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Effect of cadmium on uptake of iron, zinc and copper and mRNA expression of metallothioneins in HepG2 cells in vitro

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ABSTRACT

The intake of cadmium contaminated fish was mimicked by incubating human hepatoblastoma cells (Cell line HepG2) with a combination of different levels of cadmium (0–5 μM) plus the n-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid, which are typical for fish. Uptake of cadmium, iron, copper and zinc was measured by ICP-MS. In addition mRNA expression of two metallothioneins (*mt1 g* and *mt1 m*) was evaluated by real-time PCR.

The obtained data shows that the presence of cadmium increases the uptake of iron and zinc into the HepG2 cells while the uptake of copper remains unaffected. The presence of the chosen fatty acids did not affect the uptake of either cadmium or iron, zinc and copper. The presence of already 1 μM cadmium increased the mRNA expression of *mt1 g* and *mt1 m* significantly, while the fatty acids did not interfere with the effect of cadmium.

1. Introduction

Iron, zinc and copper are essential trace elements needed in many metabolic processes (Arredondo et al., 2006). Earlier research has shown various effects of other trace metal cations on the uptake or bioavailability of iron and zinc in different cell types (Arredondo et al., 2006; Iturri and Nunez, 1998). It has also been shown that these elements affect the uptake of cadmium (Cd) into human and rat hepatocytes (Fotakis and Timbrell, 2006). However the effect of Cd on the uptake of these metals and possible effects of a combined incubation with fatty acids (FA), has not been evaluated so far.

Cd is a pollutant that is widely present in the environment due to its use in various industries (Larregle et al., 2008). Cd is also classified as cancerogenic compound by the International Agency for Research on Cancer (IARC, 1993). Critical target organs in long-term exposure to low concentrations of Cd are the liver and the kidney (kidney cortex) where 30–60% of the ingested Cd is deposited (WHO, 1997). The major intake besides smoking is via drinking water and food (EFSA, 2009; Larregle et al., 2008). In 2012 fish were listed to be one of the sources of Cd with contents of over 100 μg/kg (EFSA, 2012). As in a normal uptake with food Cd will not enter the body alone, but in a combination with other nutrients from the food item; thus it is necessary to evaluate the combined effects on human nutrition and subsequently possible health issues.

Fish are a good source for the long chain (LC) n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have abundant metabolic functions and are essential in the human nutrition (Narayan et al., 2006). Nutrition and health organizations have therefore developed specific dietary recommendations for n-3 FA and fish intake for different countries around the world (Kitessa et al., 2014). FAO and WHO (2011) concluded that a fish consumption of 1–2 servings per week could be protective against coronary heart diseases and ischemic stroke.

However, in parallel to the discussion about nutritional benefits of fish consumption, there has also been one about the risks due to possible contamination of fish (Gochfeld and Burger, 2005; Mahaffey et al., 2011).

The aim of the present study was therefore, to evaluate the effects of increased levels of Cd in combination with or without LC n-3 PUFA on the uptake of zinc, iron and copper in hepatoblastoma cells. In addition we also studied the effects on the mRNA expression of 2 metallothioneins (*mt1 m* and *mt1 g*) as metallothioneins have been shown to have

Abbreviations: BSA, bovine serum albumin; Cd, cadmium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; HepG2, human hepatoblastoma cell line; LC, long chain; PUFA, polyunsaturated fatty acids

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some function in transport and storage of essential metals and are closely related to cell proliferation and apoptosis and heavy metal detoxification (Babula et al., 2012; Bay et al., 2001; Bourdineaud et al., 2006; Park and Yu, 2013). Metallothioneins are also sensitive to oxidative stress (Babula et al., 2012) which could be caused by Cd exposure. In addition it has been shown that among other metals, cadmium, zinc and copper can induce expression of some *mts* (reviewed by Babula et al., 2012). The combination of Cd and LC n-3 PUFA used in the present work intended to mimic the intake of contaminated fish in order to evaluate the effects on human nutrition.

2. Material and methods

2.1. Chemicals

EPA and DHA, bovine serum albumin (BSA), fetal calf serum (FCS) non-essential amino acids and glutamine for cell culture, PBS (phosphate buffer saline) and cadmium chloride were obtained from Sigma-Aldrich (Berlin, Germany). Trypsin, penicillin and streptomycin solutions were products of Sigma (Deisenhofen, Germany). The culture dishes and the culture medium (MEM) for Hep G2 cells were obtained from Biochrom (Berlin, Germany). Hydrogen peroxide solution (30%, Suprapur) and nitric acid (65%, Suprapur) were products of Merck (Darmstadt, Germany). All other pro-analysis chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) and were of the highest available grade. The cell-counting kit-8 (CCK-8s) was obtained from Dojindo Molecular Technologies (Munich, Germany) and the ICP-MS elemental standard from SPECTEC (Erding, Germany).

