Effects of 4-nonylphenol on proliferation of AGS gastric cells

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Abstract

Objectives: Nonylphenol (NP) is present ubiquitously in both aquatic and terrestrial environments. This compound is considered an important endocrine disruptor and its toxic/oestrogenic activity has been investigated in a number of *in vitro* and *in vivo* studies. Human exposure to NP may occur by cutaneous absorption, ingestion of contaminated food or water and inhalation. Moreover, while the cytotoxic effects of NP are known and studied, its effects on cell death and related mechanisms are not known. Our group decided to investigate NP effects on a gastric epithelial cell line (AGS), in particular NP effects on AGS cell cycle and apoptosis.

Materials and methods: Cell cycle was analysed by flow cytometry, p21 and p27 induction, and apoptosis was analysed by flow cytometry and annexin-V assays, Fas, Fas-L, caspase 8 and caspase 3 activation.

Results: We have demonstrated that NP affected cell cycle and apoptosis in a time- and dose-dependent manner, reaching the most notable effect at concentration of 10^{-7} M, for 48 h. Flow cytometry revealed that treatment with 10^{-7} M NP led to accumulation of cells at G₂/M transition and increased percentage population of apoptotic cells. Results have shown that NP at concentration 10^{-7} M induced marked increase in Fas and Fas-L expression. Moreover, 10^{-7} M NP induced activation of caspases 8 and 3, but not activation of caspase 9.

Conclusions: The results reported suggest that gastric cells can be useful as screening population for appropriate study of developmental toxicity.

Introduction

Endocrine disruptors (EDs) are exogenous compounds that have the ability to interfere with hormonal regulation and normal homeostasis of the endocrine system, and consequently engender effects on health of animals and humans (1).

Alkylphenol ethoxylates (APEs) are widely used in surfactants and detergents in domestic and industrial products and are commonly found in waste water. In sewage treatment of plant effluents, APEs are degraded to the more resistant alkylphenols such as 4-*n*-nonylphenol (NP) and 4-*n*-octylphenol (OP). NP and OP are ubiquitous pollutants of the environment and due to their widespread uses in many industrial and agricultural products, it is believed that high amounts of NP and OP have been discharged into aquatic ecosystems (2). These chemicals can accumulate within internal organs of fish and birds, reaching concentrations 10–100 times higher than found in the general environment, due to their high stability and lipid solubility. Interest in these substances generates considerable concern in the public domain and scientific community as they can easily spread to humans *via* the food chain, and they can mimic actions of natural hormones in the body.

NP environmental contamination is closely related to food consumption, as any substance dispersed in the environment cannot easily avoid entering the food chain. NP after use, enters the aquatic environment – it has been detected in drinking water (3,4) and has been found in water and fish from rivers in Japan (5). Moreover, NP leaches from plastics used in food processing and packing and from commercially available polystyrene tubes (6). Thus, human exposure to NP may occur by cutaneous absorption, ingestion of contaminated food or water and inhalation (7,8).

All evidence reported in recent years has emphasized the need for more effective and reliable methods for risk assessment of these substances for humans. A number of previous studies has focused on effects of toxins on late embryological developmental processes including foetal and neonatal stages of animal models (9–11); however,

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little is known of their effects on early embryonic development, particularly in humans. Although cytotoxicity of NP is well-known, effects of these chemicals on cell death and related underlying mechanisms to now, are not fully understood. Drawing from this background, we decided to investigate activity of NP on human gastric epithelial cells as this contaminant can easily be ingested. Understanding its effects on gastric epithelial cells and related molecular mechanisms that are able to regulate the cell cycle and apoptosis, is a pre-requisite to understanding mechanisms by which NP may exert its toxic activity.

Materials and methods

Cell line and 4-nonylphenol treatment

AGS human gastric epithelial cells (American Type Culture Collection, Manassas, VA) were grown in Ham's F-12 medium supplemented with 10% FBS and 50 mg/ml penicillin-streptomycin in an atmosphere of 5% CO₂ at 37 °C. Cells were collected at 24 and 48 h after treatment with 4-nonylphenol at the appropriate concentrations (10⁻⁷ and 10⁻⁸ M) and were prepared for flow cytometric analysis or prepared for protein extracts.

