Influence of prostaglandin A₃ and 2-methoxyestradiol on mitogen-activated protein kinase (MAPK) expression levels in malignant cell lines

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ABSTRACT
Mammalian cells often use the highly conserved mitogen-activated protein kinase (MAPK) or extracellular signal regulated protein kinase (ERK) cascades to transmit intracellular instructions. These signaling pathways have been proposed to regulate a diverse range of biological functions including apoptosis. Since 2-methoxyestradiol (2ME) and prostaglandin A₃ (PGA₃) play an active role in the induction of apoptosis, the influence of 1 μM 2ME or 20 μg/ml PGA₃ was investigated on ERK1/2 expression levels in three cancer cell lines. PGA₃ exposure led to a statistically significant increase in ERK1/2 expression levels of HeLa and WHCO3 cells. In contrast to the HeLa and WHCO3 cancer cell lines, no effect on ERK1/2 expression levels was observed after exposure of PGA₃ to MCF-7 cells. 2ME caused a statistically significant increase in ERK1/2 expression levels in HeLa, MCF-7 and WHCO3 cells. WHCO3 cells were shown to be more susceptible to the effects of PGA₃ and 2ME compared to the other two cancer cell lines. Since the characteristics of both PGA₃ and 2ME render them as possible anti-tumor agents when compared to conventional chemotherapeutic treatments, understanding the functional role of signaling events and their regulation by interfering pathways after exposure of cells to PGA₃ and 2ME respectively, will provide new insights into mechanisms involved in malignant cell proliferation.

When responding to extracellular signals, mammalian cells often use the highly conserved mitogen-activated protein kinase (MAPK) or extracellular signal regulated protein kinase (ERK) cascades to transmit intracellular instructions (20). These cascades consist of a three-kinase module that includes a MAPK that is activated by a MAPK/ERK kinase (MEK). The latter in turn, is activated by a MEK kinase (MEKK). The MAPK cascade that consists of Raf isoforms, MEK1/2 and ERK1/2, is regulated by Ras (25). Raf leads to the activation of MEK1 and MEK2. Once activated, either MEK1 or MEK2 can directly activate ERK1 and ERK2 by phosphorylating their conserved threonine and tyrosine activation sites. This results in the phosphorylation and regulation of several transcription factors that mediate changes in gene expression (2). c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK are also members of the MAPK family. They are activated when cells are exposed to growth factors, cytokines and/or various forms of stress (15). Activation of JNK and p38 MAPK are achieved through dual phosphorylation of the threonine and tyrosine residues in the TXY motif (7) have been proposed to regulate a diverse range of biological functions, including cytokine production, cell growth, differentiation and cell death (31).

Since we and other researchers have previously shown that 2-methoxyestradiol (2ME) and prostaglandin A₃ (PGA₃) play an active role in the induc-
tion of apoptosis and especially in cells that present with carcinogenic properties (11, 30, 37), the influence of these two endogenous metabolites was investigated on MAPK (ERK1/2) expression levels in three malignant cell lines in this study.

2ME, an endogenous metabolite of 17β-estradiol, can be regarded as an anti-mitotic drug and tubulin poison (35). This endogenous metabolite has been shown to inhibit growth and induce apoptosis in a large variety of tumor and non-tumor cell lines in vitro (16, 18, 19, 28, 30). 2ME is devoid of estrogenic effects, has both antiangiogenic and antitumor effects and is currently being evaluated in Phase I and Phase II clinical trials for the treatment of multiple types of cancer (35).

Another endogenous metabolite derived from arachidonic acid, namely PGA₃, was also shown to exhibit potent anti-proliferative activity in vitro (5, 13). PGA₁ reversibly blocked cell cycle progression of NIH 3T3 cells at G₁ and G₂/M phase. Data indicated that PGA₁ could arrest the cell cycle in G₁ by interfering with the activation of G₁ phase cyclin-dependent kinases (CDKs) (8). PGA₁ also exhibited anti-proliferative activity in vivo (3, 10, 11, 22–24).

Thus, in succession to our previous research and since MAPKs are known to regulate apoptosis (27) in various cancers (36), the purpose of this study was to investigate the effects of PGA₁ and 2ME on the expression levels of ERK1/2 in three malignant cell lines. It has already been stated that the characteristics of both PGA₁ and 2ME render them as possible anti-tumor agents when compared to conventional chemotherapeutic treatments (9). Understanding the functional role of signaling events and their regulation by interfering pathways after exposure of cells to PGA₁ and 2ME respectively will provide new insights into mechanisms involved in malignant cell proliferation.

