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Inhibition of placental 11beta-hydroxysteroid dehydrogenase type 2 by lead

Joey St-Pierre, Marc Fraser, Cathy Vaillancourt*

INRS-Institut Armand-Frappier, BioMed Research Center and Center for Interdisciplinary, Research on Well-Being, Health, Society and Environment (CINBIOSE), Laval, QC H7V 1B7, Canada

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ABSTRACT

Lead interferes with cortisol blood concentration, increases the risk of obstetrical complications, and could alter fetal development. The placenta controls maternal cortisol transfer to the fetus by the activity of the type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2), which converts cortisol into inactive cortisone. This study determines the effect of lead on the expression and activity of the placental 11β-HSD2 in human trophoblast-like BeWo cells. Cells were treated with increasing concentration (0–1000 nM) of PbCl2 for 24 h. 11β-HSD2 protein expression was reduced by 45% at 1000 nM of $PbCl_2$ compared to untreated cells, while the activity was significantly reduced by $PbCl_2$ at 10, 100 and 1000 nM. This study shows the direct inhibitory action of lead on placental 11β-HSD2 activity and suggests that this heavy metal reduces the efficiency of the placental protection against the adverse effects of high cortisol level during fetal development.

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1. Introduction

Lead (Pb), a heavy metal, is an environmental pollutant that can have several adverse health effects [1]. Human exposure to Pb may occur occupationally or environmentally through inhalation, smoking or water and dietary intake [2,3]. Maternal exposure to Pb is known to cause adverse effects in pregnancy such as an increased risk of spontaneous abortion, impaired calcium transport through the placenta and low birth weight [4,5]. Pb interferes with glucocorticoid blood levels in rats and humans during pregnancy affecting the offspring's stress response [6,7]. Prenatal exposure to Pb is also linked to several adverse effects on neonates such as learning disabilities, reduced IQ and endocrine disorders (reviewed in [2]).

Embryonic exposure to increased glucocorticoid (GC, cortisol in humans) concentrations is associated with low birth weight and metabolic disorders later in life [8]. The placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2, HSD11B2 gene) protects the fetus from adverse cortisol levels from the mother by converting cortisol to inactive cortisone [9]. Its location in the syncytiotrophoblast layer of the placenta provides a barrier and restricts the free transfer of cortisol from the mother to the fetus [10]. The role of the 11β-HSD2 is important as inhibition of 11β-HSD2 is associated with to low birth weight, intra-uterine growth retardation (IUGR) and to other pregnancy disorders, such as preterm birth and preeclampsia [11–16]. Metals, such as cadmium (Cd), manganese (Mn), nickel (Ni), cobalt (Co), and zinc (Zn), have been associated with altered placental 11β-HSD2 expression [17,18]. Moreover, Cd can reduce placental 11β-HSD2 activity in a concentration dependent manner [19]. The placenta is a known site of Pb accumulation, predominantly in the syncytiotrophoblast layer [20], however the effect of this heavy metal on the placental 11β-HSD2 has never been studied. Taken together, these studies raise the question of the mechanism by which placental Pb exposure could cause low birth weight and metabolic disorders later in the offspring's life. We hypothesize that low doses of Pb can down regulate the expression and activity of the 11β-HSD2 in trophoblast cells. The present study investigated the effect of low doses of Pb on the expression and activity of the 11β-HSD2 in trophoblast-like cell line BeWo.

2. Material and methods

2.1. BeWo cell culture and treatment

BeWo cells were acquired from the American Type Culture Collection (ATTC, Manassas, VA). Cells were cultured in Dulbecco's

* Corresponding author at: INRS-Institut Armand-Frappier, 531, boul. des Prairies, Building 18, Laval, QC H7V 1B7, Canada.
E-mail address: cathy.vaillancourt@iaf.inrs.ca (C. Vaillancourt).

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modified eagle's medium (DMEM/F12, Sigma-Aldrich, Oakville, Canada) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich). Cells were grown in vented 75 cm^2 flasks at 37° C in a humidified atmosphere (5% CO_2 and 95% air) and cells were passaged at confluence. Cells were plated in 6-well plates (11β-HSD2 expression) or 24-well plates (11β-HSD2 activity) and then treated with either $PbCl_2$ (0, 0.01, 0.1, 1, 10, 100 and 1000 nM) for 24 h or pre-treated with forskolin (FSK, 20 μM) for 24 h followed by treatment with FSK and $PbCl_2$ for another 24 h. FSK induces cell fusion and a phenotype change in BeWo cells into syncytiotrophoblast [21]. For no FSK control, vehicle was used (dimethyl sulfoxide, DMSO, 0.1%). A 24 h glycyrrhizic acid (GA, 1 μM), a potent 11β-HSD2 activity inhibitor, treatment was also used as a control for the activity assay with or without FSK pre-treatment [22].

2.2. BeWo cell, viability and function analysis

Lactate dehydrogenase (LDH) assay (Roche, Basel, Switzerland) was performed to evaluate $PbCl_2$ cytotoxicity. The assay measures the release of lactate dehydrogenase in the culture media. The assay was performed following the manufacturer's instructions. To evaluate the effect of $PbCl_2$ on cell viability, real-time proliferation assays was performed using the xCELLigence real time cell analysis (RTCA) system (ACEA, San Diego, CA). Briefly, cells were plated in 96 wells plates coated with electrodes for impedance measurement (E-Plates, ACEA). As cells adhere to the detector and grow, a higher cell index is measured which can be converted into cellular doubling time for interpretation, as previously described [23]. β-hCG secretion was analysed in culture media by ELISA (DRG diagnostics, Marburg, Germany) following the manufacturer's instructions and suggested dilutions. Results were obtained by spectrophotometry by reading plates at 450 nm in a SpectraMax M5 microplate reader (Molecular Devices, Toronto, Canada).