Nonylphenol was purchased from Sigma-Aldrich (St Louis, MO) and was dissolved in Me₂SO. Test agents were diluted with culture medium at final concentrations of 10⁻⁸ and 10⁻⁷ M. A preliminary dose-response curve (10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶ and 10⁻⁵ M) was performed to determine higher concentrations of 4-nonylphenol, not toxic to the cells. This preliminary experiment allowed us to identify doses that were not toxic to cells but that acted on cell-cycle regulation.

Flow cytometry for cell-cycle analysis

After treatment with 4-nonylphenol for 24 and 48 h, cells were collected and centrifuged at 2000 *g* at 4 °C, the pellets were fixed in 1 ml with 70% ethanol for 4 h at -20 °C. Then cells were centrifuged at 2000 *g* at 4 °C, washed in PBS and incubated in 500 ml of hypotonic buffer (0.1% TritonX-100, 0.1% sodium citrate, 50 mg/ml propidium iodide (PI) and 100 mg/ml RNase). Then, 20 000 cells were analysed in each sample using a Becton Dickinson FACSCalibur flow cytometer and percentages of G₁, S, G₂/M, and sub-G₁ (apoptotic cells) populations were calculated by ModFit version III software (Verity Software House Inc, USA).

Annexin-V assays

Apoptotic cells were analysed using annexin V FITC apoptosis detection kit (Calbiochem, Gibbstown, NJ,

USA) according to the manufacturer's instructions. For fluorescent microscopic image analysis of the apoptotic fraction of the cells, treated cells and control cells (1 × 10⁶ cells/ml) were mixed with annexin V-biotin and medium-binding reagent, and incubated in the dark for 15 min at room temperature. Cells were then centrifuged and medium was replaced with 1× binding buffer containing FITC-streptavidin. PI was added to discriminate early apoptotic cells from late apoptotic or necrotic cells. A portion of cell suspension (50 µl) was placed on to a glass slide, covered with a coverslip, and viewed immediately, using fluorescence microscopy and FITC (green) and PI (red) filters.

Protein extraction and western blotting analysis

AGS cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM Na₃VO₄, 1 mM phenylmethylsulphonyl fluoride and 10 µg/ml leupeptin] for 30 min in ice. Total extracts were cleared by centrifugation for 30 min at 4 °C at 10 000 *g* and assayed for protein content using Bradford's method. Fifty micrograms protein from each cell lysate was separated using SDS-PAGE and transferred to PVDF membranes, and filters were stained with 10% Ponceau S solution for 2 min, to verify equal loading and transfer efficiency.

Membranes were probed with primary antibodies against p21, p27, p53, cleaved caspase 3, procaspase 3, procaspase 8, procaspase 9, Fas, Fas-L, cyclin D, E2F (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with rabbit polyclonal antibodies against Rb (able to recognize the p68/Rb fragment); Hsp70 antibody (heat shock protein) (Santa Cruz Biotechnology) was used to estimate equal protein loading. Primary antibodies were incubated following suggestions of the manufacturers. Membranes were then incubated in 1:5000 peroxidase-conjugated anti-mouse immunoglobulin for 1 h at 22 °C and 1:2500 peroxidase-conjugated anti-rabbit immunoglobulin for 1 h at 22 °C. They were extensively washed and finally analysed using the ECL system (Amersham, Milan, Italy).

Results

NP inhibited AGS cell population growth

To assess whether NP induced cell population expansion inhibition by alteration in cell cycle progression in a concentration-dependent manner, we evaluated effects of this compound at different concentrations, on cell cycle phase distribution.