MATERIALS AND METHODS

Cell lines. HeLa (human epithelial cervix carcinoma) was purchased through Sterilab Services (Johannesburg, SA) from the American Tissue Culture Collection (ATCC) (Maryland, USA). MCF-7 cells were derived from a pleural effusion of human breast adenocarcinoma and supplied by Highveld Biological (Pty) Ltd. (Sandringham, SA). The WHCO3 cell line (a poorly differentiated non-keratinising squamous oesophageal cell carcinoma) was a gift from Professors Thornley and Veale (Department of Zoology, University of the Witwatersrand, Johannesburg, SA). WHCO3 cells were obtained through a biopsy from a patient with squamous oesophageal carcinoma.

Cell culture maintenance, chemicals and antibodies. PGA₁, 2ME, Eagle’s minimum essential medium (EMEM) with Earle’s salts, L-glutamine and NaHCO₃, trypsin-EDTA and trypan blue were supplied by Sigma Chemical Co. (St. Louis, USA). Heat-inactivated fetal calf serum, sterile cell culture flasks and plates were obtained through Sterilab Services. Phosphate buffered saline (PBS) was purchased from Gibco BRL through Laboratory Specialist Services (Johannesburg, SA). Penicillin, streptomycin and fungizone were obtained from Highveld Biological (Pty) Ltd. Goat-anti-mouse IgG (H+L) peroxidase conjugate and ERK1/2 (anti-MAPK) mouse monoclonal antibody were provided by Sterilab Services. The Bio-Rad Dye Reagent Concentrate protein assay was purchased from Bio-Rad Laboratories (München, Germany) and supplied by S.A. Scientific Inc. (Midrand, SA). All other chemicals were of analytical grade and supplied by Sigma Chemical Co.

Culturing of cell lines. The cells were grown and maintained as monolayer cultures in minimum essential medium, containing 10% heat inactivated fetal calf serum and a 10% mixture of 10 μg/ml penicillin, 10 μg/ml streptomycin and 25 μg/ml fungizone at 37°C in a humidified atmosphere containing 5% CO₂.

PGA₁ - and 2ME exposure of cells, cell extract preparation and protein concentration determination. 500000 viable cells were seeded in 25 cm² culture vessels after trypan blue exclusion and incubated for 24 h. Cells were harvested after 24 h of exposure to vehicle controls, 1 μM 2ME or 20 μg/ml PGA₁. Stock solutions of PGA₁ and 2ME were prepared in ethanol and dimethyl sulfoxide (DMSO) respectively. The solvent concentrations in the media never exceeded 0.05%. In all the experiments concentrations of 20 μg/ml PGA₁ and 1 μM 2ME were used, since dose-dependent studies (4, 10) showed maximum growth response at these dosages in vitro. Cells were homogenized in saline (150 mM NaCl, pH 7.4). Cell extracts were collected by centrifugation at 10000 × g for 10 min at 4°C. Protein concentrations of each cell extract were determined by means of the Bio-Rad Dye Reagent Concentrate protein assay according to the manufacturer’s instructions.
Enzyme-linked immunosorbent assay (ELISA). A 96-well microtiter plate was coated with the above-mentioned cell extract samples of known protein concentration (0.1 mg/100 μl/well). The plate was dried under a 150 W lamp and an electric fan for 2 h. Non-specific binding was excluded by incubation of each well with 300 μl of PBS (pH 7.4) containing 0.5% casein (blocking buffer), for 60 min. The latter was replaced with ERK1/2 (anti-MAPK) mouse monoclonal antibody (1 mg/ml, diluted 1:100 in blocking buffer) and incubated at 37°C for 45 min. Subsequently the plate was washed three times in blocking buffer and incubated with goat-anti-mouse IgG (H+L) peroxidase conjugate (1:500 dilution in blocking buffer) at 37°C for 30 min. After a second washing step, 100 μl of developing buffer composed of 10 ml citrate buffer (pH 4.5), 10 mg ortho-phenyl diamine and 8 mg hydrogen peroxide, was added. The reaction was monitored after 10 min at a wavelength of 450 nm with a SLT 340 ATC scanner (SLT Labinstruments, Austria).

