Effect of nonylphenol on the regulation of cell growth in colorectal cancer cells

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Abstract. Nonylphenol (NP) is a well-known endocrine-disrupting chemical (EDC), which can enhance the progression of cancer by functioning as an estrogen-like factor. In the present study, the effects of different concentrations of NP on COLO205 colorectal cancer (CRC) cells were examined. The results of flow cytometric analysis revealed that NP significantly decreased the proportion of cells in the G0/G1 phase in a dose-dependent manner, which was accompanied by a marginal increase in the proportions of cells in S and G2/M phases. NP did not induce apoptosis, whereas estradiol (E2) did induce apoptosis. To elucidate the mechanisms underlying the action of NP on COLO205 cells, the transcriptional levels of extracellular signal-regulated kinase (ERK)1, ERK2 and phosphoinositide 3-kinase (PI3K) were assessed using reverse transcription-quantitative polymerase chain reaction analysis. The expressions levels of ERK1, ERK2 and PI3K were increased by treatment with NP in a dose-dependent manner. On examining protein levels, the expression of PI3K p38 was increased by NP and E2, and the expression of ERK1/2 was increased by NP. The phosphorylation of the ERK protein was significantly increased by treatment with NP at a high concentration (10⁻⁴ M; P<0.01), but significantly decreased by E2 (P<0.01). Two key proteins in the transforming growth factor (TGF)β pathway (c-Fos and SnoN) were selected for analysis using western blot analysis in the COLO205 cells treated with NP and E2. The expression levels of c-Fos and SnoN were significantly increased by treatment with E2 (10⁻⁴ M; P<0.01) and NP (10⁻²-10⁻⁴ M; P<0.01). Taken together, these results indicated that NP affected the development of CRC via the ERK signaling pathway and TGFβ pathway.

Introduction

Endocrine-disrupting chemicals (EDCs) are exogenous compounds, which are present in the environment and food, and have an effect on the normal endocrine system (1,2). EDCs have effects on male and female reproduction, developmental disorders, the development and progression of cancer, metabolism and obesity, and cardiovascular endocrinology (1,3,4). EDCs can induce estrogen-like or androgen-like effects by binding to hormone receptors, including the estrogen receptor (ER) and androgen receptor (AR), therefore, they may interfere with the actions of endogenous steroid hormones or induce hormone-mediated responses. The abnormal activation of estrogen signaling by EDCs leads to altered gene expression in target tissues and carcinogenesis (5).

Nonylphenol (NP) is a well-known EDC. It is a degradation product of alkyphenol polyethoxylate (APE). The use of pesticides, polystyrene plastics and paints, including APE, can lead to the bioaccumulation of NP in the food chain. The accumulation of NP in the body can result in endocrine disruption, and immunological and reproductive disorders (6). It has been reported that NP has an estrogenic effect in humans (6). Previous studies have demonstrated that NP can enhance the progression of cancer by acting on the cell cycle, apoptosis and metastasis in breast, ovarian and prostate cancer (7-10).

Colorectal cancer (CRC) is the third most common type of malignancy with high mortality rates in men and women worldwide (11). In disease progression, the processes of the DNA repair system, inflammation and apoptosis are altered, and metastases are the primary cause of poor prognosis and cancer-associated mortality in patients with CRC (12). The risks of human CRC are associated with smoking, alcohol intake, dietary factors and obesity. A number of studies have suggested that estrogen has a potential role in the development of CRC (13,14). Men are more susceptible to colon cancer than women, and the use of hormone-replacement therapy reduces the risk of CRC in postmenopausal women (15). However, the evidence that high concentrations of circulating estrogen confer an increased risk for CRC in men and women is inconsistent (16-18). Estrogen concentrations are higher in CRC tissues, compared with concentrations in nonneoplastic tissues in patients with CRC, and patient prognosis is poorer when intratumoral estrogen concentrations are higher (19).

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Therefore, xenoestrogens, including NP, may also be associated with the risk of CRC.

