Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular Immunology 43 (2006) 915–926
Molecular Immunology
www.elsevier.com/locate/molimm

Nonylphenol-induced thymocyte apoptosis involved caspase-3 activation and mitochondrial depolarization

Genhong Yao [a,b], Lingsong Yang [b], Yali Hu [c], Jun Liang [b], Junfeng Liang [d], Yayi Hou [a,b,*]

[a] *Jiangsu Province Key Lab of Reproductive Medicine, Nanjing Medical University, Nanjing 210029, China*
[b] *Immunology and Reproductive Biology Lab, Medical School, Nanjing University, Nanjing 210093, PR China*
[c] *The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing 210008, PR China*
[d] *Department of Chemistry and Chemical Biology, Stevens Institute of Technology, Hokoken, NJ 07030, USA*

Received 1 May 2005
Available online 25 July 2005

Abstract

Although the effect of 4-nonylphenol on cells of immune system have long been recognized, little is known about the effect of 4-nonylphenol on the induction of apoptosis and related signaling events in the lymphoid cells. In the present study, we used cultured thymocytes of mice to investigate the ability of 4-nonylphenol to induce the apoptosis of thymocytes and to explore the role of signal transduction pathway leading to apoptosis. The results showed that the cytotoxic effects of 4-nonyphenol involved DNA fragmentation (DNA ladder), characteristic of apoptosis. Staining of 4-nonyphenol-treated thymocytes with DNA-binding fluorochrome Hoechst 33258 showed the typical apoptotic nuclei condensation and fragmentation of chromatin. The rates of apoptosis of the 4-nonylphenol-treated thymocytes increased significantly at 4 and 6 h, which were determined by analysis of hypodiploid cells and FITC-Annexin V and PI double staining. Flow cytometer analysis also revealed that the loss of mitochondrial membrane potential and increased activity of caspase-3 occurred concomitantly with the onset of 4-nonyphenol-induced apoptosis. Furthermore, a caspase-3 inhibitor, z-DEVD-fmk protected thymocytes from apoptosis induced by 4-nonyphenol. These results suggest that 4-nonylphenol induces thymocyte apoptosis via caspase-3 activation and mitochondrial depolarization.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Nonylphenol; Thymocytes; Apoptosis; Mitochondrial depolarization; Caspase-3

1. Introductions

Nonylphenol (NP) is not produced naturally. It is the major degradation products of alkylphenol ethoxylate and is one of the most common chemical contaminants. NP presents in ambient air, water, soil, sediments and biota. It enters aquatic ecosystems through sewage treatment, pulp mill effluent, industrial effluent, urban and agricultural runoff (Ying et al., 2002). NP is not water soluble because of its hydrophobic properties. It also favors anaerobic environment and is stabilized, thus there are high concentrations of the toxic compound NP in anaerobically stabilized sewage sludge compared to those of other contaminants (Giger et al., 1984). Studies showed that the concentration of NP in the aquatic environment, particularly in sediment, could reach up to 300 ppb (Nagao et al., 2001). NP is accumulated in our bodies through the food chain. Concern has increased recently about the NP because it can mimic natural hormones and the levels present in the environment may be sufficient to disrupt endocrine, immunology, and reproduction (Chapin et al., 1999; Masuno et al., 2003; Ying et al., 2002). However, most previous studies, including ours have focused on the toxicity of NP on development process and reproductive system, especially in aquatic ecosystems (Kinnberg et al., 2000; Odum and Ashby, 2000; Rankouhi et al., 2004; Sweeney, 2002; Wang et al., 2003). Little is known about the toxicological effect of NP on immunocompetent cells, animal and human immune system. To address this issue, the present study focuses on the effects of NP

* Corresponding author. Tel.: +86 25 83686441; fax: +86 25 83686441.
E-mail address: yayihou@nju.edu.cn (Y. Hou).

0161-5890/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2005.06.031

on the thymocyte, which is an important immunocompetent cell.

Apoptosis is the most common form of cell death of the immune system. Without death by apoptosis, the life of the immune cells and their precise and specific function would be impossible (Krammer, 2000). Apoptosis is a central regulatory feature of the immune system. Deregulation of the apoptotic process leading to either increased or reduced cell death can contribute to various pathologic conditions such as autoimmune diseases, AIDS or cancer.

Thymus, the primary lymphoid organ of mammals plays a critical role in immune response. It generates large numbers of functionally mature antigen specific T cells. These T cells then migrate to the peripheral lymphoid tissue to mediate protection against invading microbes. Thymocyte apoptosis has been shown to act in the immune system, including limiting over-expression of an immune response by deleting unwanted lymphocytes and eliminating auto reactive T cells during thymus selection. This may be of primary benefit in maintaining immunological and physiological homeostasis by eliminating unnecessary cells through apoptosis (Shames, 2002).

Although the immunotoxicity of NP has long been recognized, the effects of NP on cell death and its underlying mechanisms are not clear. Our previous study has demonstrated that NP induced thymocyte apoptosis in vivo (Yao and Hou, 2004). To our knowledge, the signal transduction mechanism of apoptosis induced by NP in thymocytes in vitro has not been reported. The present study was designed to investigate the ability of NP to induce apoptosis directly in thymocytes and explore its signaling pathway.

2. Materials and methods

2.1. Materials

The 4-nonylphenol was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Cell Counting Kit was from Dojin Laboratories (Kumamoto, Japan). Hoechst 33258 was purchased from Beyotime Biotechnology Inc. (Nantong, China). PI and RNAse A were obtained from Sigma (St Louis, MO, USA). FITC-Annexin V was purchased from Bender Medsystems (Vienna, Austria). JC-1 mitochondrial membrane potential detection assay kit was from Biotium Inc. (Hayward, CA, USA). CaspGLOW fluorescein active caspase-3 and caspase-8 staining kit was purchased from Bio-Vision (Research, Mountain View, California). IETD-fmk and z-DEVD-fmk were purchased from Becton Dickinson Company (Palo Alto, CA, USA). All other reagents were of the highest grade commercially available.

2.2. Thymocyte preparation and culture

Mice (3-week-old and weighing 20–30 g) were obtained from Animals Center of Jingling Hospital of Nanjing University. The animal experiments were carried out in compliance with the institutional guideline. The thymus was obtained from normal 3-week-old mice. To prepare a single-cell suspension, the cells were teased out from freshly removed thymus in chilled phosphate-buffed saline (PBS), filtered through a sterile wire mesh and centrifuged at $400 \times g$ for 5 min. Pelleted thymus cells were resuspended in Tris-buffered 0.83% NH_4Cl solution to lyse erythrocytes. The resulting thymus cells were washed in ice-cold PBS (containing 10% FBS) three times and adjusted to a cell density of 1×10^6 cells/ml. The viability was determined by trypan blue dye exclusion and was routinely more than 95%. The thymocytes were seeded in RPMI 1640 (without phenol red) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Then the thymocytes were treated with different concentrations of NP or vehicle control at 37 °C in a humidified 5% CO_2 atmosphere (final volume 100 μl/well) and were harvested at various time points after seeding.