Statistics. Data obtained from independent experiments are shown as the mean ± SD and were statistically analysed for significance using the analysis of variance (ANOVA)-single factor model followed by a two-tailed Student’s t-test. Means are presented in bar charts, with T-bars referring to standard deviations. P < 0.05 was regarded as statistically significant and indicated by means of an asterisk (*).

RESULTS
The effect of 20 μg/ml PGA₂ and 1 μM 2ME was evaluated on the expression levels of ERK1/2 in HeLa, MCF-7 and WHCO3 cells compared to vehicle-treated controls after 24 h of exposure (Fig. 1–3). PGA₂ exposure led to a statistically significant increase in ERK1/2 expression levels of HeLa and WHCO3 cells when compared to vehicle-treated controls (P < 0.05) (Fig. 1, 3). PGA₂ increased ERK1/2 expression levels in HeLa and WHCO3 cells 1.5-fold and 2.8-fold respectively over their controls. In contrast to the HeLa and WHCO3 cancer cell lines, no effect on ERK1/2 expression levels was observed after exposure of PGA₂ to MCF-7 cells (Fig. 2). The influence of 2ME on the expression levels of ERK1/2 in HeLa, MCF-7 and WHCO3 cells was statistically significant (P < 0.05) (Fig. 1–3). 2ME caused an increase in ERK1/2 expression levels in HeLa, MCF-7 and WHCO3 cells 1.5-fold, 1.6-fold and 3.5-fold respectively over their controls. WHCO3 cells were shown to be more susceptible to the effects of PGA₂ and 2ME compared to the other two cancer cell lines. (Note the difference in scale when comparing results of these three figures). P < 0.05 was regarded as statistically significant and indicated by an * on the graphs.

![Fig. 1](image_url) ERK1/2 expression levels of either PGA₂- or 2ME-exposed HeLa cells and vehicle-treated control cells after 24 h. ERK/2 expression levels were normalized with regard to vehicle-treated control cells. Both PGA₂ and 2ME exposure led to a statistically significant increase in ERK1/2 expression levels of 1.5-fold over vehicle-treated controls (P < 0.05). P < 0.05 was regarded as statistically significant and indicated by an * on the graph.
Fig. 2 ERK1/2 expression levels of either PGA2- or 2ME-exposed MCF-7 cells and vehicle-treated control cells after 24 h. ERK/2 expression levels were normalized with regard to vehicle-treated control cells. 2ME exposure led to a statistically significant increase in ERK1/2 expression levels of 1.6-fold over vehicle-treated control cells ($P < 0.05$). No statistically significant effect on ERK1/2 expression levels was observed after exposure of PGA2 to MCF-7 cells. A $P$-value of $< 0.05$ was regarded as statistically significant and indicated by an * on the graph.

Fig. 3 ERK1/2 expression levels of either PGA2- or 2ME-exposed WHCO3 cells and vehicle-treated control cells after 24 h. ERK/2 expression levels were normalized with regard to vehicle-treated control cells. PGA2 and 2ME exposure led to a statistically significant increase in ERK1/2 expression levels of 2.8-fold and 3.5-fold respectively when compared to vehicle-treated control cells ($P < 0.05$). WHCO3 cells were shown to be more susceptible to the effects of PGA2 and 2ME compared to the other two cancer cell lines. A $P$-value of $< 0.05$ was regarded as statistically significant and indicated by an * on the graph.

DISCUSSION

MAPK cascades transmit and amplify signals involved in cell proliferation as well as cell death and are known to regulate apoptosis in various cancers (6, 17). It has been shown that activation of MAPKs
plays an important role in the phosphorylation of B cell lymphomalal leukemia-2 and apoptosis induction in HL-60 and HeLa cells (21). In the human lung alveolar epithelial cancer cell line A549, reactive oxygen species caused the activation of ERK and p38, subsequent upregulation of p21 as well as cell division cycle 2 (cdc2) and degradation of cdc25C, resulting in cell growth arrest at the G1/M phase (38). However, no data are yet available on the precise relationship between MAPK and apoptosis (26).

