Aflatoxin B1 targeted gene expression profiles in human placental primary trophoblast cells

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A R T I C L E   I N F O

Keywords:
Aflatoxin B1
Estrogen signalling
Gene expression profiling
Pathway analysis
Placenta
Trophoblast

A B S T R A C T

Aflatoxin B1 (AFB1) is a mycotoxin produced by Aspergillus flavus and A. parasiticus. A high exposure (40 nM and 1 µM AFB1 for 72 h) was used to study mechanistic effects of AFB1 on gene expression patterns in human primary trophoblast cells, isolated from full term placentae after delivery. Gene expression profiling was conducted, and Ingenuity pathway analysis (IPA) software was used to identify AFB1-regulated gene networks and regulatory pathways.

In response to 40 nM AFB1, only 7 genes were differentially expressed whereas 1 µM AFB1 significantly dysregulated 170 genes (124 down- and 46 upregulated, ±1.5-fold, p < 0.05) in AFB1-exposed trophoblasts when compared to controls. The top downregulated genes were involved in endocrine signalling and biosynthesis of hormones, and lipid and carbohydrate metabolism. The top upregulated genes were involved in protein synthesis and regulation of cell cycle. The main canonical pathways identified by IPA were associated with endocrine signalling including growth hormone signalling, and corticotropin releasing hormone signalling. Furthermore, genes involved in aryl hydrocarbon receptor (AhR)-mediated estrogen receptor signalling were dysregulated in response to AFB1.

Our findings indicate that a high concentration 72 h AFB1 exposure caused relatively moderate number of changes on transcript level to human placental primary trophoblast cells. However, these preliminary results need to be confirmed with human-relevant concentrations of AFB1.

Introduction

Human placenta is an intermediary organ that is responsible for growth and development of the fetus and serves as a protective barrier that separates fetal blood from maternal blood (Burton and Jauniaux, 2015). Placenta is responsible for regulating fetal environment, providing immune protection, transfer of gases and nutrients from the maternal to fetal blood, and production of hormones, cytokines and growth factors necessary for maintenance of pregnancy (Guttmacher et al., 2014; Illidromiti et al., 2012).

Aflatoxin B1 (AFB1) is a secondary metabolite produced by Aspergillus flavus and A. parasiticus mould and contaminates crops such as rice, maize, nuts, cereals, soybeans (Alshannaq and Yu, 2017) and even animal products such as meat, eggs and milk (Pier, 1992). Aflatoxins, especially AFB1 are known to cause acute and chronic toxicity such as liver failure and liver cancer (Ostry et al., 2017; Williams et al., 2004), teratogenicity (Gong et al., 2004; Smith et al., 2017), immunosuppression (Williams et al., 2004) and potential endocrine disruptive effects (Storvik et al., 2011).

AFB1 and its metabolites can be found in breast milk, neonatal cord blood and serum of pregnant women (Lamplugh et al., 1988). These toxins can be transferred and metabolized through the placenta in which aflatoxicol (AFL) is the only metabolite from AFB1 formed in the placenta (Partanen et al., 2010). However, the effects and molecular targets of AFB1 in human placental cells are not well-known. It has been previously demonstrated that AFB1 causes disturbances in placental hormone metabolism and normal oestrogen production in placental chorion carcinoma (JEG-3) cells (Huuskonen et al., 2013; Storvik et al., 2011; Wang et al., 2015; Zhu et al., 2016). In addition, previous gene expression profiling studies with AFB1 have been done on human hepatocytes which reported alternation in genes related to p53 signalling pathway, cell cycle, apoptosis and DNA repair (Josse et al., 2012). Yip et al., (2017) reported effects on cell growth, DNA synthesis and cell cycle progression in breast cancer MCF-7 cells. Other transcriptomic studies reported that AFB1 altered genes involved in gluconeogenesis, lipid metabolism, p53 signalling pathway (Lu et al., 2013) aryl

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https://doi.org/10.1016/j.crtox.2022.100082
Received 31 December 2021; Received in revised form 29 June 2022; Accepted 30 June 2022
Available online 4 July 2022
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hydorcarbon receptor (AhR), nuclear factor erythroid 2-related factor 2 (Nrf2), glutathione-S-transferase (GSH), cell cycle, extracellular matrix, and cell differentiation networks (Merrick et al., 2013) in rat hepatocytes. Liu et al. (2020) identified AFB1 target genes in chicken liver and reported an upregulation in peroxisome proliferator-activated receptor gamma (PPARγ) signalling and apoptosis.