In the present study, the in vitro effects of NP at different concentrations on COLO205 CRC cell cycle and apoptosis were examined, and the mechanism of action was investigated by analyzing alterations in the expression of genes in the ERK pathway and TGFβ pathway.

Materials and methods

Cell culture and treatment. Human COLO205 CRC cells were obtained from the American Type Culture Collection (ATCC CCL-222; Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 medium (HyClone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., Huzhou, China), 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. NP with analytical standard purity was purchased from Aladdin Industrial Corporation (Shanghai, China), and was dissolved in absolute ethyl alcohol to 50 mmol/l.

Flow cytometric analysis of cell cycle and cell apoptosis. The effects of NP and estradiol (E2) on cell cycle progression were determined using flow cytometry. Following fixation, the cells were stained with propidium iodide (PI) solution (50 µg/ml PI and 100 µg/ml RNase A in PBS) and then subjected to cell cycle analysis. The extent of cell apoptosis was measured using Annexin V/PI double staining. Binding buffer (300 µl) was used for cell resuspension (6x10⁴ cells), and 5 µl of Annexin V-FITC was added to the cell suspension for 10 min incubation in the dark. Subsequently, 5 µl of PI was added to the cell suspension for 5 min in the dark. The samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The COLO205 cells were seeded at a density of 0.5x10⁴ cells/well in 6-well plates at 37°C in a humidified atmosphere of 5% CO₂ until >70% confluent growth. The cells were treated with medium containing E2 (10⁻⁷ M) or NP (10⁻⁷-10⁻⁴ M) and cultured for 48 h at 37°C in a humidified 5% CO₂ atmosphere. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s protocol. Total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Toyobo Co., Ltd., Dalian, China) according to the manufacturer’s protocol. The cDNA was stored at -20°C. The qPCR analysis was performed on a StepOne™ system (Thermo Fisher Scientific, Inc.) in a 10 µl volume containing 5 µl of 2X qPCR mix of SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd., Dalian, China), 1.0 µl of each forward and reverse primer (Table I; 2.5 µM), 1.0 µl of cDNA (~100 ng) and 3 µl ddH₂O. The reaction consisted of 5 min at 95°C, followed by 40 cycles of 95°C (5 sec), 72°C (40 sec) and then melting curves from 60 to 95°C (0.1°C/sec), ending with a step at 15°C. Melting curves were used to confirm the specificity of each primer, and no primer-dimer were identified. The relative gene expression levels were calculated using the 2⁻ΔΔCq method (20) and β-actin was used as an internal control. The sample containing six biological replicates was amplified in triplicate.

Western blot analysis. To measure the protein expression levels of ERK, PI3K, c-Fos, SnoN and β-actin, the COLO205 cells were cultured to a density of 1x10⁶ cells and then incubated with E2 (10⁻⁷ M) or NP (10⁻⁷-10⁻⁴ M) for 48 h at 37°C in a humidified 5% CO₂ atmosphere. Following treatment, whole cell lysates of the COLO205 cells were prepared in 1X RIPA buffer (Beyotime Institute of Biotechnology) for 30 min on ice. The total protein concentrations were determined using bichoninic acid (Thermo Fisher Scientific, Inc.). The total protein (40 µg) was separated by SDS-polyacrylamide gel electrophoresis (5% stacking gel and 10% separating gel), following which the proteins were transferred onto polyvinylidenedifluoride membranes (EMD Millipore, Billerica, MA, USA), and these membranes were blocked with 5% skim milk powder (BD Biosciences) for 60 min at room temperature. The membranes were then incubated with primary antibodies overnight at 4°C: Rabbit anti-β-actin (cat no. T2051; 1:10,000; Thermo Fisher Biotech Co., Ltd., Beijing, China) and anti-PI3K (cat no. ab40755; 1:500; Abcam, Cambridge, MA, USA) diluted in 5% skimmed milk, and anti-ERK1/2 (cat no. 4695; 1:1,000), anti-p-ERK1/2 (cat no. 4370; 1:10,000) and anti-PI3Kp85, c-Fos, SnoN and c-Fos (cat no. ab40755; 1:500; Abcam, Cambridge, MA, USA) diluted in 5% skim milk powder for 60 min at room temperature. The membranes were then incubated with primary antibodies overnight at 4°C: Rabbit anti-β-actin (cat no. T2051; 1:10,000; Thermo Fisher Biotech Co., Ltd., Beijing, China) and anti-PI3K (cat no. ab40755; 1:500; Abcam, Cambridge, MA, USA) diluted in 5% skimmed milk, and anti-ERK1/2 (cat no. 4695; 1:1,000), anti-p-ERK1/2 (cat no. 4370; 1:10,000) and anti-PI3Kp85, c-Fos, SnoN and c-Fos (cat no. ab40755; 1:500; Abcam, Cambridge, MA, USA) diluted in 5% BSA. The membranes were subsequently probed with secondary antibody (HRP-goat anti rabbit; cat no. ab6721; 1:10,000; Abcam) for 30 min at room temperature. Target proteins were detected using Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The optical density was analyzed using AlphaEaseFC software version 6.0. All experiments were performed at least three times.

