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Toxicology

Arsenic-containing hydrocarbons disrupt a model *in vitro* blood-cerebrospinal fluid barrier

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ABSTRACT

Lipid-soluble arsenicals, so-called arsenolipids, have gained a lot of attention in the last few years because of their presence in many seafoods and reports showing substantial cytotoxicity emanating from arsenic-containing hydrocarbons (AsHCs), a prominent subgroup of the arsenolipids. More recent *in vivo* and *in vitro* studies indicate that some arsenolipids might have adverse effects on brain health.

In the present study, we focused on the effects of selected arsenolipids and three representative metabolites on the blood-cerebrospinal fluid barrier (B-CSF-B), a brain-regulating interface. For this purpose, we incubated an *in vitro* model of the B-CSF-B composed of porcine choroid plexus epithelial cells (PCPECs) with three AsHCs, two arsenic-containing fatty acids (AsFAs) and three representative arsenolipid metabolites (dimethylarsinic acid, thio/oxo-dimethylpropanoic acid) to examine their cytotoxic potential and impact on barrier integrity. The toxic arsenic species arsenite was also tested in this way and served as a reference substance. While AsFAs and the metabolites showed no cytotoxic effects in the conducted assays, AsHCs showed a strong cytotoxicity, being up to 1.5-fold more cytotoxic than arsenite. Analysis of the *in vitro* B-CSF-B integrity showed a concentration-dependent disruption of the barrier within 72 h. The correlation with the decreased plasma membrane surface area (measured as capacitance) indicates cytotoxic effects. These findings suggest exposure to elevated levels of certain arsenolipids may have detrimental consequences for the central nervous system.

1. Introduction

Food chemists and toxicologists are currently paying considerable attention to arsenolipids, a heterogeneous group of lipid-soluble arsenicals that occur naturally in many seafoods, and include, among others, arsenic-containing hydrocarbons (AsHCs) [1] and arsenic-containing fatty acids (AsFAs) [2]. In contrast to inorganic arsenic (iAs), a group 1 carcinogen according to the International Agency for Research on Cancer [3], data for the hazard identification of arsenolipids is still insufficient. Recently, it could be shown that the AsHCs, an important subgroup of arsenolipids, are up to 5-fold more cytotoxic than arsenite (iAs(III)) in the *in vitro* blood-brain barrier (BBB) model; also, they caused disruption of the *in vitro* BBB at sub-cytotoxic doses [4]. It has been concluded that arsenolipids are highly bioaccessible and possess neurotoxic potential [5,6]. Previous *in vivo* studies indicated that AsHCs substantially interfere with the normal development of the fruit fly *Drosophila melanogaster*, where a disturbance of eclosion from pupae could be observed [7]. AsHCs have also been shown to accumulate in the brain area of *D. melanogaster* raising the question of possible transfer of these arsenicals across protective barrier systems [8]. Apart from the BBB, the blood-cerebrospinal fluid barrier (B-CSF-B) represents one of these cerebroprotective barrier systems in vertebrates. The B-CSF-B is located at the *plexus choroideus*, a highly branched tissue in the ventricles of the brain, and consists of tight junctions among polarized epithelial cells which are also responsible for the production of CSF [9,10]. In the current study, we investigated how selected AsHCs, AsFAs and common metabolites of arsenolipids impair the B-CSF-B in reference to arsenite with special focus on their cytotoxicity and impact on the barrier integrity.

2. Materials and methods

2.1. Reagents

L-Glutamine and Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1) were acquired by Biochrom GmbH (Berlin, Germany).

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Fetal calf serum (FCS) was obtained from PAA (Pasching, Germany). Bovine serum albumin, cytosine β-D-arabinoside, insulin (recombinant human), penicillin/streptomycin, Triton X-100, sodium deoxycholate, aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, DL-dithiothreitol (DTT) as well as cacodylic acid (dimethylarsinic acid, DMA(V)) were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Sodium (meta) arsenite (> 99% purity; iAs(III)) was supplied by Fluka Chemie AG (Neu-Ulm, Germany). Sodium chloride, hydrochloric acid, ethanol (≥99.9% purity), HEPES Pufferan®, neutral red, TRIS, acrylamide (Rotiphorese® Gel 40; 37,5:1), glycine, Tween® 20, skimmed milk powder and formaldehyde were supplied by Carl Roth GmbH (Karlsruhe, Germany) and potassium phosphate from Thermo Fisher (Kandel; Karlsruhe, Germany). Porcine trypsin 1:250 was acquired by Belger Biochemie (Kleinmachnow, Germany). Hoechst 33258, potassium chloride, sodium dodecyl sulfate (SDS), glycerin and sodium phosphate were bought from Merck KGaA (Darmstadt, Germany). Acetic acid, EDTA and methanol were acquired from VWR International GmbH (Leuven, Belgium) and the Cell Counting Kit-8 (CCK-8®) was acquired from Dojindo EU GmbH (Munich, Germany). The primary antibodies mouse anti-occludin and rabbit anti-ZO-1 were obtained from Zytomed Systems GmbH (Berlin, Germany), while the second antibodies AlexaFluor® 488 goat anti-mouse IgG (H + L) and AlexaFluor® 488 goat anti-rabbit IgG Fab2 were obtained from Invitrogen, Molecular Probes Inc. (Eugene, USA). Aqua Poly/Mount was bought from Polysciences Inc. (Washington, USA). Goat anti-mouse horseradish peroxidase (HRP) conjugate and Clarity™ Western ECL Substrate were supplied by Bio-Rad Laboratories GmbH (Munich, Germany). Mouse anti-β-actin antibody HRP conjugate was purchased from Abcam plc (Cambridge, United Kingdom). The arsenolipids, namely AsHC 332 [1-(dimethylarsinyl)pentadecane], AsHC 360 [1-(dimethylarsinyl)heptadecane], AsHC 444 [1-(dimethylarsinyl) tricosane], AsFA 362 [15-(dimethylarsinyl)pentadecanoic acid], and AsFA 388 [17-dimethylarsinyl-9-heptadecenoic acid] were synthesized in-house [11] as were the two arsenolipid metabolites thio-dimethylarseno propanoic acid (thio-DMAPr) and oxo-dimethylarseno propanoic acid (oxo-DMAPr) [12] (Fig. 1). As assessed by NMR spectroscopy, HPLC-ESI-MS and ICP-MS, the purity of the arsenic compounds is at > 99%. The arsenolipids were dissolved in ethanol and stored at stable conditions (4 °C; [13]). Sterile deionized water (Elix® Reference 15 water purification system, Merck KGaA; Darmstadt, Germany) was used to prepare aqueous arsenical stock solutions. Prior to each experiment, all stock solutions were freshly diluted.

