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Effect of 2-Methoxyestradiol on Dephosphorylation of Neuronal Nitric Oxide Synthase in Osteosarcoma 143B Cells. An in vitro study

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Abstract

Objective: 2-methoxyestradiol, an endogenous metabolite of anticancer agent, has been evaluated in ongoing advanced phases of clinical trials as an anticancer agent. The aim of the study was to determine the impact of 2-methoxyestradiol on phosphorylation status of neuronal nitric oxide synthase at position of serine 847. We determined also influence of the compound on the gene and protein expressions, phosphorylation status of PP2A phosphatase.

Methods: In the current study we used western blotting, real time PCR, and ELISA assays.

Results: We demonstrated that 2-methoxyestradiol decreases the level of neuronal nitric oxide synthase phosphorylated at position of serine 847 referred to as inactive pool of enzyme. The expression of PP2CA gene was down-regulated by 2-methoxyestradiol at concentration of 1 μM. 10 μM did not statistically significant impact on phosphatase gene expression. Moreover, 1 μM 2-methoxyestradiol up-regulated protein level of PP2A while 10 μM did not exert any effect.

Conclusions: 2-methoxyestradiol decreased an inactive pool of neuronal nitric oxide synthase. Additionally, in a concentration-dependent manner it impacts on the gene and protein expressions, as well as phosphorylation status of PP2A-α phosphatase. Increasing activity of neuronal nitric oxide synthase by reversible phosphorylation may constitute an important tool resulting in osteosarcoma cell death.

Keywords: 2-methoxyestradiol; Neuronal nitric oxide synthase; Phosphatase PP2A

Introduction

Osteosarcoma (OS) is one of the most malignant bone tumors of childhood and adolescence. OS has its origin in mesenchymal bone tissues and usually arises around knees and in the regions of long bones [1,2]. Bone tumors are commonly developed during the maximal somatic growth what strictly correlates with the peak of incidence among people aged 14-18 [3]. Nowadays, despite the fact that a novel approach combining limb-sparing surgery with adjuvant and neoadjuvant chemotherapies is employed, the 5-year survival rate has reached a plateau of 75-80% [1-3]. The main clinical problem with OS is a high degree of malignancy and resistance to chemotherapy [1-3]. Common cytostatic drugs used for the treatment of OS patients are: cisplatin, doxorubicin, methotrexate, ifosfamide and etoposide administered separately or in combination [1-3].

2-methoxyestradiol (2-ME), one of the natural 17β-estradiol derivatives, is a potentially active, novel anticancer agent currently being under evaluation in advanced phases of clinical trials [4,5]. Anticancer activity of 2-ME has been determined in various OS cells [6-8]. Previously, we have reported that 2-ME inhibit OS cell growth and induce apoptosis through induction of neuronal nitric oxide synthase (nNOS) [8]. nNOS is referred to as constitutive isoform expressed mainly in brain tissues, but similarly was found in a spinal cord, adrenal and ganglia glands, peripheral nitrergic nerves, kidneys, pancreatic cells and interestingly in vascular smooth, skeletal muscles and cardiac myocytes [9]. Moreover, we have previously demonstrated that under specific conditions e.g., osteosarcoma 143B cells treated with 2-ME, nNOS may also be an inducible isoform [8]. Up regulation of nNOS may result in nitro-oxidative stress generation what leads to cancer cell death [8]. One of the mechanisms controlling nNOS activity is a reversible phosphorylation. A negative regulation of nNOS by phosphorylation at position of serine 847 (Ser847) resulting in diminished nNOS activity has been reported. Thus, phospho-Ser847 nNOS is referred to as inactive pool of the enzyme [10,11]. Protein phosphatases, such as protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), and calcineurin (also known as protein phosphatase 2B), reverse this inhibitory phosphorylation and stimulate nNOS activity [12].

Herein, we determined the impact of 2-ME on the level of phospho-Ser847 nNOS, and phosphatase PP2A-α playing an important role in regulation of nNOS phosphorylation in OS experimental model.

