Nonylphenol induces apoptosis of Jurkat cells by a caspase-8 dependent mechanism

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Abstract

Nonylphenol is the final biodegradation product of nonylphenol polyethoxylates, which are widely used surfactants in domestic and industrial products. Although nonylphenol is well known as an endocrine disrupting chemical, its effects on cell death and the mechanisms responsible for these apoptotic effects remain unclear. In the present study, Jurkat cells were treated with 0.1, 1 and 10 μM nonylphenol for 12 and 24 h, respectively. Cell viability was assessed with a Cell Counting Kit. The effects of nonylphenol on apoptosis of Jurkat cells were determined by DNA fragmentation (DNA ladder), Hoechst33258, PI and Annexin V FITC/PI double staining. Changes in mitochondrial membrane potential were detected with JC-1 fluorescence. In addition, enzyme activity of caspase-8 was evaluated by flow cytometry. The results demonstrated that nonylphenol inhibited the proliferation and induced loss of mitochondrial membrane potential, caspase-8 activation, internucleosomal DNA fragmentation. Furthermore, a caspase-8 inhibitor, IETD-fmk, blocked loss of mitochondrial membrane potential and apoptosis. These findings suggested that nonylphenol induced apoptosis of Jurkat cells by caspase-8 dependent mechanisms.

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1. Introduction

It is well established that natural (17β-estrogen) and synthetic (e.g. diethylstilbestrol) estrogens not only affect the reproductive system, but also markedly influence the immune system. In recent years, a new class of estrogens that is abundant in the environment (in industrial chemicals, pesticides, and surfactants) has been recognized and has also been shown to influence the immune system. A convincing body of evidence indicates the nonylphenol (NP) is one of the potential endocrine disruptors and possesses estrogen-like properties [1–8]. Several studies also have shown that nonylphenol bound to estrogen receptor and activated the transcription genes, which are regulated by some estrogen-like compounds [9–13]. Nonylphenol often found in the environment. NP belongs to the group of alkylphenols, which are degraded products of alkylphenolethoxylates (APEs). APEs are widely used as detergents, emulsifiers, solubilizers, wetting agents, dispersants, cleaners, and household products [14]. In effluents from sewage, maximum concentrations of 300 ppb of NP are currently found [15].

Apoptosis is a form of cell death by a characteristic set of morphological and biochemical changes. Apoptosis is recognized as an early cellular indicator of toxicity [16]. The biochemical processes involved in apoptosis are...
highly evolutionarily conserved, including specific DNA fragmentation at internucleosomal sites. Many studies have demonstrated the role of caspases in apoptotic death, especially in cancer cells. Different members of the caspase family mediate apoptosis in different cell types and, even within a given cell type, distinct caspases have been found to mediate apoptosis, depending on the apoptotic stimulus received by the cells.

Jurkat cells are frequently employed as a T-cell lymphoma model system to study apoptotic processes. It has been reported that some endocrine disrupters act as chemical substance that caused apoptosis in cells [4,17–20]. Our previous study has demonstrated that NP induced thymocytes apoptosis in vivo [21]. However, to date, there is no detailed laboratory evidence regarding the mechanisms of nonylphenol on leukaemic Jurkat cells. The aim of this work was to study the effects of nonylphenol on apoptosis of Jurkat cells and its mechanisms.

2. Material and methods

2.1. Reagents

Jurkat cells, a cell line of human T lymphocytes, were purchased from Cell Bank of Chinese Academy of Science (Shanghai, P.R.China). 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 Sigma (St Louis, MO, USA). CaspGLOW fluorescein active caspase-3 and caspase-8 staining kits were purchased from BioVision (Mountain View, California). 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). IETD-fmk and z-VAD-fmk were purchased from Cell Bank of Chinese Academy of Science (Shanghai, P.R.China). IETD-fmk was purchased from Becton Dickinson Company (Palo Alto, CA, USA). All other reagents were of the highest grade commercially available.

2.2. Cell culture

Jurkat cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified incubator at 37 °C and 5% CO2. The cells were preincubated in 6-well plates overnight, and then the medium was replaced with RPMI-1640 with or without nonylphenol. Then cells were subsequently analyzed for viability, morphological changes, DNA degradation and capase-8 activity.