In this study, AFB1 targeted gene expression profiles were determined in human primary trophoblast cells isolated from full-term placentas immediately after delivery. Two concentrations, 40 nM and 1 μM of AFB1 were used to study mechanistic effects on placental gene expression at one time point, 72 h. To our knowledge, our microarray study is the first to report the transcriptomic changes in human primary trophoblasts in response to AFB1.

Materials and methods

Placental tissue

Four full term placentas were obtained from Kuopio University Hospital after a caesarean section. The study was approved by the ethics committee (Kuopio University Hospital, Finland) and the experiments were done according to the Declaration of Helsinki. All individual study participants signed the informed consent. The full-term placentas were from uncomplicated non-smoking pregnancies.

Isolation and culturing of human primary trophoblast cells

The isolation and culture of primary placental trophoblast cells was conducted according to Petroff et al. (2006) and described in detail in previous study by El Dairi et al., (2018). Briefly, the isolation of trophoblast cells started 20 min after caesarean sections by dissecting the placental soft villous tissue (50 × 10^5 cells/well) was treated with 40 nM and 1 μM AFB1 (Sigma-Aldrich, #A6636) for 72 h and the control was 0.1% dimethyl sulfoxide (DMSO, Sigma-Aldrich, #D8418) (six replicates).

Cell viability test

The cell viability of AFB1 treatment on the primary trophoblast cells was assessed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) test (Sigma-Aldrich/Merck, #M2128) as previously described by Storvik et al. (2011). Human primary trophoblasts (5x10^5 cells/well) were incubated with 0.01–3 μM AFB1 for 72 h at 37 °C and then the MTT reagent was added for 4 h incubation, sodium dodecyl sulfate - N-dimethylformamide (SDS-DMF) (Sigma-Aldrich/Merck, #L3771 and #D4551, respectively) was added and the plate was maintained overnight at 37 °C. The optical density was measured using a BioTek ELx800 reader (BioTek, USA) at a wavelength of 570 nm. The results were expressed as a percentage of the control cells exposed to dimethyl sulfoxide to the same concentration used as the solvent for AFB1. The significance of the differences between exposures and respective controls was analyzed by one-way ANOVA followed by Tukey’s multiple comparison post-hoc test.

DNA microarray

The extraction of total RNA from human primary trophoblast was performed with a Qiagen AllPrep mini kit (Qiagen Ltd, UK, #80284) followed by DNase treatment (Ambion Turbo DNA-Free Kit, Life Technologies, USA, #AM1907). The quality and integrity of RNA samples were monitored as previously described (El Dairi et al. 2018). DNA microarray assay with Illumina HumanHT-12 Expression BeadChip (Illumina, USA) was conducted according to the manufacturer’s instructions at the Core facility of the Estonian Genome Center, University of Tartu. The raw intensity data for the transcripts was exported using the Illumina GenomeStudio software, and normalized to the same distribution in each chip by using Chipster (CSC, Finland). The differentially expressed genes were selected when the difference in the normalized expression was statistically significant (p < 0.05, Student’s t-test with adjustment by the Benjamini-Hochberg multiple testing correction) and the fold-change between the AFB1 treated group was at least 1.5-fold as compared to the respective controls. A heatmap of the significantly up- and downregulated transcripts was created with Heatmapper (heatmapper.ca) with average linkage and Euclidian distance method. The complete data sets are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database and gene expression profiling data comply with the Minimum Information About a Microarray Experiment (MIAME) standard. The data can be obtained from the GEO database with the accession number GSE153590.