In this study the expression levels of ERK1/2 in PGAe and 2ME-induced apoptosis in HeLa, MCF-7 and WHCO3 cells were studied. Our previous research has already revealed that PGAe and 2ME caused significant decreases in cell growth and subsequent induction of apoptosis in these cell lines. We confirmed these anti-mitogenic effects of PGAe and 2ME by means of cell cycle progression analysis and morphological studies where chromatin aggregation, cell membrane blebbing and uneven distribution of chromosomes were observed (10, 11, 28, 34). Hypercondensed chromosomes in arrested metaphase cells, indicative of apoptosis, as well as nuclear fragmentation were demonstrated in 2ME-treated MCF-7, HeLa and WHCO3 cells (28, 34). In MCF-7, HeLa and WHCO3 cells, 2ME caused faulty spindle formation that resulted in a metaphase block and abnormal distribution of chromosomes (30, 34). WHCO3 cells were proven to be more susceptible to the above-mentioned effects caused by PGAe and 2ME.

In this study 2ME caused a statistically significant \( P < 0.05 \) overexpression of ERK1/2 in the three cell lines investigated when compared to the vehicle control. PGAe also led to a statistically significant \( P < 0.05 \) overexpression of ERK1/2 in the HeLa and WHCO3 cell lines. However, PGAe had no influence on the expression levels of ERK1/2 in the MCF-7 cells. The possibility exists that PGAe may follow the same mechanism of action proved in NIH 3T3 cells where it led to a reversibly block at the G1 and G2/M phase by interfering with the activation of cyclin-dependent kinases without altering mitogen-activated protein kinase stimulation (8). WHCO3 cells were also more susceptible to PGAe and 2ME-induced expression of ERK1/2 when compared to MCF-7 and HeLa cells. Subbaramaiah et al. has shown that 2ME and other microtubule-or actin-interfering agents led to the stimulation of ERK, JNK, and p38 MAPK signaling in human mammary epithelial cells (33). Furthermore, consistent with antimetotics that inhibit the dynamic instability of tubulin and initiate apoptosis, novel 2ME analogues also led to the activation of mitogen-activated protein kinase signaling pathways (35).

Previous research has shown that peptide growth factors acting through tyrosine kinase-containing receptors, are the major regulators of ERK-1 and -2. We have evaluated the effects of exogenous PGAe on total tyrosine kinase (TK) activity and tyrosine phosphorylation status in MCF-7, HeLa and WHCO3 cells. PGAe increased TK activity in the three cell lines investigated (11). Western blotting employing an anti-phosphotyrosine antibody showed only one protein of approximately 55 kDa (~55 kDa) to be phosphorylated in the MCF-7 cells, while a variety of proteins were phosphorylated in the HeLa cells, including the ~55 kDa protein. PGAe caused a decrease in tyrosine phosphorylation of the ~55 kDa protein in WHCO3. Amino acid analyses as well as Matrix Assisted Laser Desorption Ionization were conducted on this protein from these cell lines and it was shown to be similar. Comparison to p53 did not show similarities (12). Furthermore, 2ME was shown to cause an eight-fold increase in the expression of p34(cdc2) as well as an accumulation of phosphorylated cdc2 in human prostate cancer cells (14). 2ME led to unscheduled activation of p34(cdc2) in the S-phase as well as a decrease in the levels of p34(cdc2) and proliferating cell nuclear antigen (PCNA) during G2/M in MCF-7 cells (19). Premature activation of p34(cdc2) has been implicated as a prerequisite for apoptosis induction in cells arrested in G2/M (32). PGAe decreased p34 (cdc2) activity in WHCO3 cells (29).

Although p53 also controls the G2/M checkpoint and not only that of G1, cells (1), p53 can functionally interact with the MAPK pathways, including the stress-activated protein kinase SAPK/JNK, the p38 mitogen-activated protein kinase and the ERK. Upon exposure to stressful stimuli, MAPKs phosphorylate and activate p53, leading to p53-mediated cellular responses. Recent studies have suggested a role of p53 as an upstream activator to regulate MAPK signaling via the transcriptional activation of members of the dual specificity phosphatase family (36). We have shown a marked elevation in p53 levels in HeLa and WHCO3 cells after exposure to PGAe (11). 2ME increased p53 levels and apoptosis induction in transformed cells but not in normal cells (30). PGAe and 2ME may be involved in the activation of transcription factors by mediating the upregulation of ERK1/2, as well as its subsequent activation by means of tyrosine phosphorylation. Since both the p53 and MAPK signaling pathways are altered in the majority of human tumors, and no
data are yet available on the relationship between MAPK and apoptosis (26), additional studies are needed to provide new insights into the deregulated cell proliferation and survival that is characteristic of cancer.

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REFERENCES