Effects of 2-methoxyestradiol on apoptosis and HIF-1α and HIF-2α expression in lung cancer cells under normoxia and hypoxia

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Abstract. Hypoxic tumor cells are known to be more resistant to conventional chemotherapy and radiation than normoxic cells. However, the effects of 2-methoxyestradiol (2-ME), an anti-angiogenic, antiproliferative and pro-apoptotic drug, on hypoxic lung cancer cells are unknown. The aim of the present study was to compare the effects of 2-ME on cell growth, apoptosis, hypoxia-inducible factor 1α (HIF-1α) and HIF-2α gene and protein expression in A549 cells under normoxic and hypoxic conditions. To establish the optimal 2-ME concentration with which to carry out the apoptosis assay and to examine mRNA and protein expression of HIFs, cell growth analysis was carried out through N-hexa-methylpararosaniline staining assays in A549 cell cultures treated with one of five different 2-ME concentrations at different times under normoxic or hypoxic growth conditions. The 2-ME concentration of 10 mM at 72 h was selected to perform all further experiments. Apoptotic cells were analyzed by flow cytometry. Western blotting was used to determine HIF-1α and HIF-2α protein expression in total cell extracts. Cellular localization of HIF-1α and HIF-2α was assessed by immunocytochemistry. HIF-1α and HIF-2α gene expression was determined by real-time PCR. A significant increase in the percentage of apoptosis was observed when cells were treated with 2-ME under a normoxic but not under hypoxic conditions (p=0.006). HIF-1α and HIF-2α protein expression levels were significantly decreased in cells cultured under hypoxic conditions and treated with 2-ME (p<0.001). Furthermore, 2-ME decreased the HIF-1α and HIF-2α nuclear staining in cells cultured under hypoxia. The HIF-1α and HIF-2α mRNA levels were significantly lower when cells were exposed to 2-ME under normoxia and hypoxia. Our results suggest that 2-ME could have beneficial results when used with conventional chemotherapy in an attempt to lower the invasive and metastatic processes during cancer development due to its effects on the gene expression and protein synthesis of HIFs.

Introduction

Hypoxia is an event that allows neoplastic cells from the primary tumor to become metastatic cells (1,2). It also influences the formation of new vessels by upregulating the expression of VEGF (3). It has been established that cellular response to hypoxia is mediated by hypoxia-inducible factors (HIFs), which are transcriptional factors that promote the expression of genes involved in cell survival under hypoxic conditions. They also participate directly in other processes of tumoral development such as neoplastic glucose/energy metabolism, cellular growth and apoptosis (4,5). HIFs are heterodimers consisting of either HIF-1α or HIF-2α bound to the HIF-1β subunit. In normoxic conditions, the α subunit is constitutively expressed but rapidly degraded. In a low-oxygen environment, the α subunit is stabilized and translocated to the nucleus (6,7). Therefore, both HIF-α subunits are regulated by O2 availability, while HIF-1β is constitutively expressed.

2-Methoxyestradiol (2-ME) is an anti-angiogenic, antiproliferative and pro-apoptotic agent that suppresses HIF-1α protein levels and its transcriptional activity (8,9). Its effect correlates with a decrease in tubulin polymerization (10) and it also disrupts normal microtubule function and stability (11). 2-ME binds directly to the colchicine binding site and does not interact with estrogen receptors, lowering therefore its probable side-effects (12). 2-ME may have potential clinical benefit in...
the treatment of cancer since it inhibits the proliferation of many human cancer cell lines in vitro (13, 14). There is evidence that HIF-1α mediates tumoral cell survival and apoptosis resistance under hypoxic and normoxic conditions (15, 16-18). Furthermore, the pharmacological inhibition of HIF-1α, and particularly HIF-regulated genes that are important for cancer cell survival, may be more advantageous than HIF-gene inactivation therapeutic approaches (19).

