Nonylphenol promotes the proliferation of colorectal cancer COLO205 cells by upregulating the expression of protein kinase C ζ

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Abstract. Previous studies have indicated the potential role of estrogen in the development and prognosis of colorectal cancer (CRC). Nonylpheno (NP) is an endocrine-disrupting chemical, which may influence the development of estrogen-dependent types of cancer. However, the molecular mechanism of NP in the development of CRCs remains unclear. In the present study, various concentrations of NP were used to treat COLO205 CRC cells, and the expression of protein kinase C ζ (PKC ζ) was knocked down using PKC ζ small interfering RNA. The effects of NP in various concentrations on the cell cycle and apoptosis of COLO205 cells were examined, and the change in the expression level of PKC ζ was analyzed. The results indicated that NP may significantly induce proliferation of COLO205 CRC cells, and significantly reduce cell apoptosis. However, suppression of PKC ζ expression may inhibit proliferation, while NP could reduce this inhibition. The results of a western blot analysis indicated that the expression level of cyclin D1 and E were significantly increased following NP treatment, and the expression of p27 was significantly decreased. The phosphorylation of PKC ζ and extracellular-signal-regulated kinase (ERK)1/2 was significantly increased following NP treatment in a dose-dependent manner. Overall, NP induced human CRC COLO205 cell proliferation and inhibited the apoptotic rate of COLO205 cells by increasing the activity of PKC ζ and ERK1/2.

Introduction

Colorectal cancer (CRC) was the third most common carcinoma of the human digestive system worldwide in 2014 (1). Influenced by genetic, environment and life style factors, the risk of CRC

has increased annually, becoming one of the most common types of cancer with a high mortality rate reported in China (2014) (1). The current treatments of CRC are primarily surgery and chemotherapy (2). A previous study indicated that estrogen serves a potential role in the development and prognosis of CRC (3).

With the development of modern industry, numerous endocrine-disrupting chemicals (EDCs), including nonylphenol (NP) and bisphenol A, have been identified in the environment (4-7). EDCs may have an estrogen-associated or androgen-associated effect by binding to hormone receptors and promoting the development of hormone-dependent tumors (8). NP is an EDC, which could influence the development of estrogen-dependent cancer types, including breast and prostate cancer (9,10). Our previous study indicated that NP could activate extracellular-signal-regulated kinase (ERK)1/2 to induce the proliferation of CRCs cells (11). However, the underlying molecular mechanism of NP on the development of CRCs remains unclear.

Protein kinase C (PKC), which exists in various cells and tissues, is a type of multifunctional serine and threonine kinase. This protein could mediate the proliferation and differentiation of cells, and has been reported to be involved in the regulation of the cell cycle and apoptosis, promoting the development and metastasis of tumors (12). PKC ζ is a member of the PKC family, which influences proliferation and transfer of various types of cancer cells (13-15). Inhibiting the expression of PKC ζ may reduce the invasion ability of CRC, breast cancer and glioma (16). To the best of our knowledge, no evidence exists on whether NP could mediate the development of CRC by regulating the expression of PKC ζ .

To further examine the effect of NP on the proliferation of CRC cells and the expression and activity of PKC ζ , different concentrations of NP were used to treat the COLO205 CRC cells, and to knock down the expression of PKC ζ by PKC ζ small interfering (si)RNA. The *in vitro* effects of NP were further examined with different concentrations of NP on the cell cycle and apoptosis of COLO205 cells, and the altered expression of PKC ζ was analyzed.

Materials and methods

Cell culture and treatment. Human CRC COLO205 cells were obtained from the Chinese Academy of Sciences Institute

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of Cell Resource Center (Shanghai, China). The cells were cultured in RPMI-1640 (GE Healthcare Life Sciences, Little Chalfont, UK) supplemented with 10% fetal bovine serum (FBS; Procell Life Science & Technology Co., Ltd., Wuhan, China), 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in an atmosphere containing 5% CO₂. NP with analytical standard purities was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China), and was dissolved in absolute ethyl alcohol to 50 mM.

siRNA design and cell transfection. The siRNA oligo was synthesized by Sangon Biotech Co., Ltd., (Shanghai, China). Sequences were as follows: si-PKC ζ , 5'-GGAGGACCTTAAGCCAGTT-3' and siRNA negative control (NC) 5'-AGACTGTGAATCTAGATCAAG-3'.