Statistical analysis. Each experiment was repeated three times and analyzed using Excel 2013 (Microsoft Corporation, YANG et al.: EFFECT OF NONYLPHENOL ON COLORECTAL CANCER CELLS

Table I. Primer sequences and product sizes of products for reverse transcription-quantitative polymerase chain reaction analysis.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>Actin</td>
<td>5'-CGTTGACATCCGTAAGACCTC-3'</td>
<td>110</td>
</tr>
<tr>
<td>PI3K</td>
<td>5'-ATTAGCCATTTCACCC-3'</td>
<td>262</td>
</tr>
<tr>
<td>ERK1</td>
<td>5'-ACTGCTTTTCCACGGATGT-3'</td>
<td>219</td>
</tr>
<tr>
<td>ERK2</td>
<td>5'-GCACCCACCATTGAGCAT-3'</td>
<td>173</td>
</tr>
</tbody>
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PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase.
Redmond, WA, USA). Data are presented as the mean ± standard deviation. Statistical analyses were performed using a one-tail t-test, P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Effects of NP on cell cycle and apoptosis.** To evaluate the effect of NP on cell growth, COLO205 cells were cultured with E2 (10⁻⁷ M) or NP (10⁻⁷-10⁻⁴ M) for 48 h, and cell cycle distribution was analyzed using flow cytometry. Representative fluorescence histograms and the percentage of cells in each phase are shown. NP, nonylphenol; E2, estradiol.

![Figure 1. Effects of NP and E2 on COLO205 cell cycle. COLO205 cells were treated with E2 (10⁻⁷ M) or NP (10⁻⁷-10⁻⁴ M) for 48 h, and cell cycle distribution was analyzed using flow cytometry. Representative fluorescence histograms and the percentage of cells in each phase are shown. NP, nonylphenol; E2, estradiol.](image1)

![Figure 2. Effects of NP and E2 on apoptosis. (A) blank and (B) negative control (CRC cells with no treatment). COLO205 cells were treated with (C) E2 (10⁻⁷ M) or (D-F) NP (10⁻⁷-10⁻⁴ M) for 48 h, and apoptosis was analyzed using flow cytometry. The graphs show that the percentage of apoptotic cells (right quadrants) increased in COLO205 cells treated with E2 (10⁻⁷ M) and those treated with the middle dose of NP (10⁻⁵ M), but was not affected by treatment with low or high doses of NP (10⁻⁷ M or 10⁻⁴ M). NP, nonylphenol; E2, estradiol; PI, propidium iodide.](image2)
apoptotic rate increased in the E2 treatment group and in the middle dose NP (10^{-5} M) treatment group, compared with the control group, however, no significant changes were observed in the low and high dose NP (10^{-7} M and 10^{-4} M) treatment groups.

