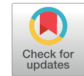
Reproductive Toxicology 81 (2018) 50–57

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Avobenzone suppresses proliferative activity of human trophoblast cells and induces apoptosis mediated by mitochondrial disruption



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ARTICLE INFO

Keywords:
Avobenzone
Trophoblast
Proliferation
Apoptosis
Mitochondria

ABSTRACT

Avobenzone is widely used in various personal care products, is present in swimming pools, and is toxic to aquatic organisms. However, it is unclear how avobenzone affects human trophoblast cells. Results of the present study demonstrated that avobenzone inhibited the proliferation of HTR8/SVneo cells, the immortalized human trophoblast cell line, and inhibited the expression of PCNA. In addition, avobenzone increased the activity of AKT and ERK1/2 in HTR8/SVneo cells. When LY294002 (AKT inhibitor) and U0126 (ERK1/2 inhibitor) were treated with avobenzone, the anti-proliferative effect of avobenzone was alleviated. Moreover, avobenzone promoted Ca^{2+} overload into the mitochondria and induced depolarization of the mitochondrial membrane. Expression of *IFI27*, which is located in the mitochondria, was elevated by avobenzone via inhibition of expression through siRNA transfection against *IFI27*, but did not alter cell properties. This study suggests that avobenzone induces mitochondrial dysfunction-mediated apoptosis leading to abnormal placentation during early pregnancy.

1. Introduction

Avobenzone (butyl methoxydibenzoylmethane) is an endocrine disruptor that directly binds to estrogen receptor β and acts as an estrogen agonist [1–3]. According to a survey in Switzerland, approximately 71% of personal care products contain avobenzone, which is the highest proportion among the various compositions [4]. The amount of avobenzone in personal care products is limited to 3% by The Food and Drug Administration (FDA) and 5% by Cosmetics Directive of the European Union, and it is toxic if products used frequently have concentrations of avobenzone greater than 5%. Avobenzone acts as an agonist or antagonist by reacting with various hormone receptors [5]. Additionally, avobenzone is found in underwater environments such as seawater swimming pools, making this potential toxin easily accessible to the human body [6]. In particular, under UV-irradiation and chlorination conditions, avobenzone produces a wide variety of eco-toxicant products which are much more reactive than avobenzone [7]. It is known to be toxic to aquatic organisms even at low concentrations [8]. Moreover, exposure of human immune cells to avobenzone promotes the release of inflammatory cytokines and reduces the viability of macrophages and monocytes at concentrations of 10 μg/mL (32 μM) [9]. However, little is known about the physiological activity of avobenzone in other types of human cells.

Human trophoblast cells form the placenta through a precisely controlled differentiation process during the early pregnancy of women. Routine exposure to various environmental factors is known to affect the viability, proliferation, and invasiveness of human trophoblast cells. The endocrine disruptor, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), induces apoptosis through mitochondrial dysfunction and reactive oxygen species (ROS) production in human trophoblast cells [10]. In addition, chemicals contained in personal care products and food additives such as propyl gallate, butyl paraben and decanoic acid can lead tomitochondrial defects and death of human trophoblast cells [11–13]. It is also known that pathways involving AKT or ERK1/2 signaling proteins are important for the survival and growth of human trophoblast cells, but it is unclear whether external environmental factors alter cell characteristics by modulating signal transduction pathways [14,15].

Therefore, the objectives of this study were to determine whether avobenzone affects the proliferation and death of human trophoblast HTR8/SVneo cells and whether AKT and ERK1/2 activities are regulated by avobenzone. We also measured mitochondrial membrane

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https://doi.org/10.1016/j.reprotox.2018.07.003
Received 16 March 2018; Received in revised form 21 June 2018; Accepted 3 July 2018
Available online 04 July 2018
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potential and mitochondrial Ca^{2+} concentration to analyze the effect of avobenzone on mitochondrial functions in HTR8/SVneo cells. Finally, we verified the expression of genes that may be targeted by avobenzone in HTR8/SVneo cells.

2. Materials and methods

2.1. Chemicals

Avobenzone was purchased from Selleckchem (Houston, TX, USA). Antibodies against phosphorylated AKT (Ser^{473}), P70S6 kinase (P70S6K, Thr^{421}/Ser^{424}), ribosomal protein 6 (S6, Ser^{235}/Ser^{236}), glycogen synthase kinase 3 beta (GSK3β, Ser^{9}), and extracellular signal-regulated protein kinase 1 and 2 (ERK1/2, Thr^{202}/Tyr^{204}), and total AKT, P70S6K, S6, GSK3β, and ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). LY294002 was purchased from Cell Signaling Technology and U0126 was purchased from Enzo Life Science (Farmingdale, NY, USA).

2.2. Cell culture

HTR8/SVneo cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 with 2.05 mM L-glutamine (Cat No: SH30027.01, HyClone, Logan, UT, USA) with 5% fetal bovine serum at 37 °C in a CO_2 incubator. For experiments, monolayer cultures of HTR8/SVneo cells were grown in culture medium to 70% confluence in 100-mm tissue culture dishes. Cells were serum-starved for 24 h, and then treated with avobenzone. In each assay, dimethyl sulfoxide was used as a vehicle.

