Supplementary file S1 related to:

Article title: Transcriptomic analysis in zebrafish larvae identifies iron-dependent mitochondrial dysfunction as a possible key event of NAFLD progression induced by benzo[a]pyrene/ethanol co-exposure

Journal: Cell Biology and Toxicology

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2. Materials and Methods

2.1. Zebrafish larvae handling and exposures

Animals were handled, treated and killed in agreement with the European Union regulations concerning the use and protection of experimental animals (Directive 2010/63/EU). All protocols were approved by local ethic committee CREEA (Comité Rennais d'Ethique en matière d'Expérimentation Animale). Zebrafish fertilized embryos—collected following natural spawning—were obtained from the Structure Fédérative de Recherche Biosit (INRA LPGP, Rennes, France). Embryos and larvae—sex unknown—were raised at 28°C according

to standard procedures and as previously described [8,10]. Briefly, from 4-dpf until last day of treatment and bath renewal—at 9-dpf—larvae were fed once daily with a high-fat diet (HFD; dried chicken egg yolk containing around 53% of fat; Sigma-Aldrich). At 5-dpf, larvae were exposed till 12 dpf with 43 mM ethanol and 25 nM B[a]P in dimethyl sulfoxide (DMSO; final proportion: 0.001% v/v; group BE) or by this vehicle only (group C: Control (DMSO)). For co-treatment experiments, 1 µM CH223191, 25 µM quercetin, 100 µM Vitamin E or 100 µM deferoxamine (Sigma-Aldrich, St. Louis, MO, USA), were respectively added along with toxicants.

2.2. Microarray experiments

a. RNA extraction, microarray hybridization and data processing

Whole larvae RNA samples were extracted from a pool of 25 zebrafish larvae using TRIzol reagent method (Thermofisher Scientific) and then purified on-column by DNAse digestion using a RNeasy Mini Kit (Qiagen, Courtaboeuf, France). Quantification of RNA was next performed using nanodrop ND-1000 spectrophotometer (Nano-Drop Technologies, Rockland, DE, USA). RNA integrity was assessed with Agilent RNA 6000 Nano kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only RNA with an RNA integrity number >9 was used for further analysis (2100 expert software, Agilent Technologies). Five RNA samples per each condition (Control, B[a]P, Ethanol and B[a]P/Ethanol) were collected. Each RNA sample was amplified and labelled using the Gene Chip ™ WT PLUS Reagent Kit; and then hybridized to GeneChip ™ Zebrafish Gene 1.0 ST array (ThermoFischer Scientific) according to manufacturer's procedures. Finally, microarrays were scanned, and images were analyzed and rigorously quality controlled for hybridization artefacts. As previously described[1]; the resulting .CEL files were processed using the oligo

package from R/Bioconductor[2]. Data were then normalized and corrected with the RMA method by using the Brainarray custom chip description files (version 22.0.0) for directly mapping Affymetrix probe to Entrez gene identifiers[3]. Raw data were uploaded to the NCBI Gene Expression Omnibus (GEO) repository under the accession number GPL16933[4]. The resulting lists of up- and down-regulated transcripts were uploaded to the TOXsIgN repository (https://toxsign.genouest.org) under the accession number TSP769.

b. Statistical filtration of differentially expressed genes and clustering

The statistical filtration of the differentially expressed genes (DEGs)was performed using the AMEN (Annotation, Mapping, Expression and Network) suite of tools[5]. Briefly, we first filtered genes with at median signal above the background expression cutoff (median signals for one gene expression ≥ overall signals median (6.42)) and an expression fold-change greater or equal to 1.3 between control and B[a]P/ethanol-exposed zebrafish. To define a set of genes displaying significant differential expression (F-value adjusted with the FDR method: *p value* ≤ 0.05), the empirical Bayes moderated t-statistics was performed using the Limma package [6]. The resulting DEGs were further partitioned into several clusters (for up- and down-regulated set of genes) by the k-means algorithm and integrating expression levels found in all 4 experimental conditions (control, each toxicant alone exposed group and together). These clusters were ordered according to peak expression levels in the 4 different exposure groups (control, B[a]P, Ethanol, B[a]P/Ethanol).

c. Functional analysis

The subsets of differentially expressed genes were used for functional analysis based on gene ontology enrichments (GOEA) (up- and down-regulated gene sets and expression pattern sets) implemented in AMEN to identify significantly enriched terms from the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases [5]. Briefly, a specific annotation term was considered enriched in a cluster, if the FDR-adjusted *p value* was ≤0.001 (Fisher exact probability).

