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Decrease of intracellular pH as possible mechanism of embryotoxicity of glycol ether alkoxyacetic acid metabolites

Jochem Louisse [a,b,c,*], Yanqing Bai [a], Miriam Verwei [b,c], Johannes J.M. van de Sandt [b,c], Bas J. Blaauboer [d], Ivonne M.C.M. Rietjens [a,c]

[a] Division of Toxicology, Wageningen University, Tuinlaan 5, 6703 HE Wageningen, The Netherlands
[b] TNO Quality of Life, PO Box 360, 3700 AJ Zeist, The Netherlands
[c] WUR/TNO Centre for Innovative Toxicology, PO Box 8000, 6700 EA Wageningen, The Netherlands
[d] Institute for Risk Assessment Sciences (IRAS), Utrecht University, PO Box 80176, 3508 TD Utrecht, The Netherlands

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ABSTRACT

Embryotoxicity of glycol ethers is caused by their alkoxyacetic acid metabolites, but the mechanism underlying the embryotoxicity of these acid metabolites is so far not known. The present study investigates a possible mechanism underlying the embryotoxicity of glycol ether alkoxyacetic acid metabolites using the methoxyacetic acid (MAA) metabolite of ethylene glycol monomethyl ether as the model compound. The results obtained demonstrate an MAA-induced decrease of the intracellular pH (pH_i) of embryonic BALB/c-3T3 cells as well as of embryonic stem (ES)-D3 cells, at concentrations that affect ES-D3 cell differentiation. These results suggest a mechanism for MAA-mediated embryotoxicity similar to the mechanism of embryotoxicity of the drugs valproic acid and acetazolamide (ACZ), known to decrease the pH_i *in vivo*, and therefore used as positive controls. The embryotoxic alkoxyacetic acid metabolites ethoxyacetic acid, butoxyacetic acid and phenoxyacetic acid also caused an intracellular acidification of BALB/c-3T3 cells at concentrations that are known to inhibit ES-D3 cell differentiation. Two other embryotoxic compounds, all-*trans*-retinoic acid and 5-fluorouracil, did not decrease the pH_i of embryonic cells at concentrations that affect ES-D3 cell differentiation, pointing at a different mechanism of embryotoxicity of these compounds. MAA and ACZ induced a concentration-dependent inhibition of ES-D3 cell differentiation, which was enhanced by amiloride, an inhibitor of the Na^+/H^+-antiporter, corroborating an important role of the pH_i in the embryotoxic mechanism of both compounds. Together, the results presented indicate that a decrease of the pH_i may be the mechanism of embryotoxicity of the alkoxyacetic acid metabolites of the glycol ethers.
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Introduction

Several members of the chemical group of glycol ethers are known for having adverse effects on the developing fetus in mice, rats and rabbits (Hanley et al., 1984; Nelson et al., 1984; Wier et al., 1987). Most attention in developmental toxicity studies of glycol ethers has gone to ethylene glycol monomethyl ether (EGME), being the most potent member of this group of chemicals (Nelson et al., 1984; Welsch, 2005). Embryotoxic effects are not caused by EGME itself, but by its metabolite methoxyacetic acid (MAA) (Fig. 1) (Brown et al., 1984; Giavini et al., 1993). Although being the most potent embryotoxic glycol ether, EGME only causes embryotoxicity in rats when MAA plasma levels in the millimolar range are reached (Sweeney et al., 2001). Also in *in vitro* embryotoxicity studies MAA concentrations in the millimolar range are needed to obtain adverse effects. For example, the BMC_5 (Benchmark concentration at which a 5% change occurs compared to the control) of MAA for the total morphological score in the whole embryo culture is 1.6 mM (Piersma et al., 2008) and the BMC_{50} for MAA in the Embryonic Stem cell Test (EST) is 2.3–2.5 mM (De Jong et al., 2009). Like several other embryotoxic compounds such as valproic acid (VPA), ethylhexanoic acid and boric acid, MAA is a weak acid, suggesting that the acidic nature of MAA and related glycol ether alkoxyacetic acid metabolites might play a role in their embryotoxicity. Nau and Scott (1986) showed that weak acids accumulate in the developing embryo because of its relatively high pH compared to maternal blood. They hypothesized that birth defects caused by these acids could be due to an alteration of the intracellular pH (pH_i) of embryonic cells (Nau and Scott, 1986). A decrease of the pH_i of embryonic cells by the drugs

Abbreviations: 5-FU, 5-Fluorouracil; ACZ, Acetazolamide; ATRA, All-*trans*-retinoic acid; BAA, Butoxyacetic acid; BMC, Benchmark concentration; EAA, Ethoxyacetic acid; EGBE, Ethylene glycol monobutyl ether; EGEE, Ethylene glycol monoethyl ether; EGME, Ethylene glycol monomethyl ether; EGPE, Ethylene glycol monophenyl ether; EST, Embryonic Stem cell Test; MAA, Methoxyacetic acid; PAA, Phenoxyacetic acid; pH_i, Intracellular pH; VPA, Valproic acid.

