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MEHP promotes the proliferation of cervical cancer via GPER mediated activation of Akt

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ABSTRACT

Cervical cancer is the fourth leading cause of cancer death in females worldwide and the second leading cause of mortality among women. Estrogenic signals can regulate the progression of cervical cancer, however, little is known about the mono-2-ethyhexyl phthalate (MEHP), an environmental xenoestrogen, on the development of cervical cancers. Our present data showed that nanomolar concentrations of MEHP can trigger the proliferation, while not invasion, of cervical cancer HeLa and SiHa cells, which was confirmed by the results that MEHP can also increase the expression of proliferating cell nuclear antigen (PCNA). MEHP treatment can increase the phosphorylation and nuclear localization of Akt, while had no effect on the activation of ERK1/2 or p65. Targeted inhibition of Akt via its specific siRNA or inhibitor can reverse MEHP induced cell proliferation. In addition, the inhibitor of G protein coupled estrogen receptor (GPER), while not estrogen receptor α (ERα), can abolish MEHP induced phosphorylation of Akt and cell proliferation, suggesting that GPER is involved in MEHP induced activation of Akt. Collectively, our data showed that MEHP can trigger the progression of cervical cancer via activation of GPER/Akt. It suggested that MEHP exposure is also an important risk factor for development and progression of cervical cancers.

1. Introduction

As the fourth leading cause of cancer death in females worldwide, cervical cancer has been reported to cause 266,000 deaths worldwide each year (Siegel et al., 2016). It is reported that about 99.7% of cervical cancers were induced by the high-risk (HR) human papillomavirus (HPV) (Forman et al., 2012). Although cervical cancer can be effectively treated with surgery or radiation at the early stage, the treatment of advanced stage of cervical cancer remains a great challenge (Schiffman et al., 2011). In addition, the overall therapy outcomes of cervical cancer are also very poor (Schiffman et al., 2011). Therefore, the identification of novel therapy targets and illustration of its related mechanisms will be great helpful for cervical cancer treatment.

Both epidemiological and laboratory studies indicated that estrogenic signals can promote the tumorigenesis and progression of cervical cancer (Chung et al., 2010). It was reported that chronic low-dose E_2 treatment will significantly increase the incidence of cervical cancer after HPV infection (den Boon et al., 2015). Estrogen receptor α (ERα), rather than ERβ, is necessary for the development of cervical cancer (Chung et al., 2013). Further studies indicated that environmental estrogenic chemicals such as phthalates and bisphenol A (BPA) can trigger the progression of cervical cancer. Recently, it has been shown that food contaminant BPA can act as agonist for ERα in breast cancer cells (Vivacqua et al., 2003). Nanomolar concentrations of BPA can promote the migration of cervical cancer cells via activation of IKK-β/NF-κB signals (Ma et al., 2015). Therefor there is great chance that other environmental estrogenic chemicals can also regulate the development cervical cancers.

Di-(2-ethylhexyl) phthalate (DEHP) is one of the most widely used environmental chemicals in the production of polyvinyl chloride. After absorption into human body, DEHP can be converted to the metabolite mono-ethylhexyl phthalate (MEHP), which has higher toxicity than DEHP and can influence reproductive and development system (Wang et al., 2012). Clinical studies revealed that higher concentrations of MEHP, while not urinary cotinine, arsenic, or phthalate monoesters, were associated with a larger change in tumor size in 37 cervical cancer patients (Neamtiu et al., 2016). However, there is no study concerning the effects and related mechanisms of MEHP on the growth and biological functions of cervical cancer cells. Our present study revealed that nanomolar concentrations of MEHP can trigger the proliferation of cervical cancer cells via activation of G-protein coupled estrogen receptor (GPER)/Akt signals.

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2. Materials and methods

2.1. Cells and reagents

The human cervical cancer cell line HeLa and SiHa were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in our lab by use the ATCC recommended medium supplemented with 10% bovine calf serum (Gibco). Cells were cultured in 37 °C in an atmosphere of 5% CO_2 with penicillin-streptomycin. The antibiotics were removed two days before experiments. Medium were changed each two-days regularly. Antibodies in the present study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were obtained from (Jackson Immunoresearch, West Grove, PA). MEHP was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Shanghai, China) with the final DMSO concentrations in cell culture medium less than 0.5% (v/v).

2.2. Cells proliferation assay

Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96 well plate and treated with MEHP for the indicated times. After treatment, cells were incubated with 20 μl of MTT (5 mg/ml) for 2 h and then 150 μl DMSO was added to each well. After equilibration for 20 min, the optical density (OD) was measured at a wavelength of 490 nm. All experiments were performed for six times.

2.3. Colony formation assay

The effects of MEHP on the colony formation of cervical cancer cells were evaluated by use of CytoSelect™ 96-Well Cell Transformation Assay, Soft Agar Colony Formation (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions. Briefly, cell suspensions prepared in 0.6% agar-containing medium were seeded in 96 well plates pre-coated with 1.2% agar solution. Cells were lysed after incubation for 7 days. The DNA (indicator of cell proliferation) were stained with CyQuant GR Dye and determined by use of a fluorescent plate reader (GloMax II, Promega Madison, WI) using a 485/520 nm filter set.