**Alterations in expression of the ERK pathway by NP.** The effects of E2 and NP on expression of the ERK pathway in the COLO205 cells were determined using RT-qPCR and western blot analyses (Fig. 3). The protein expression levels of PI3Kp85 and ERK1/2 were significantly increased by NP (P<0.01; Fig. 3A-C). Phosphorylation of the ERK protein was significantly increased by treatment with NP at a high concentration (10^{-4} M; P<0.01; Fig. 3A and D), however it was significantly decreased by E2 (P<0.01). The results of the RT-qPCR analysis showed the same expression patterns in the mRNA expression levels of PI3K, ERK1 and ERK2 (Fig. 3E).

**Alterations in expression of the TGF pathway in response to NP.** Two key proteins in the TGFβ pathway (c-Fos and SnoN) were selected for examination using western blot analysis in COLO205 cells treated by NP and E2 (Fig. 4A). The expression levels of c-Fos and SnoN were significantly enhanced.
by treatment with E2 (10⁻⁷ M; P<0.01) and NP (10⁻⁷-10⁻⁴ M; P<0.01) as shown in Fig. 4B and C.

**Discussion**

EDCs can interfere with hormone systems and produce adverse developmental, reproductive, neurological and immunological effects in mammals (21). Previous studies have shown that these substances also adversely affect human health, resulting in reduced fertility and increased progression of certain diseases, including obesity, diabetes, endometriosis and certain types of cancer (9,22-24). EDCs can interrupt the normal functions of reproductive organs by aberrantly binding to hormone receptors and further triggering hormone-responsive cancer initiation and progression, including in breast, ovarian and prostatic cancer (8-10). The present study examined whether one of the EDCs, NP, enhances the progression CRC.

The results of the present study revealed that the proportion of COLO205 CRC cells in the G0/G1 stage was significantly reduced by NP in a dose-dependent manner, which indicated that NP promoted CRC cell growth, as reported in previous cancer studies (8,25). However, despite the alterations observed in cell viability, the apoptotic rates of the cells were unaffected by NP. These results suggested that NP promoted cell proliferation, but had no effect on apoptosis.

Mutation activation of the ERK signaling pathway is frequently observed in human cancer, including CRC (26), and is important in the regulation of malignant cellular proliferation, migration and invasion. Previous studies have demonstrated that estrogens and xenoestrogens can activate the phosphorylation of ERK1/2 in rat pituitary tumor cells, and that responses are inhibited by ERK inhibitors (27,28). The present study found that the phosphorylation of ERK1/2 was significantly increased in CRC cells treated with NP in a dose-dependent manner; however, this phosphorylation was decreased by E2. This difference may be due to the two types of cell being derived from different genetic backgrounds and undergoing several other genomic hits by treatment with E2. These results indicated that NP promoted the proliferation of CRC cells through activating the ERK signaling pathway, however, the mechanism of activation and the reason underlying the different effects induced by E2 and NP require further investigation.

The TGFβ signaling pathway is also a key determinant of carcinoma cell behavior, acting as a tumor suppressor pathway, and a promoter of tumor progression and invasion (29). In ovarian cancer models, EDCs have been shown to inhibit the TGFβ signaling pathway by preventing the degradation of SnoN protein and increasing the protein expression of c-Fos (8). Members of the Fos family, including c-Fos, FosB, and its smaller splice variants, Fra-1 and Fra-2, dimerize with Jun proteins to form the activating protein 1 transcription factor complex, which is central during malignant transformation and progression (30). In addition, SnoN is a negative regulator of the TGFβ signaling pathway and is directly linked to its ability to repress the transcription of TGFβ-inducible genes (31). In the present study, it was also found that NP induced the expression of c-Fos and SnoN, as did E2. Therefore, it was hypothesized that NP may inhibit the TGFβ signaling pathway in CRC cells as it does in ovarian cancer cells.

In conclusion, the results of the present study suggested that NP may affect the growth of CRC cells by activating the ERK signaling pathway via increasing the phosphorylation of ERK1/2 and inhibiting the TGFβ signaling pathway via the upregulation of c-Fos and SnoN. This is the first report, to the best of our knowledge, to demonstrate the potential mechanism between NP and CRC. The results of the present study demonstrated the effect of NP on the progression of CRC. Further investigations are required to establish the mechanism underlying the effects of NP on the ERK and TGFβ signaling pathways.
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References