2.3. Cytotoxicity assays

Cell viability was assessed with a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan) according to Ishiyama et al. (1997) with slight modifications. Briefly, thymocytes were plated on a 96-well (100 μl/well) microplate at a density of 1×10^6 cells/ml. NP at various concentrations was added and the plate was incubated at 37 °C in humidified 5% CO_2 for 2, 4, and 6 h. 10 μl of a solution containing WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt) was added to each well and was incubated for an additional 2 h. The absorbance was measured at 450 nm on an automated microplate reader (Bio-Rad Model 550).

2.4. Analysis of nuclear morphology

Nuclear morphology was detected using the method of Araki et al. (1987). NP-treated cells were fixed with methanol acetic acid for 10 min followed by staining with Hoechst 33258 at 1 mg/ml staining at room temperature in dark for 5 min. The cells were then washed twice with PBS, examined and immediately photographed under a fluorescence microscope (Nikon Corporation, Chiyoda-ku, Tokyo, Japan) with an excitation wavelength of 330–380 nm. Apoptotic cells were defined on the basis of nuclear morphology changes, such as chromatin condensation and fragmentation.

2.5. DNA laddering detection

To examine apoptosis by electrophoresis of nucleosomal fragments, a standard procedure for precipitating cytosolic nucleic acid was used (Jenkins et al., 2001). Briefly, 1×10^6 NP-treated thymocytes were pelleted ($200 \times g$, 5 min) and lysed at 4 °C for 15 min (250 μl, 0.4% Triton-X, 20 mM Tris, and 0.4 mM EDTA). Nuclei were then pelleted (13,000 × *g*, 5 min, 4 °C) and the supernatant were transferred to a clean

microfuge tube. Nucleosomal fragments were precipitated overnight with an equal volume of isopropanol after adjustment to 0.5 M NaCl. The pellet was washed twice in 70% ethanol, dried briefly, and resuspended in 40 μl TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.8). The extracted DNA was separated on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and visualized on an UV-transilluminator and photographed. Fragmented DNA, shown as DNA ladder in the gel, indicates apoptotic cell death.

2.6. Analysis of hypodiploid cells

Analysis of hypodiploid cells was performed using PI staining (Nicoletti et al., 1991). In brief, thymocytes (1×10^6 cells) in 1 ml RPMI 1640 were added to each well of 12-well plates and treated at 37 °C with various concentrations of NP for 2, 4, and 6 h, respectively. The cells were fixed in 75% ethanol for 30 min at room temperature. Then they were stained with propidium iodide staining buffer (Trixon X-100, EDTA, RNAse A, PI) for 10 min at dark. The fluorescence of PI was monitored by a FACSCalibur flow cytometer (Becton-Dickson, Immunocytometry System, San Jose, CA) with an excitation wavelength of 488 nm and an emission wavelength of 625 nm. Apoptotic cells were determined on a PI histogram as a hypodiploid. For each sample, 20,000 cells were analyzed. The data were analyzed using Cellquest software (Palo Alto, CA, USA).

2.7. FITC-Annexin V and PI double staining

NP induced thymocytes apoptosis other than necrosis. Annexin-V FITC/PI double stain assay was used to examine NP-induced thymocyte apoptosis, not necrosis according to a procedure described by Vermes et al. (1995). Briefly, thymocytes cultured in the presence or absence of NP were washed in cold PBS and resuspended in binding buffer (HEPES buffered PBS supplemented with 2.5 mM $CaCl_2$) prior to the addition of FITC-labeled Annexin V for 10 min at room temperature. Propidium iodide (PI) was added at a final concentration of 2 μg/ml 5 min before a final wash in PBS and immediate analysis of the cells on the flow cytometer. Flow cytometry was performed on thymocytes gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. Apoptotic cells were defined as $FITC^+/PI^-$ cells. The gated thymocytes were then plotted for Annexin V-FITC and PI in a two-way dot plot to assess percentage of apoptotic thymocytes.

2.8. Determination of the mitochondrial membrane potential by JC-1 fluorescence

JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 nm) to red (590 nm). The most widely implemented application of JC-1 is for the detection of mitochondrial depolarization during the early stages of apoptosis (Salvioli et al., 1997). Briefly, 1×10^6 thymocytes were incubated with 2.5 μg/ml JC-1 in the dark for 10 min at room temperature in complete medium. Then, cells were washed twice in PBS, resuspended in 400 μl PBS and analyzed by flow cytometry. Immediately prior to the FCM analysis, the cell suspension was suctioned three times through a needle and then filtered through a 50 μm nylon mesh to minimize cell aggregation. For assessment of individual samples for JC-1 staining, a total of 10,000-gated events were analyzed per sample by flow cytometer. The sample running rates were approximately 100–300 events/s. A 488 nm filter was used for excitation of JC-1. Emission filters of 535 and 595 nm were used to quantify the population of thymocytes with green (JC-1 monomer) and orange (JC-1 aggregates) fluorescence, respectively. Frequency plots were prepared for FL-1 (green) and FL-2 (orange) to determine the percentage of the population stained green and orange. In addition, all samples were viewed by fluorescence microscopy to confirm JC-1 labeling patterns. Confirmation of apoptotic cells was done by morphological assessment of cytospin preparations of cells.

2.9. Inhibition of caspase-mediated apoptosis using z-DEVD-fmk and IETD-fmk

To determine whether NP induced apoptosis was mediated by caspase activation, the cells were treated with a caspase 3 inhibitor, z-DEVD-fmk or a caspase-8 inhibitor, IFTD-fmk (Schlesinger et al., 2000). Briefly, thymocytes cultures were treated with z-DEVD-fmk at 50 uM for the indicated duration. To block the activity of caspase-8, 50 μM IETD-fmk (synthenic peptide inhibitor of activation of caspase-8) was added to the thymocytes cultures.

2.10. Determination of active caspase-3 and caspase-8

In order to determine the potential role of the caspase-3 and caspase-8 proteases in the pathways of NP-induced apoptosis, the activities of caspase-3 and -8 were measured by GaspGLOW fluorescein active caspase-3 and caspase-8 staining Kit, respectively, according to Hoetzenecker et al. (2004) and An et al. (2004). Briefly, thymocytes (1×10^6 cells) after different treatments were incubated with fluorescein isothiocyanate-conjugated z-DEVD-fmk and IFTD-fmk. After incubation for 30 min at 37 °C, the fluorescence was analyzed by flow cytometry. Alternations in the fluorescence intensity were determined by comparing the levels of the treated cells to those of the controls.

2.11. Statistical analysis

Results were presented as the mean ± S.E.M. Statistical significance between groups was analyzed by one-way ANOVA followed by the Student–Newman–Keuls multiple comparisons tests. A P-value of <0.05 was considered significant.

3. Results

3.1. Effect of NP on the viability of thymocytes

To evaluate cytotoxicity of NP, viability tests were performed using Cell Counting Kit. As it is shown in Fig. 1, treatment of thymocytes with NP up to 10 μM did not affect cell viability significantly for 2 h. However, a significant increase in cell death was observed when cells were treated for 4 and 6 h, respectively.