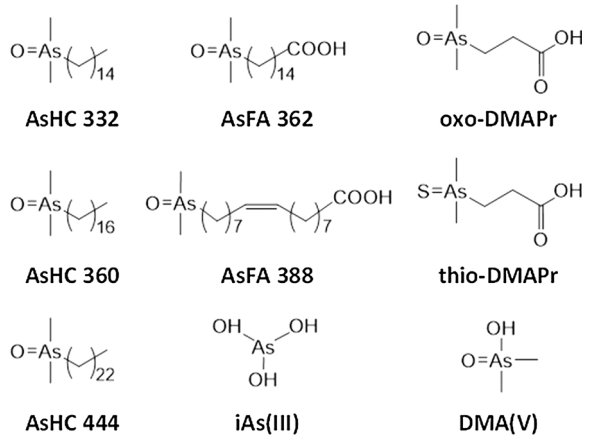

Fig. 1. Chemical structures of the investigated arsenicals. Three arsenic-containing hydrocarbons (AsHC 332, AsHC 360 and AsHC 444), two arsenic-containing fatty acids (AsFA 362 and AsFA 388), the metabolites oxo-dimethylarseno propanoic acid (oxo-DMAPr), thio-dimethylarseno propanoic acid (thio-DMAPr) and dimethylarsinic acid (DMA(V)). Inorganic arsenite (iAs(III)) was used as reference.

2.2. Cell culture

Primary porcine choroid plexus epithelial cells (PCPECs) were isolated and cultivated as described earlier [14,15]. Briefly, choroid plexus tissue obtained from freshly slaughtered pigs was enzymatically treated with trypsin to release the PCPECs from the basal lamina. After prepurification of the cells by centrifugation, PCPECs were seeded on either 96-well-plates for cytotoxicity testing or on 25 cm^2 flasks (TPP, Trasadingen, Switzerland) for western blotting. For barrier studies, PCPECs were seeded on microporous Matrigel™-coated Transwell® filter inserts (1.12 cm^2 growth area, 0.4 µM pore size; Corning, Wiesbaden, Germany). Subsequent cultivation was carried out as previously described [15].

2.3. Cytotoxicity testing

On day *in vitro* 13 (DIV 13), confluent PCPECs were treated with various concentrations of iAs(III) (1–50 µM); DMA(V) (10–1000 µM); AsHC 332, AsHC 360, AsHC 444 (1–37.5 µM); AsFA 362; AsFA 388 (10–100 µM); thio-DMAPr and oxo-DMAPr (25–500 µM). The investigated concentration range of arsenolipids spans biologically relevant levels also found in various fish oils or fish oil extracts (reviewed in [16]). Cytotoxicity testing was conducted after an incubation time of 48 h and the concentration that reduced cell viability by 30% (EC_{30}) was estimated.

2.4. Cell counting Kit-8 (CCK-8)

Confluent PCPECs were treated with the test substances for 48 h. At the end of the incubation time, a defined volume of a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium)-solution was added. Active cellular dehydrogenases reduce the yellow tetrazolium salt to an orange formazan dye, resulting in a shift in absorbance. Thus, the CCK-8 is a suitable assay to estimate the viability of cells [17]. A Tecan infinite 200 Pro multidetection microplate reader (Tecan Austria GmbH, Grödig, Austria) was used to measure the absorbance at 450 nm after incubation for 3 h at cell culture conditions (37 °C, 5% CO_2 and 100% humidity). The activity of cellular dehydrogenases was normalized to the control. In the case of the AsHCs, dehydrogenase activity was normalized to the solvent (0.5% ethanol) control.

2.5. Neutral red uptake assay

Subsequent to the incubation of confluent PCPECs with the test substances for 48 h, the culture medium was gently removed. After treatment with neutral red-containing medium (67 µg neutral red/mL) for 3 h at cell culture conditions, PCPECs were fixed with 0.5% (v/v) formaldehyde in phosphate buffered saline (PBS). An acidified ethanolic solution was used to extract the dye. Neutral red has a net charge of zero at physiological pH and is therefore able to pass across biological membranes. Once the dye reaches intact lysosomes, it will be charged [18]. Due to the binding to anionic groups of the lysosomal matrix, the cationic neutral red is trapped inside these organelles [19]. Thus, the toxicological endpoint is represented by lysosomal integrity. Absorbance of neutral red was measured using a Tecan infinite 200 Pro multidetection microplate reader at a wavelength of 540 nm. The lysosomal integrity was expressed as percent of the (solvent) control.

2.6. Barrier studies

Continuous determination of the transendothelial electrical resistance (TEER) as well as the capacitance was ensured by using a CellZscope® device (nanoAnalytics, Münster, Germany). The tightness of the barrier and the TEER are in a proportional relation, while the capacitance gives information about the plasma membrane surface