Hypoxic tumor cells are known to be more resistant to current treatment modalities and to radiation than normoxic cells (20). Hypoxia can also confer resistance against chemotherapy-induced apoptosis in numerous solid tumors such as breast and non-small cell lung cancer and pancreatic ductal adenocarcinoma (21-23). Therefore, and considering hypoxia as an important factor leading cancer cells to enhanced resistance to cytotoxic drugs, we studied the effects of 2-ME on cell growth, apoptosis, and HIF-1α and HIF-2α gene and protein expression in human lung adenocarcinoma A549 cells grown under normoxic and hypoxic conditions.

Materials and methods

The protocol was approved by the local Ethics and Research Committees.

Cell culture. The A549 human lung adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC; Rockville, Md, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and supplemented with non-essential amino acids, 50 U/ml penicillin and 50 µg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO₂.

Cell growth assay. After cells reached 80% confluence, 2x10⁴ cells/well were cultured into 48-well plates for 24 h. A stock solution of 33 mM 2-ME (Sigma-Aldrich, St. Louis, MO, USA) was prepared in dimethyl sulfoxide (DMSO). Final 2-ME concentrations were prepared by diluting the stock solution with DMEM. Medium was replaced with fresh medium with 10% FBS and with the corresponding 2-ME concentrations (0.001, 0.1, 0.1, 1 and 10 µM final concentrations). A549 cells with 10% FBS medium (non-stimulated cells) were used as growth control. Cells were also cultured with 10% FBS plus 0.03% DMSO (vehicle-control group for all experiments). Cells were cultured for 12, 24, 48, 72, and 96 h in normoxia (5% CO₂ and 95% air) and hypoxia (1% oxygen and 5% CO₂) conditions, at 37˚C in a humidified atmosphere with 5% CO₂/95% air.

Apoptosis assay. A549 cells were cultured in 6-well plates (3x10⁵ cells/well) under normoxic or hypoxic conditions for 72 h. Three different media were used: DMEM (control), DMEM with DMSO (without 2-ME) and DMEM with 10 µM 2-ME. Apoptosis was examined by flow cytometry using the Annexin V (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) staining kit. Briefly, 1x10⁶ cells in 100 µl Annexin buffer were stained with propidium iodide and FITC-Annexin V staining solutions. Cells were incubated at room temperature in the dark for 15 min, and the data were subsequently acquired through a FACSAria flow cytometer (Becton-Dickinson Biosciences). Data were analyzed using the FlowJo X.0.7 software (Stanford University, Stanford, CA, USA). Results are expressed as percentage; 100% = 5,000 cells.

Western blotting. Cells were plated into 6-well cell culture plates and were grown until they reached 60% confluence. Cells were then exposed to hypoxic conditions and lysed with 0.1% Triton (Sigma-Aldrich) in PBS without calcium to obtain total cell extracts. Protein quantification was performed by the bicinchoninic acid protein assay (BCA protein assay kit; Pierce Biotechnology, Rockford, IL, USA). Western blotting was carried out using 30 µg of cell extract proteins in 8% SDS-polyacrylamide gels (PAGE) under reducing conditions with 2-mercaptoethanol boiled for 10 min. After electrophoresis, the proteins were transferred to PVDF membranes and blocked with 2.5% non-fat dry milk in 100 mM Tris-HCl buffer, pH 7.5 with 150 mM NaCl and 0.1% Tween-20 (TTBS buffer); they were then incubated for 90 min at room temperature with the corresponding antibody: 1:500 anti-HIF-1α (mouse monoclonal antibody, NB100-479) or 1:500 anti-HIF-2α (rabbit polyclonal antibody, NB100-122) (both from Novus Biologicals, Littleton, CO, USA). β-tubulin antibody (1:500) was used as a loading control (sc-53140; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Unbound antibodies were washed with TTBS buffer and bands were detected using the Vectastain® ABC kit (Vector Laboratories, Burlingame, CA, USA). Western blotting bands were analyzed by densitometry scanning using the Kodak Digital Science ID Image analysis software (Eastman Kodak, Rochester, NY, USA). The results are expressed as densitometry units (DU).