3.2. DNA fragmentation of thymocytes

To investigate the mechanism by which NP caused cytotoxicity in cultured thymocytes, the DNA was examined. According to Hughes (Hughes et al., 2000), DNA laddering is commonly used to establish if a decrease in cell viability to due to apoptosis rather than necrosis. As shown in Fig. 2, agarose gel electrophoresis of soluble DNA of NP-treated thymocytes revealed DNA fragmentation, characteristic of apoptotic cells (DNA ladder). The results indicated that the exposure of thymocytes to 0.1, 1, 10 μM NP lead to DNA fragmentation in 4 and 6 h, suggesting that NP induced cell death by apoptosis. But no DNA fragmentation was observed in any cells in 2 h NP treatment (Fig. 2). The results are consistent with observations of decreased cellularity of thymocytes by NP treatment.

3.3. Morphological characterization of thymocytes apoptosis by Hoechst 33258 staining

Apoptosis was further confirmed by analyzing the nuclear morphology of NP-treated thymocytes. Nuclear morphology was evaluated with membrane-permeable blue Hoechst 33258. Fig. 3 showed Hoechst 33258 fluorescence photomicrographs of cultured thymocytes treated with 0.1, 1 and 10 μM NP for 2, 4 and 6 h, respectively. In control cultures, nuclei of thymocytes appeared with regular contours and were round and large in size. Rarely thymocytes with smaller nuclei and condensed chromatin were seen. A 2 h NP treatment did not lead to nuclei morphologic changes, as shown in Fig. 3. In contrast, most nuclei of NP-treated thymocytes appeared hypercondensed (brightly stained) in 4 and 6 h. Note that the numbers of apoptotic nuclei containing condensed chromatin increased significantly as the result of prolonged incubation time.

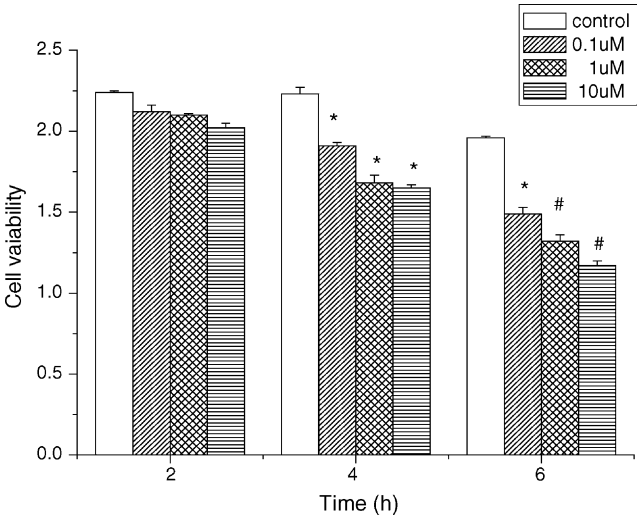

Fig. 1. NP decreased the number of viable thymocytes. The cell viability was determined by Cell Counting Kit. The results are presented as mean ± S.E. with triplicate measurement. $^{*}P < 0.05$ vs. control.

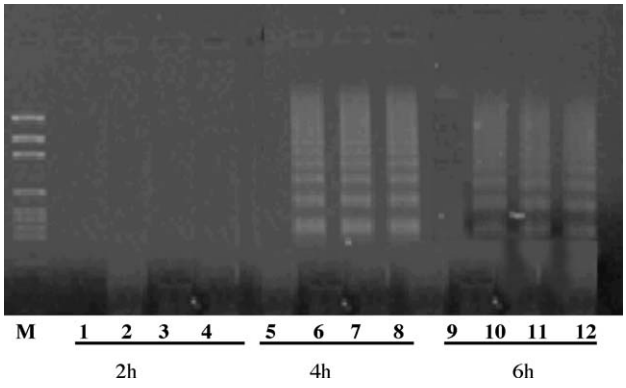

Fig. 2. Agarose gel electrophoresis of DNA fragments in thymocytes cultured for 2, 4 and 6 h. Thymocytes were pretreated with different concentrations of NP. Fragmented DNA was collected and assesses by agarose gel electrophoresis containing ethidium bromide. Data shown are representative of three separate experiments: M stands for DNA marker; 1, 5, 9 stand for control; 2, 6, 10 stand for 0.1 μM NP; 3, 7, 11 stand for 1 μM NP; 4, 8, 12 stand for 10 μM NP.

3.4. Apoptosis determination by hypodiploid cells analysis

To further confirm that NP induced apoptosis, the cells were stained with PI and analyzed for hypodiploid cells by flow cytometry. The fraction of cells in apoptosis was identified in a DNA histogram as a sub-G1 hypodiploid population. Fig. 4 showed the hypodiploid sub-G1 phase in cells treated with NP, confirming its apoptotic effect. The results indicated that thymocytes treated with NP for 4 and 6 h had sub-diploid peak and the percentage of cells with fragmented DNA increased compared with control. However, thymocytes treated with NP for relatively shorter time (2 h) appeared to have no effect on sub-G1 hypodiploid population. Once again, this observation substantiated the conclusion that NP induced thymocyte apoptosis.

3.5. Annexin-V FITC/PI assay

In the early stages of apoptosis, phosphatidylserine (PS) is translocated from the inner side of the plasma membrane to the outer layer. Annexin V, a calcium dependent phospholipid-binding protein with a high affinity for PS, can therefore be used as a sensitive probe for the exposure of PS