Quantitative real-time (qRT)-PCR

Quantitative RT-PCR was performed as previously described (Huuskonen et al. 2008) using Taqman Real Time PCR assays (Thermo Fisher Scientific, USA) for cytochrome P450 family 1 subfamily A member 1 (CYP1A1) (Hs00153120_m1) and CYP19A1 (Hs00903411_m1), and the gene expression of was normalized with β-actin (Taqman assay #4316315E).

Pathway analysis

The list of differentially expressed genes was uploaded into Ingenuity pathway analysis (IPA) software (Qiagen, USA). IPA was used to analyse the main canonical pathways, diseases and functions and upstream regulators by clustering the differentially expressed genes (≥1.5-fold, p-value < 0.05, compared to controls). The calculation of P-values for the pathways using the Fisher Exact Test, and p < 0.05 was considered statistically significant. A Z-score with a value of ≥ 2 or ≤ −2 indicates significant activation or inhibition of the biological functions and upstream regulators. The full description of pathway analysis has been described by (El Dairi et al., 2018).

AFB1 dysregulated mRNA expressions in human primary trophoblast

Primary trophoblasts were exposed to increasing concentrations of AFB1 (0.01–3 μM) for 72 h, and cell viability was measured with the MTT-assay. Cell viability was not affected significantly up to 1 μM concentration of AFB1, and at 3 μM AFB1 cell viability was decreased significantly (87 % compared to control) (p < 0.05) (Fig. 1). Based on cell viability AFB1 concentrations of 40 nM and 1 μM were selected for the gene expression profiling study. In DNA microarray study, only 7 transcripts were differentially expressed in 40 nM AFB1-treated primary trophoblasts; all these transcripts were non-significantly downregulated. Only two of the transcripts, an amino acid transporter SLC43A2 and a histone protein coding gene HIST1H4C, correspond to protein coding genes, and other 5 transcripts were non-coding RNA sequences. LOC100008589. Due to minimal impact of 40 nM of AFB1 treatment on gene expression, a higher concentration of 1 μM AFB1 was used to perform further mechanistic analyses. The higher 1 μM AFB1 concentration significantly dysregulated 170 genes (46 up- and 124 down-regulated, ≥1.5-fold, p-value < 0.05) when compared to DMSO-treated controls (Fig. 2). The top ten up- and down-regulated genes in 1 μM AFB1-treated primary trophoblasts are listed in Table 1 and the complete list of differentially expressed gene sets is presented in Supplementary Table 1. Among the top down-regulated genes were several
placental genes involved in endocrine signalling and biosynthesis of hormones, and lipid and carbohydrate metabolism whereas many cell cycle regulators and genes involved in protein synthesis were among the top up-regulated genes. We confirmed gene expression levels of CYP1A1 and CYP19A1 results by comparison with mRNA levels obtained by gene expression profiling with RT-qPCR. The normalized qPCR gene expression fold-change ratios for CYP1A1 and CYP19A1 after 72 h 1 µM AFB1 exposure were −2.9 and −1.5 respectively, when compared to controls. The corresponding microarray expression fold-change of CYP1A1 and CYP19A1 were −0.3 and −2.2, respectively.

**Pathway analysis**

To understand the mechanisms involved in AFB1 induced and repressed gene responses we explored the molecular pathways of differentially expressed genes in response to 1 µM AFB1 treatment in primary trophoblasts. First, we analyzed the canonical pathways affected by AFB1 treatment using IPA. Among the significant canonical pathways there were hormone signalling pathways inhibited including growth hormone, estrogen receptor, insulin secretion and corticotropin releasing hormone signalling pathways (Fig. 3).

IPA analysis exhibited that the main altered molecular and cellular functions in human placental trophoblast in response to 1 µM AFB1 were cell death and survival, cellular movement, development, growth and proliferation, and cell-to-cell signalling and interaction (Fig. 4). Furthermore, the most significant diseases and disorders linked to the AFB1 regulated gene set in primary trophoblasts included those related to hyperlipidemia, transport of D-glucose and impaired glucose tolerance (Fig. 3).