Immunocytochemistry. Five hundred thousand cells were cultured in a Nuncl Lab-Tek chamber slide system (Thermo Fisher Scientific, Carlsbad, CA, USA) and allowed to grow in normoxia or hypoxia with or without 10 µM 2-ME treatment for 72 h. The cells were fixed with 1% glutaraldehyde (Sigma-Aldrich) and washed thrice with distilled water for 5 min. Antigen retrieval was performed with 1:10 citrate...
buffer (Sigma-Aldrich). The slides were heated during 5 min in a microwave and they were then allowed to cool for 20 min. Endogenous peroxidase activity was quenched with 2% H₂O₂ solution (Sigma-Aldrich). Antibody blockade and incubation were performed in a humid chamber with 100 µl of blocking solution with serum, followed by the blockade of non-specific sites with 4% (wt/vol) non-fat dry milk in PBS at 4°C overnight. Cells were incubated with 100 µl of the corresponding antibody for 90 min at room temperature: 1:100 anti-HIF-1α (mouse monoclonal antibody, 1NB100-479) or 1:100 anti-HIF-2α (rabbit polyclonal antibody, NB100-122; Novus Biologicals). In another set of experiments, cells were processed with a non-immune IgG instead of the primary antibody as a negative control. Visualization of antibody localization was achieved with a Vectastain® ABC detection kit. Between each step, the slides were thoroughly washed with distilled water. Finally, the slides were manually counterstained with Harris hematoxylin and mounted with non-aqueous medium. Images were captured with an Evos-FL Auto microscope (Thermo Fisher Scientific).

RT-PCR and quantitative real-time PCR of HIF-1α and HIF-2α. Cells were incubated under normoxic or hypoxic conditions with or without 10 µM 2-ME for 72 h. After incubation, total RNA and protein were extracted. RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Thermo Fisher Scientific) and reverse transcribed into cDNA (Advantage RT-for-PCR kit; Clontech, Palo Alto, CA, USA). One microgram of total RNA was reverse transcribed using 2 µg of random primers and Moloney murine leukemia virus reverse transcriptase according to the manufacturer's protocol (Advantage RT-for-PCR kit). Real-time PCR was carried out on a One-Step system (Applied Biosystems, Thermo Fisher Scientific). The expression assay was carried out using pre-designed TaqMan gene expression assay Hs00153153 for HIF-1α labeled with FAM, Hs01026149_m1 for HIF-2α labeled with FAM and normalized with Hs99999901_s1 for 18S ribosomal RNA labeled with VIC (Applied Biosystems). The PCR duplex reactions were performed in a 20-µl reaction volume containing 10 µl of TaqMan Universal PCR Master Mix 2X, 0.5 µl of TaqMan gene expression assay Hs99999901_s1 20X used as an endogenous control (18S rRNA), 1 µl TaqMan gene expression assay of the target gene (Hs00153153 or Hs01026149_m1), 50 ng of cdNA (4 µl) and 4 µl of RNase-free water.

Relative quantitation method was used to analyze the results of two independent experiments made in triplicate. For each experimental sample, a gene was considered as not expressed if amplification was not detected by threshold cycle Ct = 40. Experimental sample, a gene was considered as not expressed of two independent experiments made in triplicate. For each

Statistical analysis. Cell growth was expressed as a percentage of their relative controls. The mean and standard deviation (SD) were obtained in triplicate. Differences between experimental assays in hypoxia, normoxia (0, 12, 24, 48, 72 and 96 h) and apoptosis assays were analyzed by the Student's t-test. Statistical analysis was conducted using the statistical software SPSS version 20.0 (IBM SPSS). p≤0.05 was considered to indicate a statistically significant result.