2.3. Cytotoxicity assay

Cell viability was assessed with a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan) according to Ishiyama et al. [22] with slight modifications. Briefly, Jurkat cells were plated on a 96-well (100μl/well) microplate at a density of 1 × 10^4 cells/ml. NP at various concentrations was added and the plate was incubated at 37 °C in humidified 5% CO2 for 12, 24, and 48 h. 10 μl solutions containing WST-8 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. [23]. 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 at 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 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 [24]. Briefly, 1 × 10^6 NP-treated Jurkat cells were pelleted (200 × 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 (13000 × 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 Propidium iodide (PI) staining [25]. In brief, Jurkat cells (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 12 and 24 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-Dickinson, Immunocytometry System, San Jose, CA) with excitation wavelength of 488 nm and 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.
2.7. FITC-Annexin V and PI double staining

Annexin-V FITC/PI double stain assay was used to examine NP induced Jurkat cell apoptosis, not necrosis according to a procedure described by Vermes et al. [26]. Briefly, Jurkat cells 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 CaCl2) prior to the addition of FITC-labeled annexin V for 10 min at room temperature. 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 Jurkat cells 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 Jurkat cells were then plotted for Annexin V-FITC and PI in a 2-way dot plot to assess percentage of apoptotic Jurkat cells.

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 detection of mitochondrial depolarization during the early stages of apoptosis [27]. Briefly, 1 × 10^6 Jurkat cells 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 filter of 535 nm and 595 nm were used to quantify the population of Jurkat cells 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. Determination of active caspase-8

In order to determine the potential role of the caspase-8 proteases in the pathways of NP-induced apoptosis, the activities of caspase-8 were measured by GaspGLOW fluorescein active caspase-8 staining Kit according to Hoetzenecher and An [28,29]. Briefly, Jurkat cells (1×10^6 cells) after different treatments were incubated with fluorescein isothiocyanate-conjugated 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.

2.10. Inhibition of caspase8-mediated apoptosis using IETD-fmk

To determine whether NP induced apoptosis was mediated by caspase-8 activation, the cells were treated with a broad specificity caspase-8 inhibitor, IFTD-fmk [30]. Briefly, Jurkat cells cultures were treated with IETD-fmk (synthetic peptide inhibitor of activation of caspase-8) at 50 μM for the indicated duration.

2.11. Statistical analysis

Results were presented as the mean±SEM. 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 Jurkat cells

To evaluate cytotoxicity of NP, viability tests were performed using Cell Counting Kit. Fig. 1a, b were representatives of clone expansion of Jurkat cells with or without NP treatment. Compared with control (Fig. 1a), the number of clones in NP-treated group (Fig. 1b) decreased significantly. As it is shown in Fig. 1c, a significant increase in

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**Fig. 2.** Agarose gel electrophoresis of DNA fragments in Jurkat cells cultured for 12 h and 24 h. Jurkat cells were pretreated with different concentrations of NP. Fragmented DNA was collected and assessed by agarose gel electrophoresis containing ethidium bromide. Data shown are representative of three separate experiments. M stands for DNA marker. C stands for control. NP1, NP2 and NP3 stand for 0.1, 1 and 10 μM NP respectively.

**Fig. 3.** Morphological changes in the nuclei (typical of apoptosis) of Jurkat cells induced by NP. Jurkat cells were treated by various concentrations of NP and stained with Hoechst 33258. Selected fields illustrating occurrence of apoptosis were shown. Cells with condensed chromatin were defined as apoptotic Jurkat cells. (a) control; (b) NP treatment.
cell death was observed when cells were treated with NP for 12 and 24 h, respectively. However, Jurkat cells treated with NP for relatively longer time (48 h) appeared to have no significant difference on cell viability.

3.2. DNA fragmentation of Jurkat cells

To investigate the mechanism by which NP caused cytotoxicity in cultured Jurkat cells, the DNA was examined. According to Hughes, DNA laddering is commonly used to establish if a decrease in cell viability due to apoptosis rather than necrosis [4]. As shown in Fig. 2, agarose gel electrophoresis of soluble DNA of NP-treated Jurkat cells revealed DNA fragmentation characteristic of apoptotic cells (DNA ladder). The results indicated that the exposure of Jurkat cells to 0.1, 1, 10 μM NP lead to DNA fragmentation in 12 and 24 h, suggesting that NP induced cell death by apoptosis. The results are consistent with observations of decreased cellularity of Jurkat cells by NP treatment.