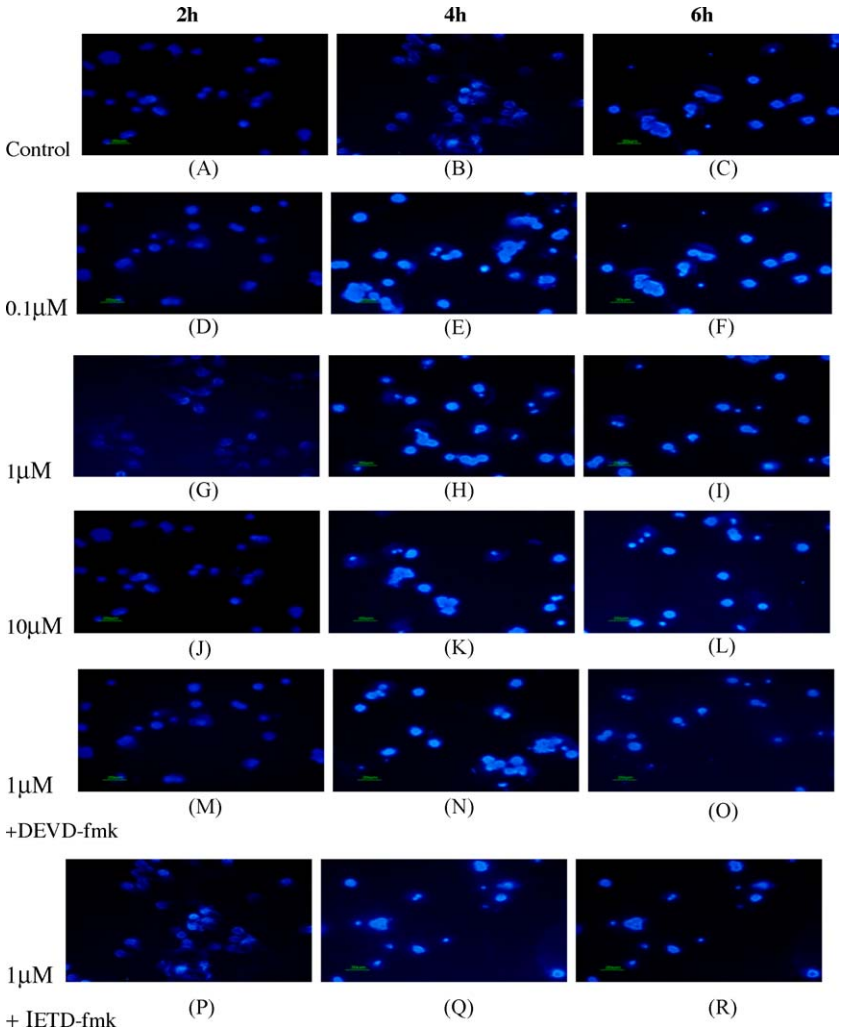

Fig. 3. Morphological changes in the nuclei (typical of apoptosis) of cultured thymocytes. Thymocytes treated by various concentrations of NP for 2, 4 and 6 h and stained with Hoechst 33258. Selected fields illustrating occurrence of apoptosis were shown. Cells with condensed chromatin were defined as apoptotic thymocytes.

on the cell membrane and hence as a marker of apoptosis. As shown in Fig. 5, analysis of the cell population showed distinct sets of population. Annexin-V^- and PI^- cells were use as controls. Annexin V^+ and PI^- cells were designated as apoptotic and Annnexin V^+ and PI^+ cells were designated as necrotic. Fig. 5 showed that the number of Annexin V-positive and propidium iodide-negative cells was increased significantly by the treatment of cells with NP for 4 or 6 h compared with control, indicating that the translocation of phosphatidylserine, an early event of the apoptotic process. However, the Annexin V^+ and PI^- cells did not increase significantly versus control for 2 h.

3.6. Assessment of changes in mitochondrial membrane potential

The loss of mitochondrial membrane potential is a hallmark for apoptosis. It is an early event coinciding with caspases activation. In non-apoptotic cells, JC-1 exists as a monomer in the cytosol (green) and accumulates as aggregates in the mitochondria, which appear red. In apoptotic and necrotic cells, JC-1 exists in monomeric form and stains the cytosol green. Fig. 6a was representative JC-1 stains of apoptotic and non-apoptotic thymocytes. In Fig. 6a(1), most thymocytes showed red and green fluorescence, which indicated that they were live cells without NP treatment. However, in Fig. 6a(2), most thymocytes showed green fluorescence, suggesting NP-induced thymocyte apoptosis. Fig. 6b showed typical FL-1/FL-2 dot plots for JC-1 staining thymocytes with and without apoptosis. Fig. 6b(1) showed thymocytes without apoptosis, which had red fluorescing J-aggregates. The green fluorescing monomers showed in Region 1 in Fig. 6b(2) indicated apoptotic cells. Fig. 6c showed the percentages of apoptotic thymocytes analyzed by flow cytometer in different NP treated groups. The results showed that the NP did not cause significantly alteration in 2 h. The increase of the percentages of apoptotic thymocytes was observed in all the doses after treatment for 4 and 6 h. In 4 h, approximately

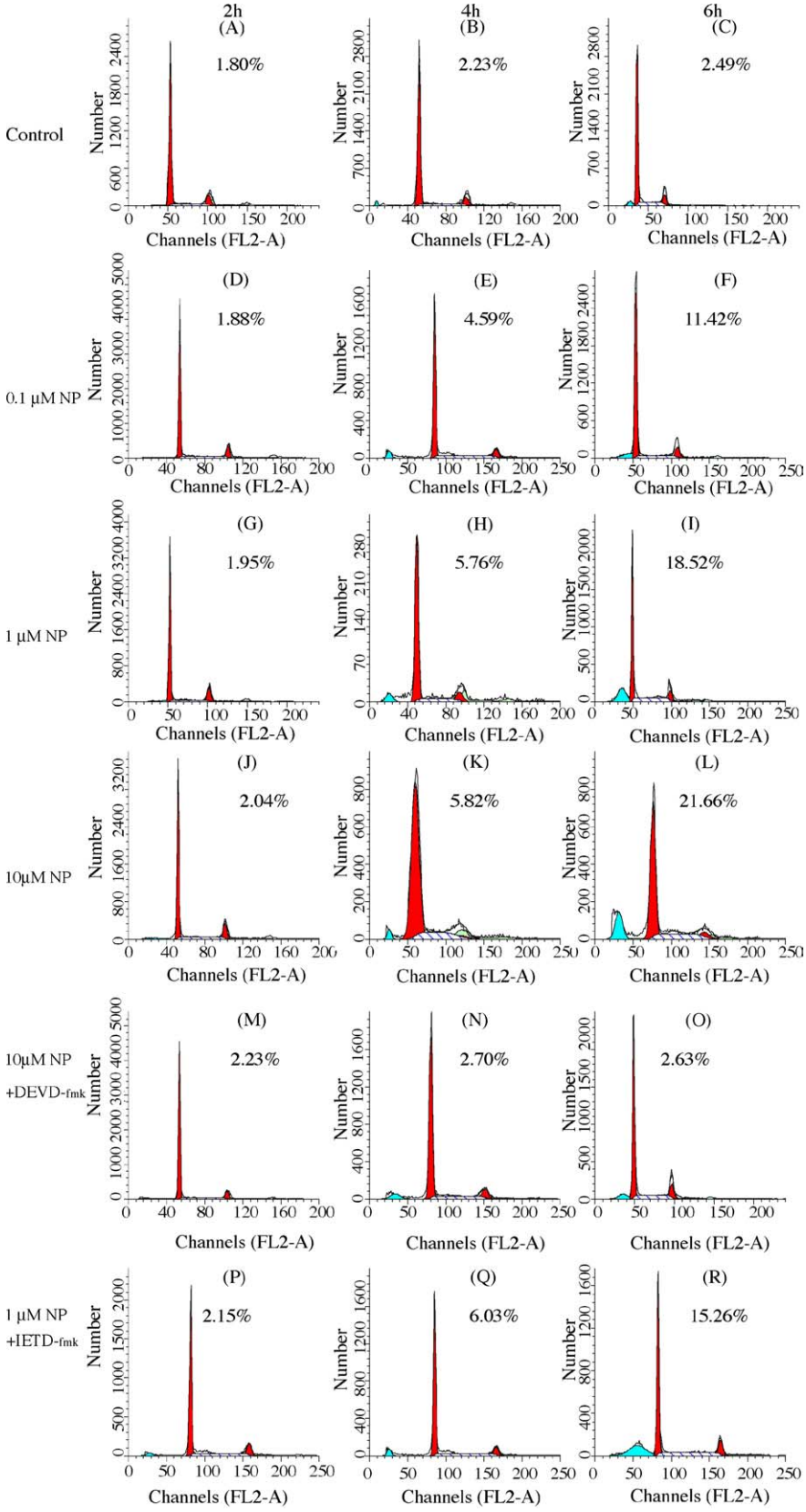

Fig. 4. Hypodiploid cells detected by flow cytometry. The thymocytes were incubated with various concentrations of NP for different time as indicated. The presence of hypodiploid cells was detected by flow cytometry after staining ethanol-fixed cells with PI. For each sample, 20,000 cells were analyzed. Results were presented as the mean ± S.E.M.