Results

2-ME inhibits cell growth and induces apoptosis in A549 cells under normoxic but not hypoxic conditions. Cell growth rates in the 2-ME-stimulated cells were decreased in comparison to the rates in the DMSO-incubated control cells, mainly at 48 and 72 h under normoxic conditions (Fig. 1A). The lowest cell growth rate was observed using a 10 µM concentration of 2-ME in comparison with the DMSO control cells: 179.4±16.6 and 552.9±12.1%, respectively (p<0.0001) at 96 h; 189.0±11.6 and 526.3±7.2%, respectively (p<0.0001) at 72 h, and 179.7±14.2 and 552.9±12.1%, respectively (p<0.0001) at 96 h. In contrast, there were no significant differences among the growth rates between the control cells and those treated with 10 µM 2-ME at 72 and 48 h under hypoxic conditions (Fig. 1B). However, a significant decrease in the growth rate was found in the 10 µM 2-ME-treated cells in comparison with the DMSO-treated cells (66.2±7.2 and 101.2±2.3%, respectively; p=0.04) at 96 h. An exponential cell growth was observed in the DMSO medium without stimulation, as expected.
2-ME at a concentration of 10 µM was used for the apoptosis and HIF-1α and HIF-2α expression assays, due to the significance found for this concentration when cells were incubated under normoxic conditions at 72 h. Longer incubation periods were not used since absence of nutrients could have biased the results. Apoptosis induced by 2-ME in the A549 cells was differentially affected by the oxygenation conditions (Fig. 2A). The presence of 10 µM 2-ME significantly increased the percentage of apoptosis (34.5±2.8%) in comparison with the DMSO control cells (12.5±6.5%) (p=0.006) and non-stimulated cells (NS) (p=4.8x10⁻⁵) in a normoxic condition. There were no significant differences among 10 µM 2-ME-treated and control cells grown under hypoxic conditions. A significant increase in apoptosis was observed in cells treated with 10 µM 2-ME in a normoxic condition in comparison with cells under lower O₂ concentration (5.8±0.2%; p=0.003) (Fig. 2B).

Western blotting for HIF-1α and HIF-2α. Western blot densitometry analysis showed differences in the protein expression of HIF-1α and HIF-2α under hypoxic conditions (Fig. 3).

HIF-1α was significantly increased in hypoxic cells (44,998.1±1,079.3 DU) in comparison with cells cultured in normoxic conditions (35,200.8±3,726.9 DU; p=0.01). HIF-1α protein expression was not modified when the cells were treated with 2-ME under hypoxic conditions. In contrast, the HIF-1α level was significantly decreased in cells treated with 10 µM 2-ME under hypoxic conditions (29,390.1±3,542.9 DU; p=0.001).

HIF-2α protein levels were significantly increased in the cells cultured under hypoxia (65,834.3±1,957.7 DU) in comparison with cells incubated under a normoxic condition (51,537±1,451.3 DU, p=0.001). The synthesis of this protein was not significantly modified by the exposure to 2-ME under a normoxic condition. In contrast, the HIF-2α level was significantly decreased in cells treated with 10 µM 2-ME under hypoxic conditions (25,921±3,5442.9 DU; p=6.8x10⁻⁵).

Significant differences were also found when HIF-1α and HIF-2α levels of cells grown under normoxic (p=0.02) and hypoxic conditions (p=8.6x10⁻⁵) were compared, but not among the same cell groups treated with 2-ME.
HIF-1α and HIF-2α immunocytochemistry in 2-ME-treated cells. HIF-1α and HIF-2α were detected in the normoxic-grown cell cytoplasm, particularly HIF-2α, using immunoperoxidase staining (Fig. 4). When cells were cultured under hypoxic conditions, HIF-1α was observed with higher intensity in the cell nucleus; however, staining for HIF-2α was low. Neither HIF-1α nor HIF-2α were noted in the cells treated with 2-ME under a normoxic condition. The incubation with 2-ME decreased the nuclear staining for HIF-1α in cells under hypoxia, while no staining for HIF-2α was observed under the same experimental conditions.

Apart from the effects of 2-ME on HIF-1α and HIF-2α protein expression in both experimental conditions, some morphological and physiological effects were also evident. Nuclear lobation (open arrows) and nuclear fragmentation (apoptosis, solid arrows) were present in cells treated with 2-ME under a normoxic condition; these phenomena were more frequent in cells exposed concomitantly to hypoxia and 2-ME.

HIF-1α and HIF-2α gene expression. The gene expression assay revealed that there were significant differences in HIF-1α mRNA expression among normoxic (5.9±0.9 ΔCt values) and hypoxic (9.2±0.8 ΔCt values; p=0.0004) cells (Fig. 5). A significant decrease in HIF-1α mRNA expression was observed when cells were cultured with 2-ME under a normoxic condition (0.63±0.3 ΔCt values; p=0.03) condition. HIF-1α mRNA was untraceable in cells exposed to 2-ME under hypoxia.