Furthermore, we used IPA to predict potential upstream regulators that could explain the observed changes in gene expression in AFB1-treated primary trophoblasts. The top predicted endogenous regulators of are shown in Table 2. The top predicted activated upstream regulators were tumour suppressor protein TP53 and several transcription factors including forkhead box O3 (FOXO3), FOXO4 and RUNX family transcription factor 1 (RUNX1). In top inhibited downstream regulators were several regulators related to hormone response such as androgen receptor, dihydrotestosterone, corticotropin releasing hormone (CRH) and beta-estradiol as well as to inflammatory and immune response (e.g., CD24 molecule, prostaglandin E2, interleukins 1, 1B and 6 as well as toll like receptor 2). In addition, AhR and aryl hydrocarbon receptor nuclear translocator (ARNT) were among inhibited upstream regulators. IPA was further utilized to evaluate the influence of AFB1 on AhR signaling in human primary trophoblasts. The analysis revealed that AFB1 dysregulated the following genes: adrenomedullin (ADM), Fos proto-oncogene, AP-1 transcription factor subunit (FOS), estrogen related receptor gamma (ESRRG), synedcan 1 (SDC1), solute carrier family 2A1 (SLC2A1, glucose transporter 1), cytochrome P450 subfamily 19A1 (CYP19A1, aromatase), actin alpha 2 (ACTA2), growth arrest and DNA damage inducible alpha (GADD45A) and cyclin-dependent kinase inhibitor (CDKN1A, P21), that are involved in AhR
signalling, that in turn is predicted to be inhibited (Z-score $-2.8$, p-value $8.25 \times 10^{-4}$) in human term trophoblast (Fig. 6).

Gene expression analysis of IPA revealed that cell cycle, cellular compromise, DNA replication, recombination, and repair gene network were among the biological functions and gene networks that were dysregulated in AFB1-treated primary trophoblasts (Supplementary Fig. 1). Furthermore, IPA analysis revealed that cell cycle processes mitogenesis and mitosis were predicted to be inhibited due to downregulation of genes such as FOS, ADM, epidermal growth factor receptor (EGFR), protein kinase C zeta (PRKCZ), cholecystokinin (CCK) and upregulation of CDKN1A, GADD45A (Supplementary Fig. 2).

**Table 1**

Top 10 up- and downregulated genes in aflatoxin B1-treated human primary trophoblasts compared to control cells.

| Entrez ID | Gene ID | Gene Symbol | Gene Name                          | Fold-change |
|-----------|---------|-------------|------------------------------------|-------------|
|           |         |             | **Up-regulated**                   |             |
| 92        | ACRV2A  |             | Activin A receptor type 2A         | 4.3         |
| 51065     | RPS27L  |             | Ribosomal protein S27 like         | 2.7         |
| 1026      | CDKN1A  |             | Cyclin dependent kinase inhibitor 1A| 2.5         |
| 5430      | POLR2A  |             | RNA polymerase II subunit A        | 2.2         |
| 64752     | ISG20L1 |             | Interferon-stimulated 20 kDa exonuclease-like 1 | 2.0 |
| 6223      | RPS19   |             | Ribosomal protein S19              | 1.9         |
| 2876      | GPX1    |             | Glutathione peroxidase 1           | 1.9         |
| 51499     | TRIAP1  |             | TP53 regulated inhibitor of apoptosis 1| 1.9 |
| 1647      | GADD45A |             | Growth arrest and DNA damage       | 1.8         |
| 51693     | TRAPPC2L|             | Trafficking protein particle complex 2| 1.8 |
|           |         |             | **Down-regulated**                 |             |
| 1442      | CSH1/2  |             | Chorionic somatomammotropin hormone 1/2| $-3.3$ |
| 1443      | GH1     |             | Growth hormone 1                   | $-3.3$      |
| 2688      | PAPPA   |             | Pappalyisin 1                      | $-3.0$      |
| 5069      | PVRL3   |             | Nectin cell adhesion molecule 3    | $-2.9$      |
| 8038      | ADAM12  |             | ADAM metallopeptidase domain 12    | $-2.9$      |
| 1444      | CSH1    |             | Chorionic somatomammotropin hormone like 1| $-2.8$ |
| 79987     | SVEP1   |             | Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1 | $-2.7$ |
| 5921      | RASA1   |             | Ras p21 protein activator 1        | $-2.6$      |
| 84525     | HOPX    |             | HOP homeobox                       | $-2.6$      |
| 10761     | PLAC1   |             | Placenta enriched 1                | $-2.6$      |