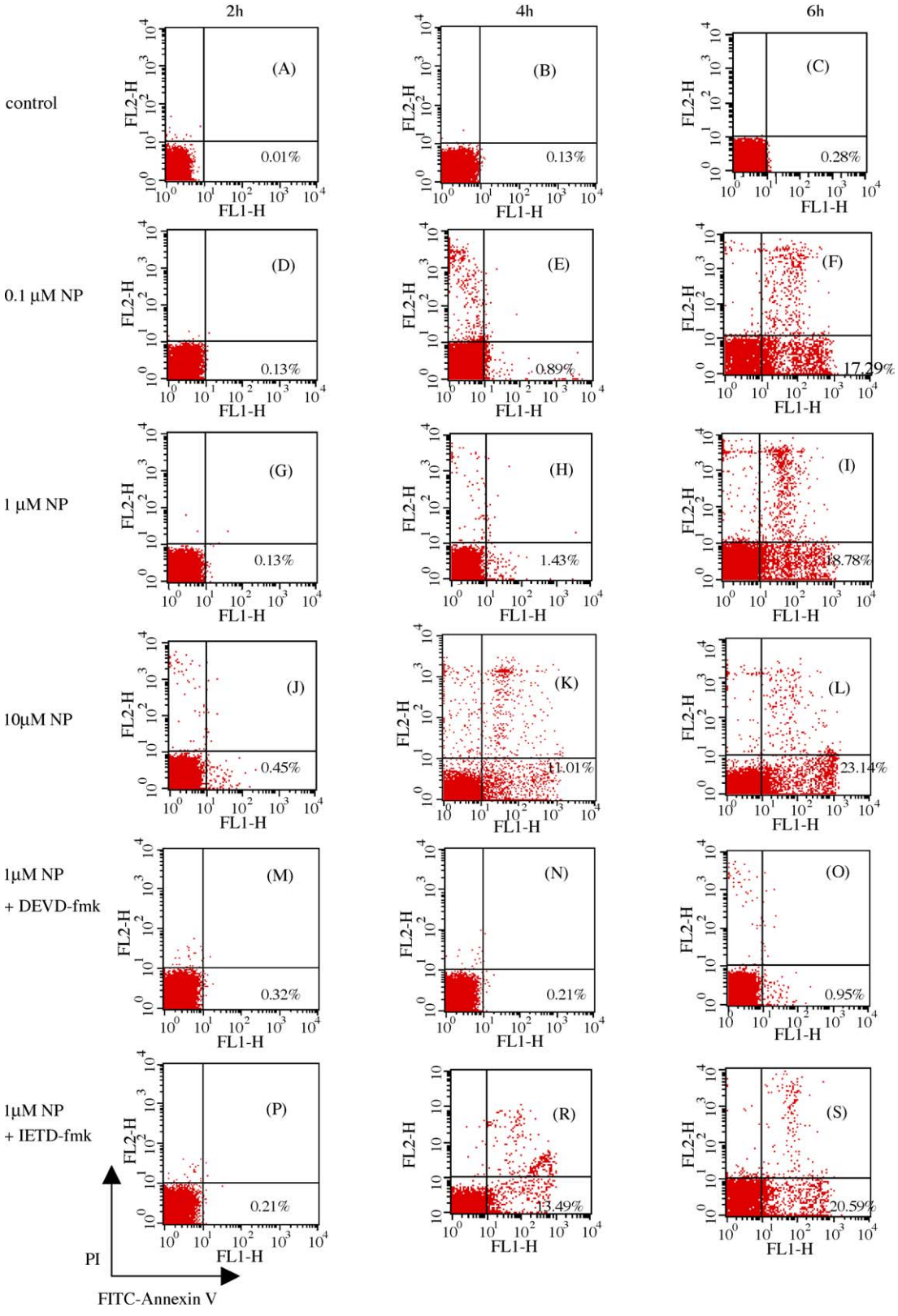

Fig. 5. Effects of NP on thymocytes discriminated by Annexin-V-FITC and propidium iodide double stain. Representative dot plots of Annexin-V/PI staining are shown. The lower left quadrant contains the vital (double negative) population. The lower right quadrant contains the apoptotic (Annexin V^+/PI^-) population. Results were presented as the mean ± S.E.M.

3.78% of thymocytes were apoptotic cells in control. The rate of apoptotic thymocytes increased to 8.55% with 0.1 μM NP treatment. When the concentrations of NP increased to 1 and 10 μM, the percentages of apoptotic thymocytes raised to 11.18 and 12.52%, respectively. The analysis of apoptotic cells in 6 h displayed the similar tendency, and the percentages were 4.45, 9.03, 15.60 and 25.10% with 0, 0.1, 1 and 10 μM of NP, respectively.

3.7. Caspase-3 and caspase-8 activity

The caspase cascade is activated during apoptosis. Therefore, we examined the activity of caspase-3 in thymocytes with or without NP treatment. Treatment of thymocytes with NP caused pronounced activation of caspase-3 as indicated by increase in the fluorescence intensity (Fig. 7). The results demonstrated that the activity of caspase-3 begin to increase after 4 and 6 h of exposure to NP. Both 4 and 6 h exposure showed similar kinetics to the occurrence of apoptosis (Fig. 7). Unlike caspase-3, the activity of caspase-8 remained unchanged in all groups in 2, 4 and 6 h (data not shown).

3.8. Effects of various protease inhibitors on NP-induced apoptosis

For further confirmation for the involvement of caspase-3, thymocytes were treated with NP and z-DEVD-fmk together. The results showed that the number of apoptotic nuclei staining decreased significantly (Fig. 3, N and O). The percentage of cells with fragmented DNA detected by PI staining decreased (Fig. 4, N and O). The rate of apoptotic thymocytes determined by Annexin-V FITC/PI assay also decreased (Fig. 5, N and O). JC-1 staining also demonstrated that NP-induced apoptotic thymocytes were inhibited by z-DEVD-fmk, suggesting z-DEVD-fmk abolished the apoptotic effects induced by NP (Fig. 6). But, for thymocytes treated with NP and ITED-fmk, the morphological changes of the cells were not inhibited and the numbers of apoptotic cells did not decreased compared with thymocytes treated with NP alone (Figs. 3–5, Q and R).