There were no differences in HIF-2α mRNA expression among cells cultured under normoxic and hypoxic conditions. When normoxic-grown cells were exposed to 2-ME, HIF-2α gene expression was significantly decreased (0.3±0.1 ΔCt values) in comparison with the untreated cells (3.9±0.4 ΔCt values; p=0.02). Neither HIF-1α nor HIF-2α mRNA was detected in hypoxic cells exposed to 2-ME.

The mRNA expression of HIF-1α was significantly higher than HIF-2α gene expression under hypoxia (p=0.04); but no differences were noted when mRNA expression for HIF-1α and HIF-2α grown in normoxic conditions was compared.

Discussion

2-Methoxyestradiol (2-ME) is a natural metabolite of 17β-estradiol that exerts antiproliferative action in
carcinoma cell lines and may play a possible antitumor and anti-angiogenesis role in vivo (8,24-26). Furthermore, it has also been used in a number of preclinical and clinical studies for the treatment of solid tumors (27-29). Likewise, it is widely known that hypoxic microenvironments inside solid tumors are one of the major causes of drug resistance (15,30,31), and that the extent of tumor hypoxia is an important prognostic factor for assessing tumor progression as well as resistance to therapy and overall patient survival (32-34). Under hypoxic conditions, some genes, such as the hypoxia-inducible factors (HIFs), are activated and their products favor tumor progression. In the present study, we analyzed whether 2-ME could inhibit the expression of HIF genes in lung carcinoma cells simultaneously exposed to this drug and hypoxia. The effect of different 2-ME concentrations on cell growth rates of human adenocarcinoma A549 cells grown under normoxia or hypoxia was analyzed first. Our results showed a dose-dependent inhibition of cell growth for 2-ME-treated normoxic cells. In contrast, a strong cell growth inhibition, probably due to the hypoxia rather than to 2-ME treatment, was observed. The effects of this compound on apoptosis were also examined, since there is evidence that HIF-1α mediates tumoral cell survival and apoptotic resistance under hypoxic and normoxic conditions (15-18). We observed that, under a normoxic condition, 2-ME stimulated apoptosis, an effect probably due to Bcl-2 and Bcl-xL phosphorylation and the subsequent inhibition of the anti-apoptotic effects (35). Contrastingly, 2-ME had no effect on cells grown under hypoxia. Notably, this 2-ME lack of effect was concentration independent. This finding correlates with the observation that hypoxia by itself prevents several agents such as the ones used in chemotherapy from inducing apoptosis (17,36,37). Likewise, the possibility that HIF-1α could display either a pro-apoptotic or an anti-apoptotic role has been raised and it has been proposed that HIF-1α acting one way or the other is probably related to the severity of the hypoxic conditions (36).

It has been determined that 2-ME inhibits the expression of HIF-1α and HIF-2α proteins and their nuclear translocation in hepatocellular carcinoma cells (31). Accordingly, we also found a significant decrease in HIF-1α and HIF-2α mRNA and protein expression in lung adenocarcinoma cells treated with 2-ME. Even though HIF-1α protein expression decreased noticeably in these cells after treatment, it was still possible to observe it in the nuclei. These findings suggest that although 2-ME stimulates apoptosis by Bcl molecule phosphorylation and HIF expression inhibition, the decline in apoptosis could be due to the HIF molecules present in the nuclei in hypoxia; it is possible that nuclear HIFs may contribute to apoptosis inhibition through the activation of cell-stress response genes (38).

According to our data, the 2-ME therapy may not be effective during the early stages of cancer in which neoplastic cells grown in a hypoxic environment. However, due to its effect on HIF expression, 2-ME may still be effective as an antmetastatic agent and it could be used in combination with other therapeutic agents. In this regard, some 2-ME analogues have been synthesized and tested in an attempt to develop drugs with improved oral bioavailability and efficacy, for example: 2-methoxyestradiol-bis-sulphamate (2-ME-BM), 2-methoxyestradiol-3,17-O,O-bis-sulphamate (2-MeOE2bisMATE), NanoCrystal Dispersion formula-

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