2.3. Proliferation assay

Proliferation assays were conducted using a Cell Proliferation ELISA BrdU kit (Cat No: 11647229001, Roche, Basel, Switzerland) according to the manufacturer's recommendations as described previously [16]. HTR8/SVneo cells were added in triplicate to a 96-well culture dish and treated with the following concentrations of avobenzone for 48 h: 0, 1, 2, 5, 10, 20, and 50 μM. Reaction products were quantified by measuring absorbance values at 370 and 492 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

2.4. Immunofluorescence microscopy

The effects of avobenzone on the expression of proliferating cell nuclear antigen (PCNA) in HTR8/SVneo cells were determined by immunofluorescence microscopy as described previously [16]. Cells were treated with avobenzone (20 μM) for 24 h. Experiments were performed in triplicate. Images were captured using an LSM710 (Carl Zeiss, Oberkochen, Germany) confocal microscope fitted with a digital microscope Axio-Cam camera with Zen2009 software. Relative fluorescence intensity was measured by obtaining the green/blue ratio using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

2.5. Determination of apoptosis by annexin V and propidium iodide (PI) staining

Induction of apoptosis in HTR8/SVneo cells by avobenzone was analyzed using a fluorescein isothiocyanate Annexin V apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ, USA) as described previously [16]. Cells were treated with different avobenzone concentrations (0, 5, 10, 20 and 50 μM) for 48 h. Fluorescence intensity was analyzed using a flow cytometer (BD Biosciences). Data are representative of three independent experiments.

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

A TUNEL assay was performed on HTR8/SVneo cells treated with avobenzone as described previously [16]. Cells were treated with avobenzone (20 μM) for 48 h, and then subjected to TUNEL staining using an *in situ* Cell Death Detection Kit, TMR red (Roche). Images were captured using a LSM710 confocal microscope fitted with a digital microscope Axio-Cam camera with Zen2009 software. Relative fluorescence intensity was measured *via* green/blue ratio using MetaMorph software (Molecular Devices). Data are representative of three different experiments conducted in triplicate.

2.7. Western blot analysis

Western blot analysis of HTR8/SVneo cells treated with avobenzone was performed as described previously [16]. Multiple exposures of each western blot were performed to ensure linearity of chemiluminescent signals. Data are representative of three independent experiments.

2.8. JC-1 mitochondrial membrane potential assay

The JC-1 mitochondrial membrane potential was determined using a mitochondria staining kit (Cat No: CS0390, Sigma-Aldrich, St. Louis, MO, USA) as described previously [16]. Cells were treated with a range of avobenzone concentrations (0, 5, 10, 20, and 50 μM) for 48 h at 37 °C in a CO_2 incubator. Fluorescence intensity was analyzed using FACS-Calibur (BD Biosciences). Data are representative of three independent experiments.

2.9. Measurement of intracellular Ca^{2+} concentration

HTR8/SVneo cells (4×10^5 cells) were seeded onto 6-well plates, grown to 70–80% confluence, and incubated for 24 h in serum-free medium. Cells were then treated with avobenzone (0, 5, 10, 20, and 50 μM) for 48 h at 37 °C in a CO_2 incubator. Vehicle was added up to the highest treated dose. Supernatant was removed from culture dishes and adherent cells were detached with trypsin-EDTA. Cells were collected by centrifugation. For intracellular Ca^{2+} analysis, collected cells were resuspended using 3 μM Fluo-4 AM (Cat No: F14201, Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C in a CO_2 incubator for 20 min. The stained cells were washed with phosphate-buffered saline. To determine mitochondrial Ca^{2+} levels, collected cells were resuspended using 3 μM Rhod-2 AM (Cat No: R1244, Invitrogen) and incubated at 4 °C for 30 min. The stained cells were washed with Hank's balanced salt solution. Fluorescent intensity was analyzed using a flow cytometer (BD Bioscience). Data are representative of three independent experiments.

2.10. siRNA knockdown experiment

For mRNA interference against *IFI27*, HTR8/SVneo cells (5×10^5 cells) were seeded in 6-well plates and transfected with nontargeting control siRNA (siCTR) (Cat No: SR30004, OriGene, Rockville, MD, USA), siRNA directed against IFI27 (siIFI27) (Cat No: 1072552, Bioneer, Daejeon, Korea) using transfection reagent Lipofectamine 2000 according to the manufacturer's instructions. Briefly, cells were cultured with siRNA and Lipofectamine 2000 diluted in Opti-MEM reduced serum medium (Cat No: 32985070, Gibco, Grand Island, NY, USA). After 6 h incubation at 37 °C in a CO_2 incubator, the media were removed and media were added containing 20 μM avobenzone or vehicle for 18 h at 37 °C.

2.11. Quantitative RT-PCR analysis

Gene expression was determined using SYBR® Green (Sigma) and a

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