4. Discussions

Concerns have been raised recently about the toxicology effect of NP. NP is a biodegrade product of nonylphenol polyethoxylates and practically ubiquitous in the natural world. In addition, NP can mimic natural hormones and disrupt the functions of endocrine, immune, and reproductive system of living beings (Laws et al., 2000; Ying et al., 2002). The result of the current study indicated that NP induced cell death of thymocytes (Fig. 1). Using a number of techniques, we demonstrated that (1) chromatin condensation by Hoechst 33258 stain, (2) DNA fragmentation (DNA ladder)

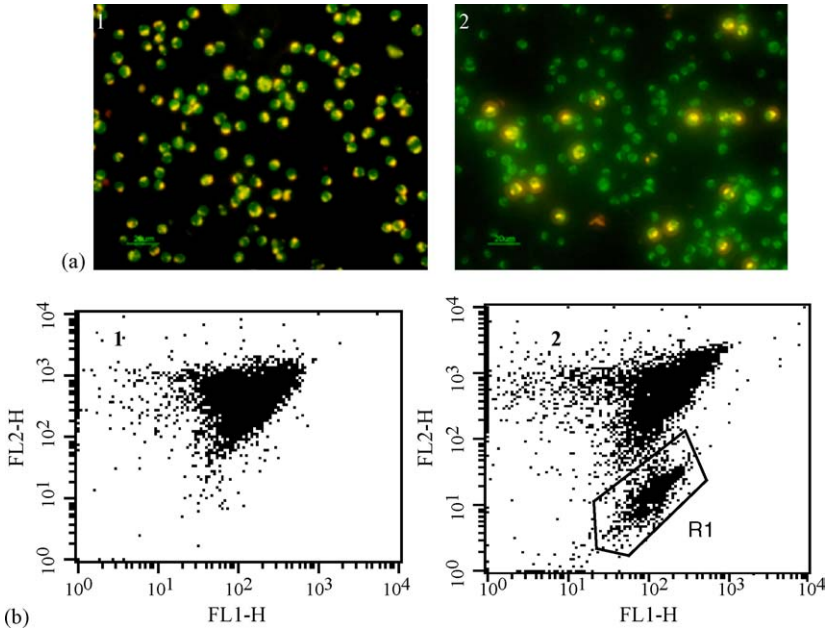

Fig. 6. Representative photos, dot plots and percentages of histogram analysis of thymocytes by JC-1 staining: (a) polarized mitochondria are marked by punctuate orange-red fluorescence staining. On depolarization, the orange-red punctuate staining is replaced by diffuse green monomer fluorescence. Apoptotic cells, showing primarily green fluorescence, are easily differentiated from healthy cells, which show red and green fluorescence. 1 represents the control without apoptosis. 2 stands for the NP-treated sample with apoptotic thymocytes. (b) Representative dot plots of JC-1 staining of thymocytes are shown. Region 1 showed thymocytes exhibiting green fluorescing monomers, which were apoptotic, cells. (c) The percentages of cells containing polarized or depolarized mitochondria were determined by histogram analysis of the ratio of the two fluorescence intensities analyzed by FCM. The numbers indicated the thymocytes expressed green monomer fluorescence only. Data represent means of three independent experiments. Results were presented as the mean ± S.E.M. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

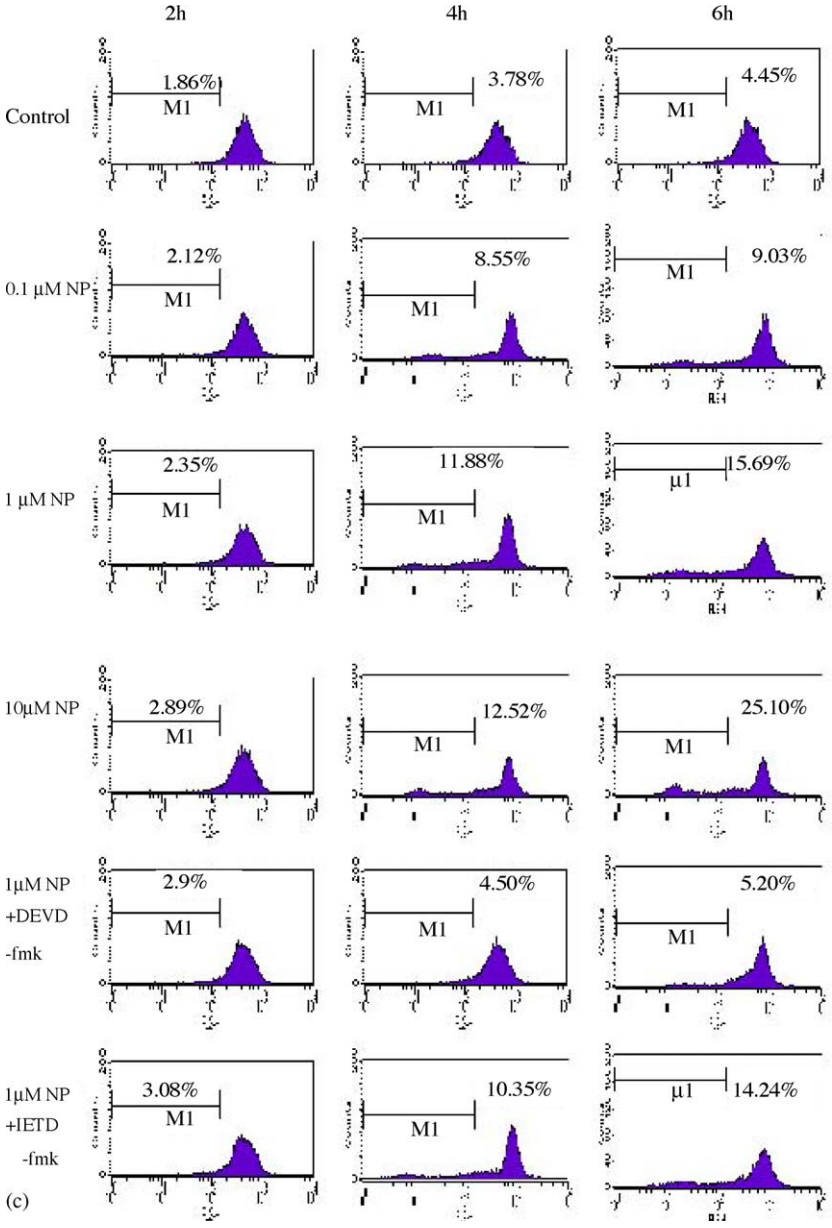

Fig. 6. (*Continued*).

by gel electrophoresis and (3) DNA cleavage (sub-diploid peak) by PI stain and biochemical marker (exposure of PS on the cell membrane) by Annexin-V FITC/PI double stain. These findings are in agreement with our previous work (Yao and Hou, 2004). In addition, Nair-Menon et al. demonstrated that exposure of splenic lymphocytes of rats and mice to octylphenol, an endocrine disrupter with estrogenic activity like NP, induced apoptosis (Nair-Menon et al., 1996). In fact NP was shown to induce apoptosis in other types of cells, such as, neuronal cells, PC12 cells, and Sertoli cells (Aoki et al., 2004; Wang et al., 2003; Negishi et al., 2003).