The human homologs to the zebrafish differentially-expressed genes after B[a]P/ethanol co-exposure was retrieved based on HomoloGene researches (when 1 human gene reference report to several gene references in zebrafish, mean of zebrafish gene expression levels was taken for this 1 human gene expression). Then, the resulting human genes were uploaded into the IPA software (IPA, Ingenuity Systems, QIAGEN, available online at https://www.ingenuity.com) for analysis of Ingenuity canonical pathways, Ingenuity Toxicity lists and Ingenuity Tox functions analysis by comparison with the Ingenuity Knowledge Databases.

2.3. HepaRG cell culture, treatment and mRNA sampling

The generation of the deficient HepaRG cell line (herein referred to as knock-out for AhR) was extensively described by Bucher et al. [7]. Human HepaRG cell lines, wild-type or knock-out for AhR, were cultivated with supplementation in fatty acids (stearic and oleic acids, 150 µM each, 2 days in pretreatment and during 2 weeks of treatment; Sigma Aldrich, France), treated or not with B[a]P (2.5 µM) and ethanol (25mM) during 2 weeks, as previously described [7,8]. After treatment, mRNA sampling was performed as reported by Bucher et al. [7,8].

2.4. mRNA extraction from zebrafish larvae

After treatment of zebrafish larvae, mRNA samples were collected from pool of 10-20 whole zebrafish larvae using TRIzol reagent, as previously described [9].

2.5. Analysis of mRNA expression by RT-qPCR

mRNA expression analyses were performed by RT-qPCR, as previously defined [8]. Briefly, mRNA samples (1 µg) were subject to reverse-transcription using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Then, quantitative polymerase chain reaction (qPCR) (5 ng of cDNA per well) was performed using SYBR Green on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The reaction conditions and cycles were : one step at 50°C, 2min; one step at 95°C, 10min followed by 40 cycles including one step at 95°C, 15s; one step at 60°C, 50s and finally melt curve analysis was performed with a temperature gradient from 65°C to 95°C with an increment of 0.5°C each 5s. Cycles threshold were taken for analysis if lower than 32. mRNA expression was normalized by means of actb2, 18s and gapdh mRNA levels for zebrafish and HepaRG samples. The $\Delta\Delta$Ct method was used to indicate the relative expression of each selected gene. Sequences of the tested primers are provided in supplementary Table S5.

2.6. In vivo assessment of mitochondrial oxygen consumption

In order to evaluate oxygen consumption rate (OCR) of mitochondria in zebrafish larvae using Seahorse XFe24 Analyzer (Agilent Technologies), we used specific exposure conditions and adapted protocol from Raftery et al. [10]. Briefly, larvae were from 4 dpf as usual but exposed to toxicants for only one day (5 to 6 dpf) using higher concentrations (1 µM B[a]P and 173 mM ethanol) or vehicule only (DMSO, final proportion: 0.001% v/v). Following

treatment, larvae were anesthetized with 31.25 mg/L tricaine (MS-222, Sigma-Aldrich) in bath water (bath water composition reported in previous article [8]). Then, larvae were placed in well bottom of 24 multi-well plate for Seahorse (1 larva/well). Larvae were fixed in place using a grid insert, and volume of bath water was adjusted to 500 µL per well. Twenty min after anesthesia onset, larvae were placed in Seahorse XFe24 analyzer for assessment of OCR (28°C, 1 read per cycle of 4 min) using following phases and inhibitors: Phase 1 : 6 cycles (24 min); Phase 2 : addition of 2.5 µM FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), 8 cycles (32 min); Phase 3: addition of 6.25 mM NaN_3 (sodium azide), 20 cycles (80 min). Using Wave software (version 2.6.0, Agilent Technologies), OCR levels were analyzed in order to obtain basal, maximal & spare mitochondrial and non-mitochondrial respiration levels with at least 7 larvae per condition.