Materials and Methods

Reagents

Tissue culture media, antibiotic cocktail, foetal bovine serum, 2-methoxyestradiol were purchased from Sigma-Aldrich (Poznan, Poland). Peroxidase-labeled rabbit anti-mouse IgG antibodies were obtained from Molecular Probes (Warsaw, Poland). Monoclonal antibodies against nNOS Ser847, horseradish peroxidase-conjugated secondary anti-rabbit antibodies were obtained from Abcam (Poland). Horseradish peroxidase-conjugated antibodies against beta-actin were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Cytoglow PP2A-α (Phospho-Tyr307) assay kit was obtained from Assay Biotechnology.

Cell line and culture conditions

The human highly metastatic OS 143B cell line (ATTC-8303) was obtained from the American Tissue Type Collection (Lomianki, Poland). The cells were cultured at 37°C in a humidified atmosphere saturated with 5% CO_2 using Dulbecco's Modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and a penicillin (100 mg/mL)/streptomycin (100 mg/mL) cocktail (Sigma-Aldrich).

Cell treatment

Before each experiment, the cells were trypsinized with a solution of 0.25% trypsin and 0.02% EDTA, and were cultured for 24 h under the above-described conditions. The medium was then replaced with the one containing 2-ME according to an experimental design. The stock solution of 2-ME was prepared by dissolving the pure compound in DMSO. The final concentration of DMSO in the working solution was 0.001%. After an appropriate incubation time, the cells were harvested and analyzed by one of the methods described below. We used charcoal-stripped FBS (Sigma Aldrich, Poland) in the medium for cell treatment. Charcoal-stripped FBS is used to elucidate the effects of hormones in a variety of *in vitro* systems.

Western blotting

Equal amounts of total cell lysates were resolved by 10% SDS-PAGE. The membranes were then incubated with primary antibodies anti-nNOSSer847 (Abcam) (1:5000) overnight at 4°C. Afterword, the membranes were incubated at room temperature for 1h with HRP-labeled secondary antibodies anti-nNOSSer847 (1:50000). Analysis has been performed as previously described [8]. Each experiment was performed at least three times.

Colorimetric cell-based ELISA – level of PP2A-α (Phospho-Tyr307)

The OS 143B cells were seeded in number of 20.000 cells/well in culture medium in each well of a 96-well plate. After 8 h incubation with 2-ME, the cells were fixed by incubating with 100 µl of 4% formaldehyde for 20 min at room temperature. After washing, the primary antibodies (anti-PP2A-α (Phospho-Tyr307), anti-PP2A-α, and anti-GAPDH Antibodies) were added to the corresponding wells and incubated overnight at 4°C. Next, secondary antibodies (HRP-Conjugated Anti-Rabbit IgG Antibody and/or HRP-Conjugated Anti-Mouse IgG Antibody) were added to corresponding wells and incubated for 1.5 h at room temperature. After adding substrate and stop solution, the absorbance was read at 450 nm. The absorbance at 450 nm values obtained for the target protein (phosphorylated and non-phosphorylated) were normalized using the absorbance at 450 nm values obtained for GAPDH. Absorbance at 450 nm was determined using a microplate reader (Bio-Tek Instruments, Inc., USA). The number of cells was calculated based on absorbance values. The results were presented as a percentage of control. Positive and negative controls were used. Mouse anti-GAPDH antibody to detect the internal positive controls for normalization of OD values of the target protein. The negative controls were HRP-conjugated anti-rabbit IgG A and HRP-conjugated anti-mouse IgG antibodies alone in different wells (without the primary antibodies). Each experiment was performed three times.