3.3. Morphological characterization of Jurkat cell apoptosis by Hoechst33258 staining

Apoptosis was further confirmed by analyzing the nuclear morphology of NP-treated Jurkat cells. Nuclear morphology was evaluated with membrane-permeable blue Hoechst 33258. Fig. 3 showed representative Hoechst 33258 fluorescence photomicrographs of cultured Jurkat cells treated with or without NP treatment, respectively. In control cultures (Fig. 3a), nuclei of Jurkat cells appeared with regular contours and were round and large in size. Rarely Jurkat cells with smaller nuclei and condensed chromatin were seen. In
contrast, most nuclei of NP-treated Jurkat cells (Fig. 3b) appeared hypercondensed (brightly stained). Note that the numbers of apoptotic nuclei containing condensed chromatin increased significantly as the result of prolonged incubation time (data not shown).

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. Jurkat cells of control group showed less sub-G1 hypodiploid population (Fig. 4a). Fig. 4b showed the hypodiploid sub-G1 phase in cells treated with NP, confirming its apoptotic effect. The results indicated that Jurkat cells treated with NP for 12 h and 24 h had sub-diploid peak and the percentage of cells with fragmented DNA increased compared with control. Once again, this observation substantiated the conclusion that NP induced Jurkat cell 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 on the cell membrane and hence as a marker of apoptosis. Fig. 5a is representative of control, which almost no apoptotic cells were detected. However, in NP-treated group (Fig. 5b), a lot of apoptotic cells were shown. As shown in Fig. 5c, analysis of the cell population had distinct sets of population. Annexin V− and PI− cells
were used as controls. Annexin V− and PI− cells were designated as apoptotic and Annexin V+ and PI+ cells were designated as necrotic. Fig. 5c showed that the number of Annexin V+ and propidium iodide-negative cells was increased significantly by the treatment of cells with NP for 12, or 24 h compared with control, indicating that the translocation of phosphatidylserine, an early event of the apoptotic process.

Fig. 6. Representative photos, dot plots and percentages of histogram analysis of Jurkat cells 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 Jurkat cells. (b) Representative dot plots of JC-1 staining of Jurkat cells are shown. Lower left quadrant showed Jurkat cells 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 Jurkat cells expressed green monomer fluorescence only. Data represent means of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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 caspase 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 Jurkat cells. In Fig. 6a, most Jurkat cells, without NP treatment, showed red and green fluorescence, indicating that they were live cells. However, in Fig. 6b, most Jurkat cells showed green fluorescence, suggesting NP-induced Jurkat cell apoptosis. Fig. 6c and d showed typical FL-1/FL-2 dot plots for JC-1 staining Jurkat cells with and without apoptosis. Fig. 6c showed Jurkat cells without apoptosis, which had red fluorescing J-aggregates. The green fluorescing monomers showed in lower left quadrant indicated apoptotic cells. Fig. 6e showed the percentages of apoptotic Jurkat cells analyzed by flow cytometer in different NP treated groups. The increase of the percentages of apoptotic Jurkat cells was observed in all the doses after treatment for 12 and 24 h. In 12 h, approximately 3.79% of Jurkat cells were apoptotic cells in control. The rate of apoptotic Jurkat cells increased to 4.98% with 0.1 μM NP treatment. When the concentrations of NP increased to 1 and 10 μM, the percentages of apoptotic Jurkat cells raised to 5.80% and 6.67%, respectively. The analysis of apoptotic cells in 24 h displayed the similar tendency, and the percentages were 3.97%, 8.7%, 9.66% and 10.5% with 0, 0.1, 1 and 10 μM of NP, respectively.

3.7. Caspase 8 activity

The caspase cascade is activated during apoptosis. Therefore, we examined the activity of caspase-8 in Jurkat cells with or without NP treatment. Treatment of Jurkat cells with NP caused pronounced activation of caspase-8 as indicated by increase in the fluorescence intensity (Fig. 7). The results demonstrated that the activity of caspase-8 begin to increase after 12 and 24 h of exposure to NP. Both 12 and 24 h exposure showed similar kinetics to the occurrence of apoptosis (Fig. 7).