* Corresponding author. Division of Toxicology, Wageningen University, Tuinlaan 5, 6703 HE Wageningen, The Netherlands. Fax: +31 317 484931.
E-mail address: jochem.louisse@wur.nl (J. Louisse).

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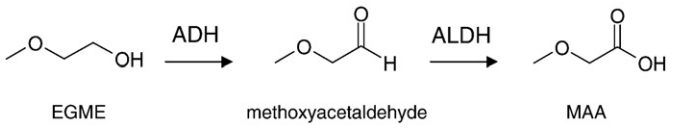

Fig. 1. EGME is metabolized to its toxic metabolite MAA by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Miller et al., 1983).

acetazolamide (ACZ) (Scott et al., 1990) and VPA (Scott et al., 1997) has been linked to their embryotoxic action in mice. ACZ inhibits carbonic anhydrase, causing indirectly an intracellular acidification (Scott et al., 1990), whereas VPA-induced intracellular acidification could be due to the acidic nature of the compound itself, carrying protons directly to the embryo (Scott et al., 1997). When amiloride, a blocker of the Na^+/H^+-antiporter in the cell membrane, was co-administered with ACZ or VPA, an exacerbation in embryotoxicity was found (Scott et al., 1990; Scott et al., 1997). This was due to the inhibition of the Na^+/H^+-antiporter by amiloride, impeding the cells to transport the excess of H^+ ions out of the cell when being exposed to an acid overload (Scott et al., 1990). The goal of the present study was to investigate the possible role of an effect on the pH_i in the mechanism of embryotoxicity induced by MAA and related glycol ether alkoxyacetic acid metabolites. This was done by measuring the changes in the pH_i of BALB/c-3T3 embryonic fibroblasts exposed to MAA and the alkoxyacetic acid metabolites of ethylene glycol monoethyl ether (EGEE), ethylene glycol monobutyl ether (EGBE) and ethylene glycol monophenyl ether (EGPE), being ethoxyacetic acid (EAA), butoxyacetic acid (BAA) and phenoxyacetic acid (PAA), respectively. Effects on the pH_i of BALB/c-3T3 cells by ACZ and VPA, both known to decrease the pH_i *in vivo*, were investigated as well. Also, the effect of MAA and ACZ on the pH_i of undifferentiated, differentiating and differentiated embryonic stem (ES)-D3 cells was measured. Furthermore, the effect of the Na^+/H^+-antiporter blocker amiloride on MAA-induced inhibition of ES-D3 cell differentiation was assessed, using ACZ as a positive control.

Materials and methods

Chemicals. ACZ, all-*trans*-retinoic acid (ATRA), amiloride, EAA, 5-fluorouracil (5-FU) and MAA were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). BAA, dimethyl sulfoxide (DMSO) and VPA were purchased from Acros Organics (Geel, Belgium) and PAA was purchased from Fluka (Zwijndrecht, the Netherlands). 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), BCECF acetoxymethyl ester (BCECF-AM) and nigericin were purchased from Molecular Probes (Invitrogen, Breda, the Netherlands). Ethanol, K_2HPO_4 and KH_2PO_4 were obtained from Merck (Darmstadt, Germany).

BALB/c 3T3 cell culture. The BALB/c 3T3 murine embryonic fibroblast cell line was cultured in 75 cm^2 cell culture flasks (Corning, Schiphol-Rijk, the Netherlands) at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (HyClone-Perbio, Etten-Leur, the Netherlands) and 100 U/ml penicillin/100 µg/ml streptomycin (Invitrogen). Cells were subcultured three times a week, using trypsin-EDTA (Invitrogen) to detach cells.

Undifferentiated ES-D3 cell culture. The murine ES-D3 cell line was cultured according to the method described by De Smedt et al. (2008). Briefly, cells were cultured in cell culture Petri dishes (35 mm × 10 mm, Corning) at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM supplemented with 20% fetal calf serum, 2 mM glutamine (Invitrogen), 50 U/ml penicillin/50 µg/ml streptomycin, 1% v/v non-essential amino acids (Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma-Aldrich). To keep cells undifferentiated, 1000 U/ml murine leukemia inhibiting factor (mLIF) (ESGRO®, Chemicon International, Temecula, CA, USA) was added to the culture medium. Cells were subcultured three times a week, using non enzymatic dissociation buffer (Sigma-Aldrich) to detach cells.