**Discussion**

In this study, AFB1 target genes were investigated in human primary trophoblasts with gene expression analysis and pathway analysis was performed to identify the main molecular and cellular functions with the dysregulated genes in the dataset. This study demonstrates that unlike the liver cells, primary trophoblast cells are resistant to AFB1-induced damage in in vitro platform. The downstream analysis data

![Fig. 3. The significant canonical pathways associated with differentially expressed genes in aflatoxin B1-treated (1 μM) primary trophoblasts by the IPA software. The most significant biological functions with the highest number of significantly altered genes (≥1.5-fold upregulation or downregulation in AFB1-treated cells compared to controls; p < 0.05) are shown.](image)

![Fig. 4. Functional analysis of differentially expressed genes in aflatoxin B1-treated primary trophoblasts by the IPA software. The most significant biological functions with the highest number of significantly altered genes (≥1.5-fold upregulation or downregulation in AFB1-treated cells compared to controls; p < 0.05) are shown.](image)
demonstrated that AFB1 disrupts endocrine signalling, lipid and carbohydrate metabolism, and cell cycle and DNA repair processes in human placental cells.

**AFB1 sensitivity of human primary trophoblasts**

Gene expression profiling analysis was performed for 40 nM and 1 µM AFB1-treated trophoblast cells. In response to 40 nM exposure, only 7 genes were changed, whereas at a dose of 1 µM AFB1 170 genes were differentially expressed. A moderate number of significant transcript changes with a high AFB1 dose could indicate low sensitivity of primary cells. AFB1 is mainly metabolised by the liver, especially by CYP1A2 and CYP3A4 enzymes into the carcinogenic DNA reactive epoxide, aflatoxin B1-8,9-epoxide (Rushing and Selim, 2019). However, these CYP enzymes are not expressed at protein level in human placenta at term. In addition, the CYP expression profile of term human placenta is restricted to the liver (Hakkola et al., 1998). Furthermore, only a less potent AFB1 metabolite, AFL, was detected in the human placenta (Partanen et al., 2010). This difference in CYP expression profile and activity between the liver and the placenta was also reported in rat in which no placental activities of CYP1A, CYP2B, 2C and 3A, UGT1, UGT2 and GST were found (Fabian et al., 2016). Therefore, because of placental cells lack the capacity to form DNA reactive AFB1 metabolites, the 1 µM dose of AFB1 did not affect cell viability on the MTT test. This is in line with results on JEG-3 cells where 1 µM of AFB1 did not decrease cell viability and a

### Table 2

Upstream analysis of aflatoxin B1 (AFB1) regulated genes. The top endogenous upstream regulators that were predicted to be activated or inhibited in response to AFB1 exposure in primary trophoblasts are shown. Z-scores ≥ 2 or ≤ 2 indicate that the upstream regulator was predicted to be activated or inhibited, respectively. The p-value calculated by a Fisher’s Exact Test was used to determine the significance of the overlap (p-value < 0.05 [i.e., −log10 ≥ 1.3] between the regulator and stretch-responsive genes. Only functional annotations that obtained a significant regulation Z-score (≥2) are presented.