AGS cells were treated with DMSO vehicle controls and with various concentrations of NP (10⁻⁹, 10⁻⁸, 10⁻⁷,

10^{-6} and 10^{-5} M) at 24 and 48 h, and FACS analysis was performed. Figure 1a displays cell cycle distribution after NP treatment. Particularly cell cycle at 10^{-9} M 4-nonylphenol concentration was comparable to vehicle control (DMSO) both at 24 and 48 h, while at 10^{-6} and 10^{-5} M concentrations 4-nonylphenol, no apoptotic peak was distinguishable and cells appeared to be necrotic (Fig. 1b). This result lead us to suppose that 4-nonylphenol effect was dose- and time-dependent. On the basis of this observation, we conducted all other experiments with 10^{-7} and 10^{-8} M concentration of NP and control AGS cells were treated with DMSO vehicle collected at 48 h.

The AGS cells were treated with concentrations of NP (10^{-7} – 10^{-8} M) for 24 and 48 h, and FACS analysis was performed. After 10^{-7} – 10^{-8} M NP treatment for 48 h, AGS cells in both cases arrested cell-cycle progression in G₂/M phase. Figure 2a shows that inhibition of cell population expansion by NP was concentration-dependent. Also, we observed that NP caused significant inhibition of cell proliferation at 10^{-7} M concentration with increase in G₂/M. Treatment with 10^{-7} M NP caused increase in the sub-G₁ peak, indicative of apoptotic cell death, compared to AGS cells, vehicle controls and cells treated with 10^{-8} M NP. On the contrary, treatment of AGS with 10^{-8} M NP induced the same accumulation of cells in the G₂/M phase with concomitant reduction in the S phase population, but no increase in sub-G₁ peak was evident. These data seem to indicate that AGS cells were more responsive at high concentration of NP (10^{-7} M) in terms of induction of apoptosis. We observed that apoptotic pathways were induced by increased dose of NP treatment, by approximately 20% (10^{-7} M treatment) of cells after 48 h treatment (Fig. 2a,b).

NP induced apoptosis in AGS cells

To best verify apoptotic induction by NP treatment, we used the annexin V assay (12,13), on 10^{-7} M NP-treated AGS cells in comparison with vehicle control cells. Vehicle control and 10^{-7} M NP AGS cells were prepared for analysis using the annexin V stain that detects cells undergoing apoptosis using PI to detect non-viable cells. We analysed cells by immunofluorescence staining with annexin V for apoptosis after 10^{-7} M NP treatment at 48 h. We observed that cells treated with 10^{-7} M NP were positive for annexin-V (Fig. 3IV, V, VI) in contrast to vehicle-treated cells (Fig. 3I, II, III).

Cell cycle molecular pathways analysis

To evaluate which molecular pathways were involved in cell cycle arrest and in apoptotic events induced by NP treatment, we performed western blot assays to analyse

important proteins involved in the cell cycle and in apoptosis. DMSO was our standard control, used over all. In our preliminary dose–response curve treatment, we did not observe any effects on the cells from time 0 of treatment until the end of the experiment (48 h). In our western blot figures, we present only the latest time point of the experiment as control (time 48 h, always in lane 1). We observed marked accumulation of p27 cells exposed to 10^{-7} M NP for 24 and 48 h compared to 10^{-8} M NP-treated AGS cells and AGS cells serving as vehicle control (Fig. 4).

We also evaluated possible involvement of p21, another cyclin-dependent kinase inhibitor reported to induce cell cycle arrest and apoptosis. Western blot analysis showed that in AGS cells exposed to 10^{-8} M NP for 24 and 48 h, p21 expression was modulated and in particular its expression was higher in 10^{-8} M NP-treated cells for 24 h than in 10^{-8} M NP treated cells for 48 h. The same modulation was observed in p21 expression when cells were exposed to 10^{-7} M NP for 24 and 48 h. As shown in Fig. 4, p21 protein had accumulated by 48 h following treatment to 10^{-7} M NP compared to both vehicle control cells and 10^{-8} M NP cells at 48 h. We also studied whether p21 accumulation was dependent on p53 induction. To study this, we performed p53 western blot analysis. We demonstrated that p53 had low level of expression at 24 h and an almost undetectable level of expression at 48 h both in 10^{-7} M NP and 10^{-8} M NP cells (Fig. 4). As shown in Fig. 4, p53 was not involved in p21 accumulation. These results indicate that cell population expansion arrest observed in 10^{-7} and 10^{-8} M NP-treated AGS cells was associated with p53-independent activation of p21.