BCECF calibration curves. To assess the effects of the test chemicals on the pH_i, BCECF-AM was used. BCECF-AM passively diffuses through the cell membrane and is cleaved intracellularly by cellular esterases, resulting in the free acid BCECF, which is trapped inside the cell. BCECF fluorescence at $\lambda_{ex} = 490$ nm, $\lambda_{em} = 535$ nm is pH-dependent, whereas BCECF fluorescence at $\lambda_{ex} = 440$ nm, $\lambda_{em} = 535$ nm is not (Owen, 1992). The BCECF fluorescence ratio ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 535$ nm)/($\lambda_{ex} = 440$ nm, $\lambda_{em} = 535$ nm) is a measure for the pH_i: the higher the ratio, the higher the pH_i. The fluorescence ratio measured in the cells can be translated to actual pH_i values using a BCECF calibration curve. Two types of calibration curves were made: a series of calibration curves using increasing concentrations of the free acid BCECF in potassium phosphate of different pH values and a calibration curve using BCECF-loaded BALB/c-3T3 cells subsequently incubated with potassium phosphate of different pH values and 10 µM nigericin to equilibrate the pH_i with the extracellular pH (Thomas et al., 1979). To obtain calibration curves for the free acid BCECF, 0.1 M potassium phosphate solutions were prepared with a pH range from 5 to 8 and supplemented with BCECF at concentration from 0.05 to 1 µM. Solutions containing BCECF were added to a ViewPlate-96 well plate (Perkin Elmer, Groningen, the Netherlands; 200 µl/well). BCECF fluorescence at $\lambda_{ex} = 490$ nm, $\lambda_{em} = 535$ nm and $\lambda_{ex} = 440$ nm, $\lambda_{em} = 535$ nm was measured using a Varian Cary Eclipse Fluorescent Spectrophotometer (Varian, Middelburg, the Netherlands). To obtain a calibration curve for the BCECF-loaded BALB/c-3T3 cells, a cell suspension of BALB/c-3T3 cells was made and cells were washed with HBSS. The cells were incubated for 30 min with 1 µM BCECF-AM in HBSS and subsequently washed with HBSS. Then, cells were transferred to 0.1 M potassium phosphate of pH values ranging from 5 to 8 supplemented with 10 µM nigericin to final cell concentrations of 0.5×10^6 cells/ml. From these solutions, 200 µl was transferred to a ViewPlate-96 well plate. Subsequently, BCECF fluorescence was measured. The calibration curve in BALB/c-3T3 cells appeared to resemble the curve obtained at 1 µM BCECF (see results section), revealing that cellular uptake and hydrolysis of BCECF-AM appear to be efficient. The calibration curve obtained with BALB/c-3T3 cells was used to translate measured fluorescence ratios to actual pH_i values.

pH_i measurements in BALB/c-3T3 and undifferentiated ES-D3 cells. A cell suspension of either BALB/c-3T3 or ES-D3 cells was made and cells were washed with HBSS. Then, the cells were incubated for 30 min with 1 µM BCECF-AM in HBSS and subsequently washed with HBSS. Cells were diluted to a concentration of 1×10^6 cells/ml in HBSS of which 100 µl was transferred to the inner 60 wells of a ViewPlate-96 well plate. Subsequently, 100 µl exposure medium containing the test compounds, added directly to HBSS (BAA, EAA, MAA), added from a 100 times concentrated stock solution in DMSO to HBSS (5-FU, ACZ, ATRA, PAA), or added from a 100 times concentrated stock solution in ethanol (VPA), was added to the cells (final solvent concentrations 0.5% v/v). After 30 and 60 min incubation, BCECF fluorescence at $\lambda_{ex} = 490$ nm, $\lambda_{em} = 535$ nm and $\lambda_{ex} = 440$ nm, $\lambda_{em} = 535$ nm was measured using a Varian Cary Eclipse Fluorescent Spectrophotometer. Fluorescence measurements of cells exposed to ACZ were also performed after 2 and 4 h exposure.

ES-D3 cell differentiation assay. To study the effects of ACZ and MAA in the presence or absence of amiloride on the differentiation of ES-D3 cells into contracting cardiomyocytes, the differentiation assay described by De Smedt et al. (2008) was used, with small modifications. Briefly, on day 1, droplets of 20 µl cell suspension (3.75×10^4 cells/ml) were seeded on the inside of the lid of a 96 well

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