RNA extraction and real time PCR analysis

Reverse transcription (1 µg RNA/reaction) was performed with First Strand cDNA Synthesis Kit (Thermo Scientific) according to manufacturer's protocol. Real time PCR reactions were performed with the initial denaturation at 95°C for 5 min (phase I) and then 40 cycles at 95°C for 15 sec, 57°C for 60 sec and 72°C for 20 sec, ending with fluorescence reading at 78°C for 10 sec (phase II). Melt Curve stage started with 95°C for 15 sec, next cooling to 70°C (redenaturation of present products) for 60 sec with temperature increasing to 95°C and fluorescence read step each 0.3°C. The primers were designed using primer-blast algorithm at www.ncbi.nlm.nih.gov on the basis of Refseq mRNA database option. Real time PCR reactions were performed with Applied Biosystems StepOnePlus Real-Time PCR thermocycler and analyzed using Step One Software. Results obtained for the gene of interest were normalized against RPL37A reference gene run. Subsequently, the numbers were related to the control sample (set to 1). All qPCR reactions were repeated at least 3 times to ensure the replicability of the results. The primers used were as follows: PP2CA (F: CGTTGTGGTAACCAAGCTGC, R: GTGCTGGGTCAAACTGCAAG).

Statistical Analysis

The results represent the mean ± SD from at least three independent experiments. All microscopic evaluations were done on randomized and coded slides. Differences between control samples versus 2ME-treated samples were assessed with one-way analysis of variance (ANOVA) with *post hoc* testing using a Dunnett's multiple comparison test. A p-value of less than 0.01 was considered to correspond with statistical significance. Data were analyzed using GraphPad Prism (GraphPad Software, Inc., version 6, USA).

Results

Impact of 2-ME on level of inactive pool of nNOS in OS cell death model

The concentrations of 2-ME and time of incubation were based on our previous study [8]. We observed the highest protein expression of nNOS after 8 h incubation with 1 µM and 10 µM 2-ME [8]. Herein, we determined the protein expression of phospho-Ser847 nNOS after 8 h treatment of 143B cells with 2-ME (1 µM or 10 µM) by Western blotting. As demonstrated, 1 µM and 10 µM 2-ME diminished protein expression of phospho-nNOS-Ser847 by 43% and 48% as compared to control cells, respectively (Figure 1A).

Impact of 2-ME on gene and protein expression of PP2A-α (Phospho-Tyr307)

Protein phosphatase PP2A has been reported to reverse this inhibitory phosphorylation and stimulate nNOS activity [12]. We determined the gene expression of PP2CA, the gene encoding PP2A, by real time PCR. As demonstrated in Figure 1B, 8 h treatment with 1 μM 2-ME significantly decreased gene expression of PP2CA while 10 μM 2-ME did not exert any significant impact. Next, we determined the protein expression of phosphatase PP2A-α and PP2A-α (Phospho-Tyr307) in OS 143B treated with 2-ME by ELISA assay (Figures 1C, 1D). As demonstrated 1 μM 2-ME significantly affected the protein expression of phosphatase PP2A-α and its and PP2A-α (Phospho-Tyr307) (Figures 1C,1D). Precisely, 1 μM 2-ME increased protein expression of PP2A-α by 30.6% (Figure 1C), while PP2A-α phosphorylated at position Tyr307 by 50.2% (Figure 1D) as compared to the control (100%). In contrast, 10 μM 2-ME did not exert any significant effect.

Discussion

Bone tumors signify heterogeneous groups of tumors with OS being the most common one [1-3]. 2-ME, one of the principal physiological 17β-estradiol derivatives, is a novel and potentially active anticancer agent being evaluated in ongoing advanced clinical trials [3-8]. It seems that 2-ME may be applied to both monotherapy and combination of therapies with popular chemotherapeutics as doxorubicin [3-8]. Previously, we have demonstrated that 2-ME induces OS cell death via increase in the level of total nNOS and generation of nitric oxide (NO) in OS cells [8]. However, multiple mechanisms are involved in the regulation of nNOS activity including reversible phosphorylation/dephosphorylation [10,11].