Rb dephosphorylation accompanied G₁ arrest and apoptosis in 10^{-7} M NP treated AGS cells

We also investigated whether Rb protein was involved in NP-induced population growth arrest and apoptosis in AGS cells. Cells had been treated with NP for 24 and 48 h at the two different concentrations (10^{-8} and 10^{-7} M). At indicated times, aliquots of cells were used for measurement phosphorylated Rb. We performed immunoblot analysis using anti-Rb antibody that reacts with p120/Rb, p115/Rb and with p68/Rb as described previously (14). In 10^{-7} M NP treated cells at 48 h, we observed conversion of phosphorylated Rb into non-phosphorylated Rb and expression of cleaved p68 band, typically indicating the apoptotic process, compared to 10^{-8} M NP treatment and AGS cell vehicle control (Fig. 5a). We observed reduction in E2F-1 (data not shown) and cyclin D protein levels in 10^{-7} M NP treatment at 48 h compared to both 10^{-8} M NP treatment and AGS cell vehicle control (Fig. 5b).

Figure 1. Effects of different NP concentrations treatment on cell cycle distribution. (a) Preliminary dose–response curve (10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} M) of cells treated with NP for 24 and 48 h respectively. Data are presented as means $\pm$ standard deviations of the results of three independent experiments. *Significantly different compared to control ($P < 0.05$). (b) Cells vehicle controls and treated with NP (10^{-6} and 10^{-5} M) for 24 and 48 h respectively. Cells were fixed, stained and flow cytometric analysis was conducted. Data shown are representative of three different experiments. G₀/G₁ phase; S phase; G₂/M phase; Ap, apoptotic cells; Nc, necrotic cells.

Figure 2. Effects of 10^{-7} and 10^{-8} M NP treatment on cell cycle distribution. (a) Cells treated with NP (10^{-7} and 10^{-8} M) for 24 and 48 h respectively. Cells were fixed, stained and flow cytometric analysis was conducted. Data shown are representative of three different experiments. G₀/G₁ phase; S phase; G₂/M phase; Ap, apoptotic cells. (b) Cells treated with NP (10^{-7} and 10^{-8} M) for 24 and 48 h respectively. Data are presented as means $\pm$ standard deviations of the results of three independent experiments. *Significantly different compared to control ($P < 0.05$).

Activation of caspase 3 in AGS cells undergoing apoptosis

To elucidate molecular pathways involved in NP-induced apoptosis, expressions of procaspase 8 and procaspase 9 were measured in the AGS cells following NP exposure (10^{-7} , 10^{-8} M). Western blot analysis showed reduction in procaspase 8 in cells exposed to 10^{-7} M NP for 48 h compared both to cells exposed to 10^{-8} M NP and vehicle controls. In contrast, immunoblotting analysis did not show procaspase 9 activation (Fig. 6). This result suggests that caspase 9 was not involved in the apoptotic pathway

activated by NP treatment, whereas caspase 8 was activated. To confirm activation of the apoptotic event, we performed western blot analysis to detect procaspase 3 and its expression. Figure 6 shows reduction of procaspase 3 and increase in cleaved caspase 3 in cells exposed to 10^{-7} M NP for 48 h compared both to cells exposed to 10^{-8} M NP and vehicle controls.

Changes in Fas and FasL expressions after NP exposure

To further investigate whether NP-induced apoptosis occurred *via* the caspase 8-dependent pathway, we

Figure 3. Annexin V FITC apoptosis detection kit. Apoptosis induced by 10^{-7} M NP after treatment for 48 h (IV, V, VI) respected to the cells control (I, II, III). Annexin V-positive signals (green) were detected after exposure to 10^{-7} M NP for 48 h. Cells were counterstained with DAPI (blue).