The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During this process, the electrochemical gradient across the mitochondrial membrane collapses. The collapse is thought to occur through the formation of pores in the mitochondria by dimerized Bax or activated Bid, Bak, or Bad proteins. Activation of these pro-apoptotic proteins is accompanied by the release of cytochrome c into the cytoplasm (Mayer and Oberbauer, 2003). In this study, we investigated the effects of NP on the mitochondrial membrane potential in order to elucidate the mechanism. According to studies of Bradbury, green fluorescence is indicative of compromised mitochondria with a low transmembrane potential by JC-1 stain (Bradbury et al., 2000). In the present study, the percentage of thymocytes with green fluorescence increased after NP treatment. In 6 h, the population of thymocytes with green fluorescence after 0.1 μM NP treatment increased to

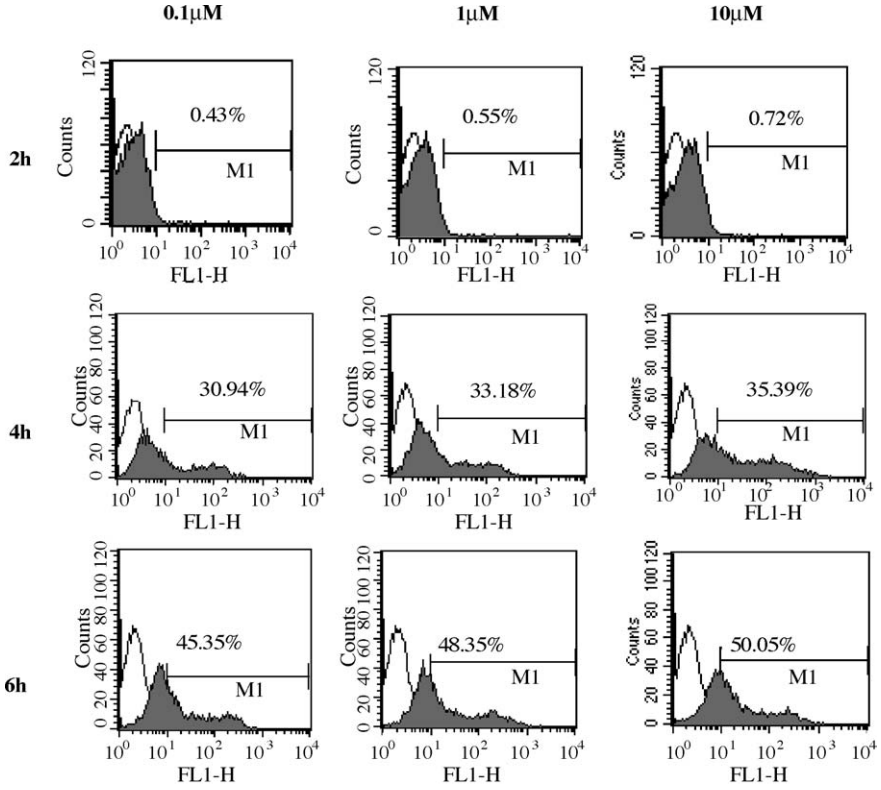

Fig. 7. NP increased caspase-3 activity. Thymocytes were treated with NP and vehicle for 2, 4 and 6 h. The caspase activity was determined by flow cytometry. The 10,000 cells were acquired and the results were shown as a histogram. Data are representative of three experiments. Results were presented as the mean ± S.E.M.

9.03% versus 4.45% in control. When the concentrations of NP were 1 and 10 μM, the percentages of thymocytes with green fluorescence dramatically increased to 15.60 and 25.10%, respectively. Although disruption of mitochondrial membrane potential is thought to be a common event in the induction of apoptosis by many chemicals (Troyan et al., 1997; Mathur et al., 2000; Barbu et al., 2002), to the best of our knowledge, our observation is the first report on the disruption of mitochondrial membrane potential by NP-induced apoptosis.

Activation of the caspase cascades has been demonstrated to be essential in the signaling pathway in the induction of apoptosis in many cells (Sylte et al., 2003; Ivins et al., 1999). In this study, the results indicated that NP induced activation of caspase-3 in association with induction of thymocyte apoptosis in both 4 and 6 h. Furthermore, we demonstrated that abrogation/inhibition of caspase-3 protease activity with z-DEVD-fmk significantly suppressed NP-induced apoptosis. This result offers a strong support for the significant mediator role of caspase-3 in molecular events of NP-induced thymocyte apoptosis. The activities of caspase-8 remained unchanged in all NP treated groups (data not shown). And block of caspase-8 with its specific inhibitor, IETD-fmk, did not protected thymocytes from NP-induced apoptosis (Figs. 3–5, Q and R), implying that NP-induced thymocyte apoptosis was independent of caspase-8. The result is in agreement with study of Kudo, which demonstrated that nonylphenol-induced neural stem cells via activation of caspase-3 (Kudo et al., 2004).

However, there is increasing evidence that apoptosis relates to a variety of signal transudation events lading to stimulation of calcium flux, cAMP production, PLC activation, inositol phosphate generation. The extent to which each pathway contributes to the thymocyte apoptosis induced by NP remains to be explored. Further studies are also needed to test if estrogen receptors participate in the response.

Apoptosis is a regulated physiological cell death, which is essential for maintenance of tissues homeostasis and crucial for defense against diseases and cancers. It is known that apoptosis has an essential role in the immune system. Aberrations in genes encoding pro- or anti-apoptotic proteins or defects in apoptotic process in thymocytes have been implicated in the initiation of conditions such as immunodeficiency, autoimmunity and cancer (Opferman and Korsmeyer, 2003; Dulos and Bagchus, 2001). Therefore, it is likely that an alteration of the immune system by environmental estrogens, including NP, could change the individual's ability of regulating immune homeostasis and host resistance to infection and tumor growth.

In summary, our findings demonstrated that the 4-NP directly induces thymocyte apoptosis through the activation of the mitochondrial and caspase-3 pathway. The findings

are in good agreement with our previous study, which demonstrated that 4-NP induced thymocyte apoptosis in vivo (Yao and Hou, 2004). These results suggested that exposure to nonylphenol might affect immune system through the induction of apoptosis in immnuocompetent cells, like thymocytes.

Acknowledgements

We thank our colleagues in our lab, for scientific contribution and discussions. This work was supported by the Opening Grant of Province Key Lab of Nanjing Medical University and the Key Medicine Program of Nanjing city (ZKG0002).

References

An, J., Chen, Y., Huang, Z., 2004. Critical upstream signal of cytochrome c release induced by a novel Bcl-2 inhibitor. J. Biol. Chem. 279, 19133–19140.

Aoki, M., Kurasaki, M., Sainto, T., Seki, S., Hosokawa, T., Takahashi, Y., Fujita, H., Iwakuma, T., 2004. Nonylphenol enhances apoptosis induced by serum deprivation in PC 12 cells. Life Sci. 74, 2301–2312.

Araki, T., Yamamoto, A., Yamada, M., 1987. Accurate determination of DNA content in single cell nuclei stained with Hoechst 33258 fluorochrome at high salt concentration. Histochem. J. 87, 331–338.

Barbu, A., Welsh, N., Saldeen, J., 2002. Cytokine-induced apoptosis and necrosis are preceded by disruption of the mitochondrial membrane potential in pancreatic RINm 5F cells: prevention by Bcl-2. Mol. Cell. Endocrinol. 190, 75–82.

Bradbury, D.A., Simmons, T.D., Slater, K.J., Crouch, S.P., 2000. Measurement of the ADP:ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. J. Immunol. Meth. 240, 79–92.