For transfection, COLO205 cells were cultured for 12 h in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, and the fragment of siRNA (50 nM) was transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. After 72 h of transfection, cells were washed by PBS and then lysed with 1X radioimmunoprecipitation (RIPA) buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with 0.2 mM phenylmethylsulfonyl fluoride protease inhibitor (cat. no. 36978; Thermo Fisher Scientific, Inc.) for 30 min on ice for western blot analysis.

MTT analysis. Cell viability was detected using the CellTiter 96[®] Non-Radioactive Cell Proliferation assay (MTT; cat. no. G4000; Promega Corporation, Madison, WI, USA). COLO205 cells (1x10⁴ cells/ml) were seeded in 96-well plates for 24 h prior to treatment, with normal saline and cell culture mediums serving as the control, and three wells were prepared for each of the following groups: 0; 1x10⁻⁶; 1x10⁻⁷ and 1x10⁻⁸ mol/l NP alone (17,18); 1x10⁻⁶ mol/l NP with si-PKC ζ ; and 1x10⁻⁶ mol/l NP with NC. Following treatment for 0, 24, 48 and 72 h, a total of 15 μ l provided dye solution was added to each well, and the 96-well plate was incubated at 37°C for 4 h, subsequent to adding 100 μ l Solubilization/Stop Solution from the kit. Viability was recorded at a wavelength of 570 nm on a microplate reader (Multiskan; Thermo Fisher Scientific, Inc.). The assay was repeated in triplicate.

Flow cytometry analysis of cell cycle and apoptosis. The effect of NP and si-PKC ζ on cell cycle progression was determined by flow cytometry. After 24 h of treatment, the cells were digested and fixed with 70% ethanol for 24 h at 4°C. Following fixation, cells were stained with 50 μ g/ml propidium iodide (PI) solution and 100 μ g/ml RNase A in PBS for 30 min in the dark on ice and then subjected to cell cycle analysis. The apoptotic rate was measured using Annexin V/PI double staining (Annexin V-FITC Apoptosis Detection kit; cat. no. C1062; Beyotime Institute of Biotechnology). A total of 300 μ l binding buffer was used for cell resuspension 1x10⁶, and 5 μ l Annexin V-fluorescein isothiocyanate was added to the cell suspension for 10 min in the dark at room temperature. A total of 5 μ l PI was subsequently added to the cell suspension for 5 min in the dark on ice. The samples were analyzed with a FACSCalibur flow cytometer and the FACSCalibur system (both BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Total protein was extracted from COLO205 cells following treatment using RIPA buffer, and the concentrations were determined by bicinchoninic acid (Thermo Fisher Scientific, Inc.). A total of 40 μ g total protein was separated by SDS-PAGE (10% spacer gel and 5% separation gel). Proteins were transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA USA), and membranes were subsequently blocked with 5% skim milk powder (BD Biosciences) for 60 min at room temperature. The membranes were subsequently incubated with the following primary antibodies: Cyclin D1 (dilution, 1:800; cat. no. ab134175); cyclin E (dilution, 1:800; cat. no. ab33911), B-cell lymphoma 2 (Bcl-2) associated agonist of cell death (Bad; dilution, 1:500; cat. no. ab62465), Bcl-2 (dilution, 1:1,000; cat. no. ab32124), cyclin-dependent kinase inhibitor (p27; dilution 1:1,000; cat. no. ab32034), PKC ζ (dilution 1:1,500; cat. no. ab59364), phosphorylated (p)-PKC ζ (dilution 1:1,500; cat. no. ab62372) (all from Abcam, Cambridge, MA, USA), ERK1/2 (dilution 1:1,000; cat. no. 4695; Cell Signaling Technology, Inc., Danvers, MA, USA), p-ERK1/2 (dilution 1:1,000; cat. no. 4370; Cell Signaling Technology, Inc.) and GAPDH (dilution 1:1,000; cat. no. ab8245; Abcam) overnight at 4°C. The membranes were subsequently probed with goat anti-rabbit horseradish peroxidase-labeled secondary antibody (dilution 1:10,000; cat. no. ab6721; Abcam) for 1 h at room temperature. Target proteins were detected with Clarity[™] Western enhanced chemiluminescence substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocols. The optical density was analyzed by AlphaEaseFC software (version 5.0, ProteinSimple, San Jose, CA, USA). GAPDH was used as the internal control. All experiments were conducted in triplicate.