Figure 4. Western blot analysis for p21, p27, p53 and Hsp70 proteins expression. Lane 1, AGS cells treated with vehicle (DMSO) for 48 h; lane 2–3, AGS cells treated with 10^{-8} M NP for 24 and 48 h respectively; lane 4–5, AGS cells treated with 10^{-7} M NP for 24 and 48 h respectively.

examined changes in Fas and Fas ligand (FasL) expressions in AGS cells after NP treatment. Western blot analysis demonstrated that expression of Fas increased in cells exposed to 10^{-7} M NP at 48 h compared to 24 h, and compared to 10^{-8} M NP AGS treated cells and vehicle control (Fig. 7). Moreover, we demonstrated that expression of Fas-L was higher in AGS cells exposed to 10^{-7} M NP compared to 10^{-8} M NP treatment and vehicle control.

Discussion

The problem of NP environmental contamination is closely related to food, as any such substance dispersed in the environment cannot avoid entering the food chain in one way or another. The European Commission and international organizations responsible for monitoring environmental health and food safety, have highlighted potential serious consequences posed by this heterogeneous group

Figure 5. Western blot analysis for Rb, Cyclin D and Hsp70 proteins expression. (a) Western blotting was performed using anti-Rb antibody. Upper part shows, in a 6% gel, the phosphorylated Rb form and low part shows, in a 10% gel, the cleaved Rb fragment. Lane 1, AGS cells treated with vehicle (DMSO) for 48 h; lane 2–3, AGS cells treated with 10^{-8} M NP for 24 and 48 h respectively; lane 4–5, AGS cells treated with 10^{-7} M NP for 24 and 48 h respectively. (b) Western blotting was performed using anti-cyclin D antibody. Lane 1, AGS cells treated with vehicle (DMSO) for 48 h; lane 2–3, AGS cells treated with 10^{-8} M NP for 24 and 48 h respectively; lane 4–5, AGS cells treated with 10^{-7} M NP for 24 and 48 h respectively.

of substances. Food is an important pathway to exposure to EDs and various studies have demonstrated that NP is able to exert its oestrogenic activity at low concentrations. Most research to date on NP has focused on development of reproductive organs in rats (15,16) and fish (17). Multi-generation studies in rats has shown that NP affects not only reproductive organs but also non-reproductive organs such as kidney and liver (18,19).

Thus, deleterious effects of NP on several organs have been reported, but there have been no reports on effects of

Figure 6. Western blotting was performed using anti-procaspase 9, anti-procaspase 8, anti-procaspase 3 and caspase 3. Lane 1, AGS cells treated with vehicle (DMSO) for 48 h; lane 2–3, AGS cells treated with 10^{-8} M NP for 24 and 48 h respectively; lane 4–5, AGS cells treated with 10^{-7} M NP for 24 and 48 h respectively.

Figure 7. Western blotting was performed using anti-Fas and Fas-L antibody. Lane 1, AGS cells treated with vehicle (DMSO) for 48 h; lane 2–3, AGS cells treated with 10^{-8} M NP for 24 and 48 h respectively; lane 4–5, AGS cells treated with 10^{-7} M NP for 24 and 48 h respectively.

NP on human gastric tissue. The purpose of this study was to determine such effects of NP (a metabolite of alkylphenol ethoxylates) in human gastric cells as human exposure to NP may occur by cutaneous absorption, ingestion of contaminated food/water or by inhalation (8,9).

In our study, we investigated whether NP would interfere with the normal cell cycle of human gastric epithelial (AGS) cells. Our results indicate that NP was able to inhibit cell proliferation and to induce apoptosis in a time- and dose-dependent manner.