| Upstream Regulator | Description | Z-score | p-value |
|--------------------|-------------|---------|---------|
| TFRC               | Ferritin receptor | 2.449   | 1.11E-07 |
| TP53               | Tumor protein P53 | 2.278   | 6.11E-10 |
| FOXO4              | Forkhead box O4 | 2.201   | 9.35E-05 |
| RUNX3              | RUNX family transcription factor 3 | 2.200   | 1.42E-03 |
| IKZF1              | IKAROS family zinc finger 1 | 2.200   | 2.52E-02 |
| FOXO1              | Forkhead box O1 | 2.077   | 2.57E-04 |
| I                  | Inosine | 2.000   | 4.24E-04 |
| MLXIPL             | MLX interacting protein like | 2.000   | 1.42E-03 |
| HIC1               | HIC ZBTB transcriptional repressor 1 | 2.000   | 6.75E-04 |
| AGT                | Angiotensinogen | -3.221  | 1.27E-08 |
| AR                 | Androgen receptor | -2.929  | 4.30E-05 |
| DHT                | Dihydrotestosterone | -2.896  | 4.11E-07 |
| AHR                | Aryl hydrocarbon receptor | -2.793  | 8.24E-04 |
| Creb               | CAMP responsive element binding protein 1 | -2.589  | 1.81E-06 |
| HGF                | Hepatocyte growth factor | -2.513  | 6.48E-03 |
| TCF4               | Transcription factor 4 | -2.449  | 4.82E-02 |
| TEAD4              | TEA domain transcription factor 4 | -2.433  | 3.89E-06 |
| CD24               | CD24 molecule | -2.429  | 6.95E-06 |
| PGE2               | Prostaglandin E2 | -2.419  | 6.34E-05 |
| CRH                | Corticosteroid releasing hormone | -2.408  | 1.98E-05 |
| FGF2               | Fibroblast growth factor 2 | -2.317  | 5.60E-06 |
| LT3                | L-triiodothyronine | -2.275  | 1.08E-07 |
| ARNT               | Aryl hydrocarbon receptor nuclear translocator | -2.229  | 2.45E-05 |
| IL1B               | Interleukin 1 beta | -2.223  | 8.49E-07 |
| AURK               | Aurora kinase B | -2.219  | 1.3E-05 |
| EGFR               | Epidermal growth factor receptor | -2.218  | 4.06E-05 |
| IL13               | Interleukin 13 | -2.157  | 1.70E-01 |
| PPARα              | Peroxisome proliferator activated receptor alpha | -2.150  | 1.40E-02 |
| LEP                | Leptin | -2.148  | 7.99E-06 |
| EGF                | Epidermal growth factor | -2.093  | 4.98E-07 |
| E2                 | Beta-estradiol | -2.087  | 4.43E-10 |
| Vegf               | Vascular endothelial growth factor A | -2.086  | 2.03E-03 |
| IL6                | Interleukin 6 | -2.059  |         |

(continued on next page)
significant reduction was seen only at the concentration of 3 \( \mu \)M (Storvik et al., 2011).

**AFB1 dysregulated genes involved in AhR and estrogen receptor signalling**

Our analysis revealed that estrogen receptor (ER) signalling is among the main pathways that were disrupted by 1 \( \mu \)M AFB1 in term human trophoblasts. Genes involved in ER signalling are highly expressed in the placenta (Su et al., 2004). ESRRG was downregulated in our analysis. ESRRG is highly expressed in the placenta, it plays a role in the placental development, and is induced during trophoblast differentiation and induction of CYP19A1 gene expression (Kumar and Mendelson, 2011).

Consistent with ESRRG repression, CYP19A1 was downregulated in 1 \( \mu \)M AFB1 exposed primary cells compared to controls. CYP19A1 plays a vital role in placental estrogen biosynthesis from C19 steroids (Nelson and Bulun, 2001). Conversely, an upregulation of CYP19A1 activity by AFB1 exposure with similar high dose in placental secondary cell line JEG-3 has been reported in our earlier studies (Huuskonen et al., 2013; Storvik et al., 2011). However, in this study primary trophoblast cells were utilized which can possess different characteristics compared to immortalized cell lines.