Statistical analysis. Each experiment was repeated in triplicate. Differences between different groups were evaluated by one-way analysis of variance, followed by Duncan's multiple range post-hoc test using GraphPad prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Difference between two groups were analyzed by a Student's t-test using Microsoft Excel 2017 (Microsoft Corporation, Redmond, WA, USA). Results are presented as means $\pm$ standard deviation and P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of NP and si-PKC ζ on the proliferation of COLO205 cells. The results of MTT indicated that NP (1x10⁻⁶-1x10⁻⁸ mol/l) could significantly induce the proliferation of COLO205 cells (F=48.66; P<0.01) in a time- and dose-dependent manner, compared with the control group (Fig. 1). Compared with the NC group, the proliferation of COLO205 cells demonstrated was significantly reduced by si-PKC ζ transfection in a time-dependent manner (P<0.01); however, recovery of proliferation was indicated following NP treatment (Fig. 1).

Effects of NP and si-PKC ζ on the cell cycle. Flow cytometry was used to investigate the influence of NP and PKC ζ on the cell cycle. Compared with the control group, the ratio of G0/G1 phase cells was significantly reduced by NP treatment (t=9.25, 13.17 and 14.74 for 1x10⁻⁸, 1x10⁻⁷ and 1x10⁻⁶ mol/l NP,

Figure 1. The effect of NP and si-PKC ζ on the proliferation rate. COLO205 cells were treated with NP (1×10^{-6} - 1×10^{-8} mol/l), si-PKC ζ , si-NC, and si-NC or si-PKC ζ with NP (10^{-6} mol/l). NP, nonylphenol; si, small interfering; PKC ζ , protein kinase C ζ ; NC, siRNA negative control. ** $P < 0.01$ vs. Control at 72 h.

respectively; all $P < 0.01$; Fig. 2A), and significantly increased by the suppression of PKC ζ expression ($t = 11.29$; $P < 0.01$), however, this elevation was suppressed by NP treatment ($t = 33.35$; $P < 0.01$) (Fig. 2B). Furthermore, NP treatment increased the ratio of S phase cells, compared with the control group ($t = 22.67$, 37.02 and 47.41 for 1×10^{-8} , 1×10^{-7} and 1×10^{-6} mol/l NP, respectively; all $P < 0.01$; Fig. 2A). Following si-PKC ζ transfection, the ratio of S phase cells was significantly decreased compared with the NC group ($t = 1.83$; $P < 0.05$), however, it was significantly increased following NP treatment ($t = 51.31$; $P < 0.01$) (Fig. 2B).

Effects of NP and si-PKC ζ on cell apoptosis. To evaluate the influence of NP and PKC ζ on cell apoptosis, flow cytometry was utilized to identify any changes in the apoptotic rate of cells following NP treatment or si-PKC ζ transfection. The results indicated that the ratio of viable to non-viable apoptotic

Figure 2. The effect of NP and si-PKC ζ on the cell cycle. COLO205 cells were treated with the following: NP (0, 1×10^{-6} , 1×10^{-7} or 1×10^{-8} mol/l), si-PKC ζ , si-NC, and si-PKC ζ or si-NC and NP (10^{-6} mol/l). (A) The effect of NP on the cell cycle. (B) The effect of NP and si-PKC ζ on the cell cycle. PKC ζ , protein kinase C ζ ; NC, siRNA negative control; NP, nonylphenol; si, small interfering.

