expression, which is involved in CORT-induced Leydig cell apoptosis. This process may be blocked by inhibition of calcineurin by Cyclosporin A (CsA).

cytoplasm in a hyper-phosphorylated latent form. However, following an increase in the intracellular Ca^{2+} concentration, activated CaN directly dephosphorylates NFAT proteins, inducing their translocation to the nucleus and increasing their intrinsic DNA binding activity. Once located in the nucleus, NFAT is then free to bind to its target promoter elements and activate the transcription of specific NFAT target genes, either alone or in combination with other nuclear partners [7].

Although NFAT has recently received considerable attention outside the immune system [9], the existence of NFAT in Leydig cells had not been demonstrated. In the present study, our preliminary results suggested that NFAT1, NFAT2, NFAT3 and NFAT4 all are expressed in rat Leydig cells. But the NFAT2 is the predominant isoform expressed in Leydig cells. NFAT1 showed the lowest level of expression in this type of cell. Both NFAT3 and NFAT4 had a middle level of expression compared with NFAT1 and NFAT2 (personal unpublished data). In addition, NFAT2 was identified in Leydig cells by immunohistochemical staining. Thereby, we further confirm the expression of NFAT2 in rat Leydig cells. Our present work is the first to report that NFAT is expressed in Leydig cells.

As substrates for CaN, NFAT proteins are major targets of the immunosuppressive drug CsA that has revolutionized transplant surgery since the introduction of CsA in 1983 [22]. In activated T-cells, the predominant outcome of CsA action is the inhibition of CaN, which prevents the dephosphorylation of NFAT and its translocation to the nucleus, and thus abolishes its transcriptional activity [23, 24]. In present experiment we selected CsA as an inhibitor to observe whether NFAT is potentially implicated in CORT-induced Leydig cell apoptosis. The high level of CsA could induce apoptosis in the rat hepatocyte [25] and in U937 cells [26]. Based on this condition, in the present experiment, the Leydig cells were treated with CsA at a concentration of 100 ng/mL, which could antagonize CORT-induced Leydig cell apoptosis but not induce apoptosis by itself. Our present results

showed that CsA blocked the CORT-induced Leydig cell apoptosis (Figure 3A). Since CsA also regulates NFAT intracellular localization, these data suggest that NFAT could be involved in CORT-induced Leydig cell apoptosis.

The increase in intracellular Ca^{2+} concentration is presumed to be required for NFAT dephosphorylation by the Ca^{2+}/CaM-dependent protein CaN [7]. In present experiment, there was increased Ca^{2+} concentration in the Leydig cells following CORT treatment for 5 min (Figure 4). The result was similar to Tong's report, which showed that dexamethasone rapidly increases intracellular Ca^{2+} within 3 min in Jurkat T cells through a transcription-independent mechanism [27].

Our recent work has established that high stress levels of CORT could induce Leydig cell apoptosis through the activation of Fas/FasL and the subsequent caspase family [4]. In the present study, the expression of NFAT2, the effect of CsA, which can inhibit NFAT activation by blocking the signal passage of NFAT activation and increased Ca^{2+} concentration were observed in the CORT-treated Leydig cells. All these data further suggest the involvement of NFAT in CORT-induced Leydig cell apoptosis.

As mentioned above, NFAT proteins are dephosphorylated by CaN, i.e. translocated from the cytoplasm to the nucleus, and become transcriptionally active [7]. Indeed, we found that the level of NFAT2 expression in the nuclei was dramatically increased at 12 h of Leydig cells treatment with CORT. The inhibition of CaN by CsA can prevent the nuclear translocation of NFAT2, therefore, when Leydig cells were treated with CsA, NFAT2 remained in the cytoplasm (Figure 5A). The present findings suggest that NFAT2 expressed in Leydig cells can be activated, i. e. from the cytoplasm into the nuclei by stimulation with CORT. These increases in nuclei paralleled the timing of the onset of apoptosis as detected by annexin-v labeling (Figure 3). This further suggests that NFAT2 is implicated in CORT-induced Leydig cell apoptosis.

Although NFAT has been found be associated with apoptosis in several types of cell [10], the transcription factors show various roles in different cells. In immune cells, for instance, NFAT activates apoptotic genes whose products activate the process of apoptosis. However, NFAT is a critical survival factors that inhibit cardiomyocyte apoptosis [28]. In addition, it was reported that methamphetamine induces neuronal apoptosis via FasL up-regulation, which is partially due to the activation of Ca^{2+}-CaN-NFAT signaling system [29]. What kind of function NFAT exerts in CORT-mediated Leydig cells apoptosis was not known. In present study, we found that the transient overexpression of NFAT2 in Leydig cells enhanced the expression of FasL when the cells were treated with a high concentration of CORT for 12 h and this promoted the Leydig cell apoptosis. When the NFAT2 nuclear translocation was inhibited by blocking CaN, we observed an alleviation of the CORT-induced up-regulation of FasL expression and a strong decrease of Leydig cell apoptosis (Figure 7A). These facts suggest that NFAT2 is involved in the CORT-induced Leydig cell apoptosis through the up-regulation of FasL (Figure 8).

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