2.2. Culture of HepG2 cells

Human hepatoblastoma cell line HepG2 cells (No. HB-8065, ATCC) were purchased from the American Type Culture Collection (ATCC, Manassas, V C, USA). HepG2 cells were grown as a monolayer in culture dishes in Minimum Essential Medium Eagle (MEM) supplemented with FCS (10%, *v*/v), non-essential amino acids (1%, *v*/v), glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL). The HepG2 cultures were incubated at 37 °C with 5% CO_2 in air with 100% humidity. Cells were passaged every 3 days. The amount of 1.5 million cells were seeded on 10 cm (in diameter) sterile petri dishes in 10 mL of sterile culture medium (MEM). Suspensions of HepG2 cells were produced from confluent cultures using trypsin/EDTA solution. Before the transfer experiments, cells were three times sub-cultured to achieve a stable phenotype. For the transfer experiments, cells were seeded at a density of 66.7 cells per µL for 96 well plates and 1.5 Mio (million) per petri dishes (10 cm in diameter). Seeded HepG2 were cultured for 24 h and 37 °C and subsequently prepared for pre-incubations and post-incubations with FA and Cd. HepG2 cells where used from passage Nr. 20 at least 3–4 weeks (till passage Nr. 35). HepG2 cells can be used from the third passage to Nr. 130. HepG2 were cultured in 6 replicates (6 different petri dishes) for each treatment for 24 h. Subsequently HepG2 cells were incubated without any additives (control), with BSA dissolved in PBS (phosphate buffer saline), in order to evaluate if the BSA alone would have any effects, with different concentrations Cd only and finally with the different concentrations of Cd and the FA (EPA + DHA, 5 + 10 µM) as a BSA-FA complex. The BSA-FA complex was prepared as follows: BSA was dissolved in PBS. EPA and DHA were dissolved in extra pure 98% EtOH to a final volume of 50 µL. Then 20 µL of EPA and DHA solutions were added to 1 mL of a mixture of 0.1 M NaOH and BSA solution (1/5; *v*/v) each. The two solutions of FA were then combined and the pH was adjusted to 7.1 using 0.1 M HCl. After 24 h pre-incubation with or without FA complex, Hep G2 liver cells were post-incubated for 24 h with 1 µM Cd, 2.5 µM Cd; and 5 µM Cd.

Viability was determined with the resazurin assay as described previously by Linhartova and Sampels (2015) and calculated as percentage of living cells in each treatment compared to the control (HepG2 only) where viability was set as 100%. Subsequently cells were pelleted by trypsinising and centrifuging several times in PBS containing 5% FCS (Sigma Aldrich), snap frozen in liquid nitrogen and subsequently stored in − 80 °C until further analyses.

The number (n) of tested samples was 6 per treatment (analysed in duplicates).

2.3. Uptake of metals into the cells

The content of Cd and the chosen trace metals (iron, copper and zinc) was analysed via inductively coupled plasma mass spectrometry (ICP-MS) as described earlier (Meyer et al., 2014) in the medium and the pelleted cells. The trace metals were not added, but present in the medium and its different components. Average cell volume in the control group was 3.93×10^{-12} mL^{-1} and did not differ among treatments. Quantification was performed with a multi-element calibration standard (M) for ICP MS, dissolved in HNO_3 (SPECTEC, Erding, Germany). The obtained values were always adjusted by the cell number of the respective pellet. Number (n) of tested cell pellets was originally 6 for all treatments, however due to analytical failure we finally evaluated n = 6 for control, 5 for 1 µM Cd and 4 for 2.5 and 5 µM Cd (analysed in duplicates).

2.4. Gene expression analyses

Total RNA from pellets of HepG2 (with the same number of viable cells) was extracted using the RNeasy Mini-Kit (Qiagen) including on-column treatment with DNase I (Qiagen) according to the manufacturer's instructions. High integrity of the RNA was verified with an Agilent 2100 Bioanalyzer using RNA 6000 Nano Kit. The annealing of 7.5 pmol of oligo(dT) primers (sequence: 5′-CCTGAATTCTAGAGCTCA$(T)_{17}$-3′, Sigma Aldrich) to 5 µg of total RNA extracted from each pellet sample was carried out at 65 °C for 5 min. The transcription with, 10 × buffer, 20 nmol of each dNTP, 10 mM DTT, 1 U Affinity Script reverse transcriptase (RT, Agilent Technologies) in a reaction volume of 20 µL was conducted at 42 °C for 60 min, followed by 15 min at 70 °C. A − RT control, where the RT had been replaced by pure water, was included to rule out possible contamination of samples by genomic DNA.

Relative real-time PCR assays [20-µL reaction volume: 2 µL of diluted cDNA, 7.5 pmol of each primer, and 1 × FastStart Universal SYBRGreen Master (Rox) (Roche)] were conducted with gene-specific primers according to the literature in a Mx3005p qPCR cycler (Agilent

Table 1
Primer sequences for the used primers.

Primers		5′–3′ sequence	References	PCR efficiency
cyclophilin A (reference gene)	s as	GGTTGGATGGCAAGCATGTG TGCTGGTCTTGCCATTCCTG	(Portolesi et al., 2008)	2.02
mt 1 m	s as	TCCGGGTGGGCCTAGCAGTCG AATGCAGCAAATGGCTCAGTATCGTATTG	(Cartularo et al., 2015)	1.92
mt 1 g	s as	GCCAGCTCCTGCAAGTGCAA TCTCCGATGCCCTTTGCAG	(Cartularo et al., 2015)	1.92