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

For further confirmation for the involvement of caspase-8, Jurkat cells were treated with NP and IETD-fmk together. The results showed that the percentage of cells with fragmented DNA detected by PI staining decreased (Fig. 4). The rate of apoptotic Jurkat cells determined by Annexin-V FITC/PI assay also decreased (Fig. 5). JC-1 staining also demonstrated that NP-induced apoptotic Jurkat cells were inhibited by IETD-fmk, suggesting IETD-fmk abolished the apoptotic effects induced by NP (Fig. 6).

4. Discussions

Our previous study has demonstrated that NP induced thymocyte apoptosis. The thymocytes are important immunocompetent cells. It contains large numbers of
functionally mature antigen specific T cells. The Jurkat cell line is established from a T-cell lymphoma. If NP induces thymocyte apoptosis, NP is also assumed to affect Jurkat cells.

The result of the current study indicated that NP induced cell death of Jurkat cells (Fig. 1). Using a number of techniques, we demonstrated that (1) chromatin condensation by Hoechst 33258 stain, (2) DNA fragmentation (DNA ladder) by gel electrophoresis, (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. 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 [31]. In fact NP was shown to induce apoptosis in other types of cells, such as, neuronal cells, PC12 cells, and Sertoli cells [17,32,33]. The present study further extends these findings by reporting that NP also can induce apoptosis of Jurkat cell.

Moreover, in this study, we investigated the effects of NP on the mitochondrial membrane potential. Studies showed that the mitochondrial permeability transition was 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 [34]. According to studies of Bradbury, green fluorescence is indicative of compromised mitochondria with a low transmembrane potential by JC-1 stain [35]. In the present study, the percentage of Jurkat cells with green fluorescence increased after NP treatment. In 24 h, the population of Jurkat cells with green fluorescence after 0.1 μM NP treatment increased to 8.7% vs 3.97% in control. When the concentrations of NP were 1 and 10 μM, the percentages of Jurkat cells with green fluorescence dramatically increased to 9.66% and 10.5%, respectively. Although disruption of mitochondrial membrane potential is thought to be a common event in the induction of apoptosis by many chemicals [36–38], to the best of our knowledge, our observation is the first report on the disruption of mitochondrial membrane potential by NP-induced apoptosis in Jurkat cells.

Caspase activation is the final common pathway to induction of apoptosis in many systems. The caspses are a family of intracellular cysteine proteases with specificity for aspartic acid residues. Members of the caspase family of cysteine proteases have been firmly established as playing key roles in signal transduction cascades that culminate in apoptosis. Therefore, the role of caspase-8 on Jurkat cell apoptosis was explored in this study. The results demonstrated that the activity of caspase-8 begin to increase after 12 and 24 h of exposure to NP, which was shown similar kinetics to the occurrence of apoptosis. Furthermore, we demonstrated that inhibition of caspase-8 protease activity with IETD-fmk significantly suppressed NP-induced apoptosis. This result offers a strong support for the significant mediator role of caspase-8 in molecular events of NP-induced Jurkat cell apoptosis. The results of Kudo demonstrated that nonylphenol induced neural stem cells via activation of caspase-3 [39]. Akoi group also suggested that nonylphenol enhances apoptosis by serum deprivation in PC12 cells through caspase-3 [17]. The different data may be explained by the possibility that NP induce apoptosis in different cells through distinct mechanisms.

Apoptosis is a regulated physiological cell death, which is essential for maintenance of tissues homeostasis and crucial for defense against diseases and cancers. Dysregulation of normal apoptotic mechanisms provides advantage to cancer cells. Our findings that NP induced apoptosis in Jurkat cells may provide new insight on the toxicity of NP.

In summary, the results indicate that nonylphenol has marked apoptotic effects on Jurkat cells in vitro, and that activation of caspase-8 in Jurkat cells is an important apoptosis-inducing mechanism.

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References


[18] Salvioli S, Ardizzone A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(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 1997;411(1):77–82.