Based on IPA analysis, AhR was predicted as one of the most important regulators of AFB1 dysregulated genes. In this data set, AhR activity was observed to be among top suppressed upstream regulators inhibited by AFB1 although previous studies have reported that AFB1 caused a significant increase in CYP1A1/A2, AhR, constitutive androstane receptor (CAR) and pregnancy X receptor (PXR) mRNA expression in human primary hepatocytes (Mary et al., 2015) and AhR-mediated increase of CYP1A1 activity and transcription in H4IE rat hepatoma cells (Ayed-Boussema et al., 2012). CYP1A1 and AhR are highly expressed and inducible in the human placenta, whereas CAR and PXR are not remarkably expressed (Storvik et al., 2014). Furthermore, several studies have reported the involvement of AhR with steroid hormone receptors, mainly the AhR cross-talk with ERs (NR3A1/A2) (Ohtake et al., 2003; Pocar et al., 2005; Safe et al., 2018). The crosstalk between the liganded AhR and ER signalling was reported to be through the inhibition of estrogen signalling by inhibiting the binding of estrogen with ER (Klinge et al., 1999) or AhR binding to ER-chaperones and inhibiting the transcription of estrogen downstream target genes such as cathepsin D and c-fos (Duan et al., 1999). The AhR anti-estrogenic effects were also shown through the AhR-mediated activation of rapid proteasomal degradation of ER that leads to reduction in ER protein and transcriptional activity (Wormke et al., 2003). In line with this, our previous studies (Huuskonen et al., 2016, 2008) demonstrated that maternal smoking during pregnancy resulted in increased placental CYP1A1 activity but decreased CYP19A1 activity, thus confirming placentocrosstalk between AhR and ER signalling.

ER signalling is predicted to be inactivated in this dataset, which supports the anti-estrogenic effects of AFB1-regulated AhR, however, the AhR-ER crosstalk also depends on the availability of estrogen that maybe linked to repressed CYP19A1 activity after AFB1 exposure. For instance, in line with the data, in the presence of estrogen, AhR possess anti-estrogenic effects and inhibit ER DNA-binding (Ohtake et al., 2003).

**AFB1 disrupts endocrine signalling in human primary trophoblast**

According to our analysis, growth hormone 1 (GH1) and corticotropic releasing hormone (CRH) signalling are predicted to be downregulated due to downregulation of choric hormon somatomammotropin hormone 1 (CSH1), choric hormon somatomammotropin hormone 1 (CSH1), FOS, GH1, PRKCZ, and nitric oxide synthase 3 (NOS3). Maternal GH1 gene is silenced in the pituitary gland during pregnancy, whereas placental syncytiotrophoblasts express GH1 (Handwerger and Freemark, 2000). GH1 is involved in fetal growth, placental development, maternal adaptation to pregnancy, and it stimulates maternal insulin-like growth factor (IGF1) production resulting in glucose and amino acids availability to the fetus (Lacroix et al., 2002; P´erez-Ibave et al., 2014). CRH is one of the vital hormones that is produced mainly by the placenta and involved in human pregnancy and parturition (Challis et al., 2000; Sasaki et al., 1987; You et al., 2014). The downregulated CSH1 and CSHL1 (placental lactogen) are members of

![Fig. 6. The aryl hydrocarbon receptor (AhR) regulated genes in aflatoxin B1 (AFB1) treated (1 µM) term trophoblasts. Based on IPA analysis, AhR is predicted as one of the most important regulators of AFB1 dysregulated genes. Green colour indicates downregulated genes and red colour upregulated genes, blue colour indicates the inhibitory effects, and orange colour indicates predicted activation. ACTA2, actin alpha 2; ADM, adrenomedullin; CDK11A, cyclin dependent kinase inhibitor 1A; CYP19A1, cytochrome P450 family 19 subfamily A member 1; ESRRG, estrogen related receptor gamma; FOX, Fox proto-oncogene; GADDS5A, growth arrest and DNA damage inducible alpha; SLC2A1, solute carrier family 2 member 1; SDC1, syndecan 1.](image-url)
somatotropin/prolactin hormone family which are solely expressed in the placental villi (Chen et al., 1989; Fagerberg et al., 2014) and play important role in fetal growth, regulation of maternal carbohydrate, lipid and protein metabolism (Handwerger and Freemark, 2000; Kim and Felig, 1971). The activity of placenta enriched 1 (PLAC1), which is important for placentar development, has been reported to be reduced in human placentas in cases of fetal growth restriction (Sun et al., 2021). Interestingly, AFB1 downregulated PLAC1 in placentary trophoblasts. Altogether, these findings suggest that a high AFB1 exposure among women of childbearing potential may result in poor prognosis for conception and further development of pregnancy and well-being of fetus.