Figure 3. The effect of NP and si-PKC ζ on the apoptotic rate of COLO205 cells. (A) The effect of NP on the apoptotic rate of COLO205 cells. **P<0.01 vs. control group. (B) The effect of NP and si-PKC ζ on the apoptotic rate of COLO205 cells. **P<0.01 vs. NC group. PKC ζ , protein kinase C ζ ; si, small interfering; NC, siRNA negative control; NP, nonylphenol; PI, propidium iodide; FITC, fluorescein isothiocyanate.

cells in the NP treatment group was lower than that in the control group (P<0.01; Fig. 3A). Suppression of PKC ζ expression significantly increased the ratio of viable apoptotic and non-viable apoptotic cells (P<0.01 vs. NC group), however, the ratio of apoptotic cells decreased following NP treatment (Fig. 3B).

Expression change of cell cycle-associated proteins. Compared to the control group, NP treatment significantly upregulated the expression of cyclin D1 and cyclin E, and downregulated the expression of p27 (all P<0.05; Fig. 4A). Following siRNA transfection, the expression of cyclin D1 and cyclin E was significantly reduced by PKC ζ suppression, and the expression of p27 was increased (all P<0.01 vs. NC group). Following NP treatment in the si-PKC ζ -transfected cells, the expression of cyclin D1 and E increased, whereas the expression of p27 decreased (Fig. 4B).

Expression change of apoptosis-associated protein. Additionally, the expression of apoptosis-associated proteins Bcl-2 and Bad was examined (Fig. 5A). The results of the western blot analysis indicated that NP treatment had no significant effect on the expression of Bcl-2, but significantly reduced the expression of Bad (P<0.01 for all concentrations of NP vs. control). The expression of Bad was significantly increased following si-PKC ζ transfection (P<0.01 vs. NC group) and significantly reduced by subsequent NP treatment (P<0.01 vs. si-PKC ζ group) (Fig. 5B).

Expression change of PKC ζ and ERK1/2. The results of the western blot analysis indicated that the expression of PKC ζ and the phosphorylation of ERK1/2 were significantly increased by NP (P<0.05 for 1×10^{-8} ; P<0.01 for 1×10^{-7} - 1×10^{-6} ; Fig. 6A). Following si-PKC ζ transfection, the expression of PKC ζ and the phosphorylation of ERK1/2 were significantly reduced

Figure 4. The effect of NP and si-PKC ζ on the expression of cell cycle-associated proteins. (A) The effect of NP on the expression of cyclin D1, cyclin E and p27. *P<0.05 and **P<0.01 vs. control group. (B) The effect of NP and si-PKC ζ on the expression of cyclin D1, cyclin E and p27. **P<0.01 vs. NC group; *P<0.05 and #P<0.01 vs. si-PKC ζ . PKC ζ , protein kinase C ζ ; si, small interfering; NC, siRNA negative control; NP, nonylphenol; p27, cyclin-dependent kinase inhibitor.

(both P<0.01 vs. NC), however, this was significantly recovered following NP treatment (both P<0.01 vs. siPKC ζ alone) (Fig. 6B). The phosphorylation of PKC ζ was also influenced by NP treatment. Following NP treatment, the phosphorylation of PKC ζ was significantly increased for all concentrations of

NP, compared with the control (P<0.01; P<0.05; Fig. 7A). The phosphorylation level of PKC ζ did not change significantly following si-PKC ζ transfection, however, subsequent NP treatment significantly increased the phosphorylation (P<0.01 vs. si-PKC ζ alone; Fig. 7B).

Figure 5. The effect of NP and si-PKC ζ on the expression of apoptosis-associated proteins. (A) The effect of NP on the expression of Bcl-2 and Bad **P<0.01 vs. control group. (B) The effect of NP and si-PKC ζ on the expression of Bcl-2 and Bad **P<0.01 vs. NC group; and ##P<0.01 vs. si-PKC ζ . Bcl-2, B-cell lymphoma-2; Bad, Bcl-2 associated agonist of cell death; PKC ζ , protein kinase C ζ ; si, small interfering; NC, siRNA negative control; NP, nonylphenol.