For the first time, we demonstrated that 2-ME decreases the protein expression of phospho-nNOS-Ser847 in OS experimental model. One of the mechanisms controlling nNOS activity leading to NO production is its phosphorylation/dephosphorylation processes. In particular, phosphorylation of nNOS at serine 847 suppresses the binding of nNOS with calcium-calmodulin (Ca^{2+}-CaM) and decreases the catalytic activity of the enzyme [11]. Phosphorylation of nNOS at Ser847 inhibits production of NO by about 50% through modulating of Ca^{2+}-CaM binding [11]. Due to the fact that Ser847 is present in the autoinhibitory loop of nNOS, phosphorylation of this residue prevent against CaM-Ca^{2+} binding and keep the enzyme inactive even at high concentrations of CaM-Ca^{2+} [10,12]. Thus, reversible phosphorylation of Ser847 constitutes a rapid turnover between active and inactive forms of nNOS [10,12]. Herein, we determined also the impact of 2-ME on PP2A phosphatase in 143B cells. Expression of PP2A in OS Saos-2 cells has been previously published [13]. As demonstrated, the effect of 2-ME on the level and expression of PP2A-α is concentration dependent. We have previously demonstrated activity of 2-ME in OS 143B cells in a concentration-dependent manner [14]. Herein, 1 μM 2-ME increased the level of phosphatase PP2A-α and its phosphorylated form at position of Tyr307. While decreased expression of PP2CA gene by 1 μM 2-ME. mRNA levels and protein levels do not always correlate. It is known that due to posttranslational modifications or/and stability, mRNA levels and protein levels do not always correlate. Nancy Kendrick in the article titled 'A gene's mRNA level does not usually predict its protein level' presented that especially the regulators of cellular division and differentiation would be expected to have a poor correlation between mRNA and protein [15]. The opposite expression patterns of mRNA and protein have been also previously reported [16]. It could be hypothesized, that down-regulation of mRNA concurrent with up-regulation of protein expression may occur when a protein half-life is increased due to - disruption of the components involved in PP2A-α turnover or the protein may be stabilized through protein-protein interactions. It may also be caused by a negative feedback loop. PPP2A has been previously reported to reverse inhibitory phosphorylation at Ser847 of nNOS and stimulate nNOS activity [12]. Interestingly, high pharmacological concentration of 2-ME did not affect level of phosphatase PP2A and its phosphorylated form at position Tyr307, while occurrence of dephosphorylated nNOS-Ser847. The obtained results may suggest that another kinases than PP2A are engaged in the process of nNOS dephosphorylation at position of Ser847 induced by 2-ME.

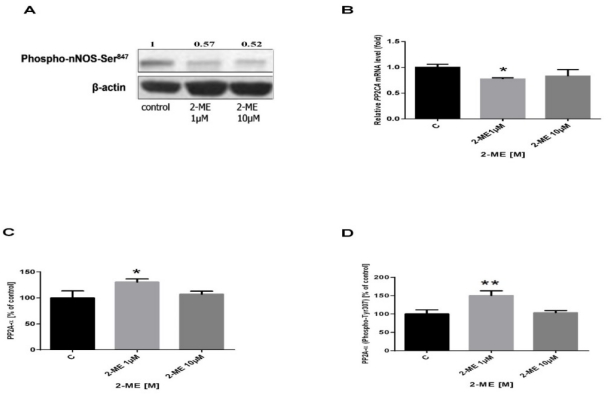

Figure 1: Effects of treatments with 2-ME on inactive pool of nNOS in OS 143B cells were treated with 2-ME (1 μM, 10 μM) for 8 hours. (A): Afterword, the proteins were separated and analyzed by western blot method. 2-ME was demonstrated to decrease protein level of phospho-nNOS-Ser847. A representative experiment of three performed is shown. (B,C): Level of PP2A-α (B) and PP2A-α (Phospho-Tyr307) (C) were determined by ELISA assay. Values are the mean ± SE of three independent experiments (N=6 replicate cultures). The absence of error bar denotes a line thickness greater than the error. *P<0.01, **P<0.001 versus control cells (C). (D): The levels of PP2CA transcript was determined by means of Real time PCR. Values are mean ± SE of three independent experiments, relative mRNA levels of PP2CA/RPL37A are presented. *P<0.01 versus control cells (C).

Conclusions

Phosphorylation/dephosphorylation processes are one of the important modes of regulation nNOS activity. nNOS despite its neuromediatory function in the central nervous system, may also constitute a novel molecular target in anticancer therapy.

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