2.4. Cell invasion assay

Transwell assay was used to measure the effects of MEHP on the in vitro invasion of cervical cancer cells according to previous study (Sanchez-Diaz et al., 2017). The transwell insert was coated with Matrigel (BD Biosciences). Then 50,000 cells were seeded into transwell insert with 0.1% FBS, while the bottom side of transwell was filled with medium containing 10% FBS. After incubation for 48 h, the membranes of insert were fixed and stained by hematoxylin-eosin staining. The invaded cells were counted by use of fluorescent microscope.

2.5. Quantitative RT-PCR (qRT-PCR)

After treatment, total RNA was extracted by use of phenol-free total RNA extraction kit (Norgen Biotek, ON, Canada). The concentration of RNA was measured by nanodrop (NanoDrop 8000 UV–Vis Spectrophotometer, Thermo Scientific, Waltham, MA). Then, 1000 ng total RNA were used for reverse transcription by use of iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). The qPCR was conducted by use of a standard SYBR Green PCR kit (Takara, Japan) and a BIO-chromo4 (Bio-Rad, USA) quantitative Real-Time PCR System with the following primers: GAPDH, forward 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse 5′-TGG TGA AGA CGC CAG TGG A-3′; Akt, forward 5′-AAT GTG GGC TCA TGG GTC TG-3′ and reverse 5′-AGA GGG AGA GGG CCA GTT AG-3′; GPER, forward 5′- ACA CAC CTG GGT GGA CAC AA-3′ and reverse 5′- GGA GCC AGA AGC CAC ATC TG-3′. GPADH was used as the reference gene for normalization. The relative changes for each gene was calculated by comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$).

2.6. Western blot analysis

After treatment, cells were lysed by CelLytic (Sigma-Aldrich, St. Louis, MO) buffer. The total protein concentrations were quantified by use of the Bradford assay. Then 30 μg of total protein were separated by precast NuPAGE® 4–12% Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membrane (Life Technologies) using the semi-dry iBlot system (Life Technologies). The membrane was blocked with 5% BSA in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) at room temperature for 1 h and further incubated with specific primary antibody against PCNA, Bcl-2, MMP-2, MMP-9, p-Akt, Akt, p-ERK, ERK, p-p65, p65, ERα, and GPER, respectively, at 4 °C overnight. After washed with TBST for 3 times, the membrane was further incubated with HRP conjugated secondary antibodies at room temperature for 2 h. The blots were visualized by use of SuperSignal® West Pico Chemiluminescent Substrate detection kit (Thermo Fisher Scientific Pierce Protein Biology Products, Rockford, IL).

2.7. siRNA transfection

The knockdown of Akt expression was conducted via transfection of siRNA purchased from RiboBio Co., Ltd. (Guangzhou, China) according to the instructions of manufacturer. Briefly, cells were seeded on a 6-well plate (2×10^5 cells/well) and transfected with 50 nM siRNA mixed with lipofectamine 2000 reagent in serum reduced medium. Medium was changed to complete culture medium 4 h later, and the cells were incubated at 37 ℃ in a CO_2 incubator for further experiments.

2.8. Immunofluorescence

HeLa cells were seeded in Millicell EZ 8-well glass slides (Merck Millipore, Germany) and treated with MEHP for the indicated times. After washed, cells were fixed by 4% paraformaldehyde for 10 min and treated with 0.5% Triton X-100 for 20 min. Then, cells were blocked with 10% BSA for 30 min and incubated with primary antibody overnight at 4 °C. Cells were further washed three times with PBS, incubated with Alexa Fluor 488 donkey anti-mouse IgG for 2 h at room temperature, and mounted in Vectashield with DAPI (Life Technology, NY, USA). The expression of p-Akt was visualized with an immunofluorescence microscope (Carl Zeiss, Jena, Germany).

2.9. Statistical analysis

Each experiment was repeated three times except specifically stated. All data were displayed as means ± standard deviation (S.D.) and analyzed by use of GraphPad Prism (GraphPad Software version 5.0c). Student's *t*-test was used to compare the control and MEHP treated groups. P value $< .05$ was considered to be significant.

3. Results

3.1. MEHP triggered the proliferation of cervical cancer cells

We investigated the effects of increasing concentrations of MEHP on the proliferation of HeLa and SiHa cells. Our data showed that MEHP concentrations ranged from 1 to 100 nM can significantly increase the proliferation of both HeLa and SiHa cells after treated for 48 h (Fig. 1 A). However, concentrations of MEHP less than 1 nM have no significant effects on the proliferation of cervical cancer cells. In addition, MEHP greater than 10 μM can inhibit the cell proliferation (Fig. 1 A). Furthermore, our data showed that 10 nM MEHP can promote the