2.3. RT-qPCR analysis

Total RNA was extracted using the Aurum total RNA mini kit (Bio-Rad, Missauga, Canada) using the manufacturer's spin protocol. Briefly, cells were rinsed in cold phosphate buffered saline (PBS), RNA extraction kit's lysis solution was added to the wells and cells were released from the plates before being transferred to the spin columns as per the manufacturer's instructions. From this step, all manipulations were performed at room temperature. Genomic DNA was removed by DNase I digestion for 15 min. RNA was eluted in 40 μl of elution buffer pre-heated to 70 °C. Extracted RNA was then stored at −80 °C until analysis. RNA quantity was measured by spectrophotometry, measuring OD_{260} and RNA purity was analysed by measuring $OD_{260/280}$ using a SpectraMax M5 microplate reader (Molecular Devices). Only RNA with an $OD_{260/280}$ ratio of 1.8:2.0 was used for further analysis. RNA integrity was assessed in at least 2 random samples in each experiment using the Experion automated electrophoresis system with the RNA standard sensitivity kit (Bio-Rad). Samples tested showed no meaningful degradation of extracted RNA. Reverse transcription reaction was performed with the iScript cDNA synthesis kit for qPCR (Bio-Rad) following the manufacturer's instructions. qPCR reactions were performed on a CFX-96 using Sso-Fast EvaGreen supermix (Bio-Rad) as previously described [24]. Reference genes were selected using GeNorm software (BioGazelle, Zwijnaarde, Belgium) [24,25]. Reference genes used for normalization (ΔΔCT) were TATA-binding protein (TBP) and type 1 topoisomerase (TOP-1) for BeWo cells treated with $PbCl_2$. For BeWo cells treated with FSK and $PbCl_2$, reference genes used were hypoxanthine phosphoribosyltransferase 1 (HPRT1) and succinate dehydrogenase complex, subunit A (SDHA). Primer sequences are presented in Table 1.

2.4. Western blot analysis

Proteins were extracted by rinsing cells in cold PBS, releasing them by scraping on ice and agitating them in cold radioimmunoprecipitation (RIPA) buffer containing Halt protease and Halt phosphatase inhibitor cocktails (Thermo scientific, Waltham, MA) for 30 min. Cells were then sonicated and centrifuged at 4 °C (10 min, 13,000 x *g*). Sample protein concentrations were determined by bicinchoninic acid assay (BCA) assay following the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). Proteins (40 μg) were separated on 10% SDS-PAGE gel and transferred to a Polyvinylidene fluoride (PVDF) membrane using trans-blot Turbo transfer system (Bio-Rad). Membranes were blocked using 5% skimmed milk in 0.5% PBS-Tween. Anti-HSD11B2 (#AB80317, Abcam, Toronto, Canada) primary antibody diluted 1:1000 in blocking solution was used for overnight exposition at 4 °C. Membranes were then washed in 0.5% PBS-Tween 3 times for 10 min. Anti-rabbit HRP (#AP307P, Millipore, Billerica, MA) was used as secondary antibody diluted 1:25,000 in blocking solution for 1 h at room temperature. Chemiluminescence was detected using Immobilon Western chemiluminescent HRP substrate (Millipore) and revealed under Chemidoc MP imaging system (Bio-Rad). To normalise protein expression, amido black stain was used, as previously described [26]. Briefly, membranes were stained in amido black (Sigma-Aldrich) for 30 s and then washed in destaining solution (50% methanol, 10% acetic acid) for 30 min. Blots were scanned using Chemidoc MP imaging system (Bio-Rad). Quantification of protein expression and reference blotting was performed using Image Lab 4.1 software (Bio-Rad). Briefly, protein expression was evaluated by carefully integrating the signal intensity for each band, making sure not to take background into account. For amido black protein stain, rectangular boxes were used to integrate the signal as previously described [26,27].

2.5. 11β-HSD2 activity assay

Radioenzymatic conversion assay was performed to assess 11β-HSD2 activity. Briefly, treated cells were rinsed in PBS, and then incubated 30 min with 0.5 μCi of ^{3}H-Cortisol (Perkin Elmer, Waltham, MA) in PBS at 37 °C. The reaction was stopped by adding one volume of diethyl ether. Steroids were extracted by freezing the aqueous phase by dipping the tube in dry-ice cooled ethanol. The liquid solvent phase containing the steroids was then removed. The extracts were then dried using an evaporator system with nitrogen and suspended in dichloromethane. A fraction of this suspension was then loaded on a thin layer chromatography plate (silica gel HLF 250 μm, Analtech, Newark, DE) and migrated with a solution of dichloromethane: methanol (95:5, v:v). Bands were identified under UV light, with unlabeled cortisol and cortisone as reference, and then the silica bands were released from the plates by scraping and placed into scintillation vials. Scintillation was measured in a Tri-Carb 2100TR (Perkin Elmer). Results are expressed as a percentage of control treatment (no $PbCl_2$) for the cortisone conversion where background values (the amount of conversion without cells) were subtracted.

2.6. Statistical analysis

Statistical analyses were performed with Prism 5.0 (Graphpad, San Diego, CA). One-way analysis of variance was used for statistical analysis with Dunnet's multiple comparison post-test. P values lower than 0.05 were considered statistically significant. Results from 3 different cell passages were used for statistical analysis. Assays were performed in technical triplicates for cell real time

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