Discussion

Globally, CRC is the third leading cause of cancer-associated morbidity and is the fourth leading cause of cancer-associated mortality (2014) (1). In China, the incidence of CRCs presents an annual rising trend (1), bearing a serious threat to human health. The risk of human CRC is associated with environment, dietary and genetics factors (19). A previous study indicated that CRC was an estrogen-dependent tumor type, and the level of estrogen in patients was directly associated with the development and prognosis of CRC (2). NP is an EDC, which has the ability to induce endocrine disruption, reproduction disorders and the development of various types of cancer (20-23).

In the present study, NP was indicated to significantly induce the proliferation of COLO205 cells by promoting cells from the G1 phase into the S phase. Cyclin D1 and E are two proteins that have been demonstrated to induce cells transforming from the G1 phase to the S phase, and p21 and p27 are two kinases, which have been reported to inhibit the cell cycle transformation (24-26). In the present study, it was indicated that NP could upregulate the expression of cyclin D1 and E, and downregulate the expression of p27.

Abnormalities in the cell apoptosis mechanism can induce cell proliferation, which may result in the development of tumors (27). Bcl-2 is an important anti-apoptosis protein, which is considered as an oncogene. Bad is a type of pro-apoptosis protein, which binds to Bcl-2 to prevent apoptosis (28). The dynamic balance of these aforementioned proteins serve an important role in maintaining a normal function of cells. The present study indicated that NP slightly affected the expression of Bcl-2, however, NP could significantly reduce the expression of Bad. Therefore, NP may inhibit apoptosis by inhibiting the pro-apoptotic function of Bad.

PKC ζ is involved in the proliferation and transfer of various tumor cells (13-15). Inhibiting the expression of PKC ζ has been reported to suppress the invasion ability of CRC, breast cancer and glioma (16). A previous study indicated that PKC ζ promotes tumor cell proliferation and the regulation of apoptosis by phosphorylation of STAT3 (29). The results of the present study indicated that NP could induce the expression of PKC ζ , in addition to the phosphorylation of ERK1/2. However, suppression of PKC ζ significantly reduced the phosphorylation of ERK1/2. The aforementioned results indicated that NP may induce the proliferation of CRC cells by upregulating the expression of PKC ζ and increasing the phosphorylation of

Figure 6. The effect of NP and si-PKC ζ on the expression of PKC ζ and ERK1/2. (A) The effect of NP on the expression of PKC ζ and p-ERK/ERK1/2. *P<0.05 and **P<0.01 vs. control group. (B) The effect of NP and si-PKC ζ on the expression of PKC ζ and p-ERK/ERK1/2. **P<0.01 vs. NC group; and #P<0.01 vs. si-PKC ζ . p, phosphorylated; PKC ζ , protein kinase C ζ ; si, small interfering; NC, siRNA negative control; NP, nonylphenol; ERK, extracellular-signal-regulated kinase.

Figure 7. The effect of NP and si-PKC ζ on the phosphorylation of PKC ζ . (A) The effect of NP on the phosphorylation of PKC ζ . *P<0.05 and **P<0.01 vs. control group. (B) The effect of NP and si-PKC ζ on the phosphorylation of PKC ζ . *P<0.05 vs. NC group; and #P<0.05 vs. si-PKC ζ group. p, phosphorylated; PKC ζ , protein kinase C ζ ; si, small interfering; NC, siRNA negative control; NP, nonylphenol.

ERK1/2. Further investigation is required in order to examine the underlying mechanism of PKC ζ overexpression by NP.

In conclusion, the present study identified the effect of NP on the expression and activity of PKC ζ using RNAi technology. The results indicated that NP could induce the proliferation of COLO205 cells by activating the ERK pathway through PKC ζ activation. The present study provides new direction for CRC prevention and therapy. However, the underlying mechanisms of NP on the development of CRCs requires further study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HH and MW cultured the COLO205 cells, and performed the NP treatment and siRNA transfection. JX examined the proliferation, cell cycle and apoptosis of the COLO205 cells. MX and XZ designed the experiment. XY performed the western blot analysis and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interests.

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