Flow cytometric analysis showed that treatment with 10^{-7} M NP led to accumulation of cells at the G_2/M transition and increased percentage population of apoptotic cells. Our findings suggest that NP caused cell cycle arrest through accumulation of AGS at G_2/M transition after 48 h. This inhibition of proliferation by NP may be partly due to apoptosis *via* activation of the caspase cascade and to G_2/M transition accumulation. FACS analysis clearly

revealed that NP at 10^{-7} M concentration arrested proliferation in G_2 phase. Cells exposed to NP at 10^{-7} M concentration for 48 h, had marked increase in p21 and p27 expression.

p21 protein can be induced by DNA damage and during apoptosis in specific cell types, as a function of wild-type p53 activation (20). It can also be induced in a p53-independent manner following mitogenic stimulation of population growth-arrested cells (21). Furthermore, when DNA damage occurs during G_2 , cells are able to initiate cell cycle arrest in absence or presence of p53. Our data showed that p21 induction was p53-independent. In particular, in our experimental model, we observed that induced accumulation of p21 in the first 24 h of treatment, and probably this event is at least partly responsible for cell population growth inhibition at both concentrations. Later, expression decreased probably as a consequence of activation of apoptosis. Such modulation was the same in both treatments because lanes 2 and 4, and lanes 1 and 3 are comparable.

A variety of studies suggest that retinoblastoma protein Rb, an important tumour suppressor and cell cycle regulator, also plays a regulatory role in the process of apoptosis. Hyperphosphorylated Rb (p120Rb) is converted into hypo-phosphorylated (p115Rb), and this new form is immediately cleaved by a certain protease that has properties in common with the caspase family (22). The hypophosphorylated form is cleaved into p68 and p48 fragments and interior cleavage of Rb is tightly associated with initiation of apoptotic execution (15). In our experimental model, we observed that there was an increase in cleavage of pRB in 10^{-7} M NP-treated AGS cells compared to both 10^{-8} M NP-treated cells and vehicle controls, thus suggesting involvement of Rb in regulation of the apoptotic process.

Activation of caspase cascades has been demonstrated to be essential in induction of apoptosis in many different cell types (23–25). There are two apoptotic pathways: the receptor-mediated pathway, initiated by binding of FasL, Fas and caspase 8, and the mitochondrial pathway, mediated by certain members of the Bcl-2 family, Apaf-1 and caspase 9. Among the various caspases, caspase 3 is considered to be a major player in apoptosis initiation as it generally participates in all major apoptotic signal transduction pathways.

Our results showed that NP at concentration of 10^{-7} M induced activation of caspases 8 and 3, but not activation of caspase 9; which lead to increased apoptosis in AGS cells. Caspase 8, which binds Fas-associated death domain, is activated by self-cleavage as a result of oligomerization and subsequently, activates effector caspases such as caspase 3 (22). This result is in agreement with the study of Kudo *et al.* (24), which demonstrated that NP

induced apoptosis in mouse neural stem cells through activation of caspase 3. In this study, we showed that protein expression of Fas and FasL was markedly increased in the AGS cells exposed to NP at 10^{-7} M concentration and that involvement of the Fas/FasL pathway in NP-induced apoptosis was found in association with activation of downstream caspase 8.

Involvement of Fas/FasL pathway in apoptosis induced by NP has also been reported in thymocytes and rat test (25,26). Our results show that NP exposure altered survival of AGS cells, causing deregulation of the cell cycle and apoptosis in a time- and dose-dependent manner. The important role of NP as oestrogenicities (27–29) has been studied, but its potential effects on early embryonic development, differentiation and cell cycle have not been examined extensively. In the present study, we provide additional evidence that gastric cells can be useful for investigation of developmental toxicity. In particular, we have demonstrated for the first time that environmental toxin NP, induces apoptosis in AGS cells in a dose-dependent manner and that the Fas/FasL pathway is involved in this process. These findings offer new perspectives to understanding fundamental mechanisms of chemical-induced apoptosis in AGS cells and may contribute to development of new predictive screening methods for hazard assessment of developmental toxicity using cell technology.

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