Chapin, R.E., Dalaney, J., Wang, Y., Lanning, L., Davis, B., Collins, B., Mintz, N., Wolfe, G., 1999. The effects of 4-nonylphenol in rats: a multigeneration reproduction study. Toxicol. Sci. 52, 80–91.

Dulos, G.J., Bagchus, W.M., 2001. Androgens indirectly accerate thymocyte apoptosis. Int. Immunopharmacol. 1, 321–328.

Giger, W., Brunner, P.H., Schaffner, C., 1984. Nonylphenol in sewage sludge:accumulation of toxic metabolites from nonionic surfactants. Science 225, 623–625.

Hoetzenecker, W., Meingassner, J.G., Ecker, R., Stingl, G., Stuetz, A., Elbe-Burger, A., 2004. Corticosteroids but not pimecrolimus affect viability, maturation and immune function of murine epidermal langerhans cells. J. Invest. Dermatol. 122, 673–684.

Hughes, P.J., McLellan, H., Lowes, D.A., Khan, S.Z., Bilmen, J.G., Tovey, S.C., Godfrey, R.E., Michell, R.H., Kirk, C.J., Michelangeli, F., 2000. Estrogenic alkylphenols induce cell death by inhibiting tesis endoplasmic reticulum Ca^{2+} pumps. Biochem. Biophys. Res. Co. 277, 568–574.

Ishiyama, M., Miyazono, Y., Sasamoto, K., Ohkura, Y., Ueno, K., 1997. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta 44, 1299–1305.

Ivins, K.J., Thornton, P.L., Rohn, T.T., Cotman, C.W., 1999. Neuronal apoptosis induced by β-amyloid is mediated by caspase-8. Neurobiol. Dis. 6, 440–449.

Jenkins, J.K., Suwannaroj, S., Elbourne, K.B., Ndebele, K., McMurray, R.W., 2001. 17-β-estradiol alter Jurkat lymphocyte cell cycling and induces apoptosis through suppression of Bcl-2 and cyclin A. Int. Immunopharmacol. 1, 1897–1911.

Kinnberg, K., Korsgaard, B., Bjerregaard, P., 2000. Concentration-dependent effects of nonylphenol on testis structure in adult platyfish. Mar. Environ. Res. 50, 169–173.

Krammer, P.H., 2000. CD95's deadly mission in the immune system. Nature 407, 789–795.

Kudo, C., Wada, K., Masuda, T., Yonemura, T., Shibuya, A., Fujimoto, Y., Nakajima, A., Niwa, H., Kamisaki, Y., 2004. Nonylphenol induces the death of neural stem cells due to activation of caspase cascade and regulation of the cell cycle. J. Neurochem. 88, 1416–1423.

Laws, S.C., Carey, S.A., Ferrell, J.M., Bodman, G.J., Copper, R.L., 2000. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol. Sci. 54, 154–167.

Masuno, H., Okamoto, S., Iwanami, J., Honda, K., Shiosaka, T., Kidani, T., Sakayama, K., Yamamoto, H., 2003. Effects of 4-nonylphenol on cell proliferation and adipocyte formation in cultures of fully differentiated 3T3-L1 cells. Toxicol. Sci. 75, 314–320.

Mathur, A., Hong, Y., Kemp, B.K., Barrientos, A.A., Erusalimsky, J.D., 2000. Evaluation of fluorescent dyes for the detection of mitochondrial membrane potential changes in cultured cardiomyocytes. Cardiovasc. Res. 46, 126–138.

Mayer, B., Oberbauer, R., 2003. Mitochondrial regulation of apoptosis. News Physiol. Sci. 18, 89–94.

Nagao, T., Wada, K., Marumo, H., Yoshimura, S., Ono, H., 2001. Reproductive effects of nonylphenol in rats after gavage administration: a two-generation study. Reprod. Toxicol. 15, 293–315.

Nair-Menon, J.U., Campbell, G.T., Blake, C.A., 1996. Toxic effects of octylphenol on cultured rat and murine splenocytes. Toxicol. Appl. Pharmacol. 139, 437–444.

Negishi, T., Ishii, Y., Kyuwa, S., Kuroda, Y., Yoshikawa, Y., 2003. Inhibition of staurosporine-induced neuronal cell death by bisphenol A and nonylphenol in primary cultured rat hippocampal and cortical neurons. Neurosci. Lett. 353, 99–102.

Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F., Riccardi, C., 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Meth. 139, 271–279.

Odum, J., Ashby, J., 2000. Neonatal exposure of male rats to nonylphenol has no effect on the reproductive tract. Toxicol. Sci. 56, 400–404.

Opferman, J.T., Korsmeyer, S.J., 2003. Apoptosis in the development and maintenance of the immune system. Nat. Immunol. 4, 410–415.

Rankouhi, T.R., Sanderson, J.T., van Holstejin, I., van Leeuwen, C., Vethaak, A.D., van den Berg, M., 2004. Effects of natural and synthetic estrogens and various environmental contaminants on vitellogenesis in fish primary hepatocytes: comparison of bream (*Abramis brama*) and carp (*Cyprinus carpio*). Toxicol. Sci. 81, 90–102.

Salvioli, S., Ardizzoni, A., Franceschi, C., Cossarizza, A., 1997. JC-1, but not $DiOC_6(3)$ or rhodamine 123, is a reliable fluorescent probe to assess ψ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS 411, 77–82.

Schlesinger, M., Jiang, J.D., Roboz, J.P., Denner, L., Ling, Y.H., Holland, J.F., Bekesi, J.G., 2000. 3-m-Bromoacetylamino benzoic acid ethyl ester: a new cancericidal agent that activates the apoptotic pathway through caspase-9. Biochem. Pharmacol. 60, 1693–1702.

Shames, R.S., 2002. Gender differences in the development and function of the immune system. J. Adolescent Health 30s, 59–70.

Sweeney, T., 2002. Is exposure to endocrine disrupting compounds during fetal/post-natal development affecting the reproductive potential of farm animals? Domest. Anim. Endocrin. 23, 203–209.

Sylte, M.J., Leite, F.P., Kuckleburg, C.J., Inzana, T.J., Czuprynski, C.J., 2003. Caspase activation during *Haemophilus somnus* lipooligosaccharide-mediated apoptosis of bovine endothelial cells. Microb. Pathol. 35, 285–291.

Troyan, M.B., Gilman, V.R., Gay, C.V., 1997. Mitochondrial membrane potential changes in osteoblasts treated with parathyroid hormone and estradiol. Exp. Cell. Res. 233, 274–280.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin-V. J. Immunol. Meth. 184, 39–51.

Wang, X., Han, X., Yao, G., Wang, Y., 2003. Effect of nonylphenol on apoptosis of sertoli cell in vitro. B. Environ. Contam. Tox. 70, 898–904.

Yao, G., Hou, Y., 2004. Nonylphenol induces thymocyte apoptosis through Fas/FasL pathway by mimicking estrogen in vivo. Environ. Toxicol. Phar. 17, 19–27.

Ying, G.G., Williams, B., Kookana, R., 2002. Environmental fate of alkylphenols and alkylphenol ethoxylates—a review. Environ. Int. 28, 215–226.