To summarize, AFB1 affected the expression of genes involved in endocrine signalling in placentary trophoblasts. AFB1 have been demonstrated to cause fetal toxicity or teratogenicity (Smith et al. 2017) which can be expected by crossing the placental barrier (Partanen et al., 2010). These results suggest that decreased endocrine signalling in trophoblasts may contribute to AFB1 caused toxicity for feto-placental unit.

AFB1 disrupts carbohydrate and lipid metabolism in human placental cells

The gene expression analysis exhibited that AFB1 downregulated the expression of several genes involved in glucose and fatty acid homeostasis, including SLC2A1, pyruvate dehydrogenase (PDK4), leptin (LEP), low density lipoprotein receptor (LDLR) and insulin induced gene 1 (INS1G1), indicating AFB1 is interfering in regulation of placental carbohydrate and lipid metabolism as well as energy production. SLC2A1 is a major transporter in glucose uptake (Mueckler and Makepeace, 2008), whereas PDK4 is a mitochondrial protein that plays a key role in glucose and fatty acids metabolism and homeostasis (Abbott et al., 2005; Kul-karni et al., 2012). Leptin induces trophoblast cells proliferation and survival, and plays a key role in placental adaptation to stimuli such as hypoxia (Cervero et al., 2005; Dos Santos et al., 2015; Henson and Castracane, 2006; Schanton et al., 2018). INS1G1 is an important regulator in glucostate homeostasis and lipid metabolism (Dong and Tang, 2010), similarly to LDLR (Dato and Chiabrando, 2018). Previous studies have also reported that AFB1 alters lipid, amino acid and carbohydrate metabolism in other experimental animal models (Baldwin and Parker, 1985; Cheng et al., 2017; Kessl, 1986). So, in line with previous studies, our data indicates that AFB1 exposure during pregnancy may disturb also human placental lipid and glucose metabolism.

AFB1 disrupts cell cycle and DNA repair processes

Human trophoblastic cells at term cannot produce DNA reactive AFB1 metabolites via metabolic activation. However, our study demonstrates that AFB1 disrupts cell cycle and DNA repair process by dys-regulating genes such FOS, ADM, CDKN1A, GADD45A and activating transcription factor 6 (ATF6) in human primary trophoblast. In the dataset, one of the downregulated genes was FOS, which is an oncogene that belongs to AP-1 family of transcription factors and it is implicated in many tumours (Curran and Morgan, 1987; Milde-Langosch, 2005). FOS plays significant role in cell proliferation, cell cycle entry and progression (Angel and Karin, 1991; Brown et al., 1998; Christmann et al., 2006; Kovary and Bravo, 1991), similarly to ADM (Miyashita et al., 2003; Ouafik et al., 2009; Shichiri and Hirata, 2003; Withers et al., 1996). CDKN1A, and GADD45A are known regulators of apoptosis, cell cycle progression and DNA repair (Barreto et al., 2007; Duto et al., 2015; Kleinsimon et al., 2018). Also, ATF6 is known to play significant role in apoptosis, cell cycle arrest progression and steroid hormone production (Xiong et al., 2017). Consequently, our data supports the previous data that AFB1 plays a role on cell cycle arrest, progression, DNA damage checkpoint, and inhibition of DNA repair systems in different human and animal cell lines (Engin and Engin, 2019; Ricordy et al., 2002; Weng et al., 2017; Yin et al., 2016; Yip et al., 2017).
exhibited significant negative effects on placental endocrine function on transcript level including disruption of GH, CRH and ER-signalling. Although, the data is in line with previous studies reporting the endocrine disruptive effects of AFB1, the genes and the signalling pathways dysregulated by AFB1 in human term trophoblasts should be confirmed and studied further. In addition, these preliminary findings should be confirmed with concentrations reflecting real-life exposure to AFB1.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This paper belongs to the studies carried out by Kuopio Birth Cohort consortium (www. KuliCo.fi). We thank Ms. Pirjo Hänninen for expert laboratory assistance. We also thank the staff of the Department of Obstetrics and Gynecology in Kuopio University Hospital for skilful collection of the placental specimens.

Funding

This work was supported by the Finnish Cultural foundation.

Data availability

Complete microarray data sets are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database, accession number GSE153590.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2022.100082.

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