2.7. Transmission electronic microscopy (TEM)

After exposure, 12 dpf larvae were fixed in 2% paraformaldehyde + 2% glutaraldehyde in cacodylate buffer for 1 hour at room temperature. Then, after 3 washes in cacodylate buffer, larvae were impregnated in heavy metal solution (1% osmium tetroxide, 1,5% potassium ferrocyanide) for 1 hour. Next, samples were dehydrated with graded alcohol series following standard procedures and embedded in eponate resin. Following section cutting (0.5 µM on a Leica UC7 microtome) and staining (with toluidine blue), liver was localized by optical microscopy for further imaging by TEM. Afterwards, ultrathin sections of 70 nm were cut, collected on copper grids, poststained with 2% uranyl acetate solution and finally imaged using a JEOL 1400 transmission electron microscope (JEOL Co, Ltd, Tokyo, Japan) operated at 120 kV).

2.8. Biochemical assessment of heme metabolism-related compounds

To evaluate the content of heme, hemin and bilirubin, commercial kits (Heme Assay kit and Bilirubin Assay Kit from Sigma-Aldrich; Hemin Assay Kit from Abcam, Cambridge, UK) were used. Briefly, from a pool of 50 larvae, homogenized in 300 µL of PBS buffer, 100 µL of homogenate were used for heme detection, 2 µL for hemin test and 150 µL for bilirubin test, and procedures were performed according to manufacturer's instruction.

2.9. Lipid peroxidation assays

Assessment of lipid peroxidation in liver of 12 dpf larvae was performed by fluorescent microscopy using C11-Bodipy $^{581/591}$ (Molecular Probes, Life Technologies, Courtaboeuf, France), as previously described [11].

2.10. *In vivo* assessment of mitochondrial labile iron content

In order to estimate the level of labile iron (Fe^{2+}) content at 12 dpf, living larvae were incubated with 5 µM Mito-FerroGreen probe during 2h (Dojindo EU GmbH, Munich, Germany). After staining, larvae were euthanized and mounted in PBS solution for imaging on confocal microscope (LEICA DMI 6000 CS; Leica Microsystems, Wetzlar, Germany). Briefly, fluorescent intensities of Mito-FerroGreen in liver were acquired by laser excitation and photomultiplier tube (PMT) (excitation at 488 nm; PMT range 500-550 nm). Liver localization was also confirmed by imaging larva with transmitted light. Finally, quantification of fluorescent intensity of Mito-FerroGreen was performed using Fiji imaging processing software (ImageJ, [12]).

2.11. Histological liver damage evaluation

Histological staining of paraffin-embedded zebrafish larvae and counting of damaged liver cells were performed as previously described [8].

2.12. Statistical analysis

All values were given as mean ± SEM (standard error of the mean) from at least three independent experiments. Detailed data and sample size could be found in Table S6. All values were checked to pass Shapiro-Wilk normality test. To assess the effect of B[a]P/ethanol co-exposure (BE) toward HFD control (C), two-tailed Student t-tests were performed (Figure 2, 3, 5a to 5c, 6b). Differences were considered significant when $p < 0.05$ (*), $p< 0.01$ (**), $p< 0.001$ (***). Multiple comparisons among groups were performed using two-way analysis of variance (ANOVA) followed by Bonferroni post-tests using GraphPad Prism5 software (GraphPad Software, San Diego, CA, USA). * indicates a statistically significant effect of BE co-exposure *vs* control counterpart [BE vs control; CH223191+BE vs CH223191 alone (Figure 4a-d, f,g; Figure 7); Quercetin+BE vs Quercetin alone (Figure 5d,e); Vitamin E+BE vs vitamin E (Figure 5d,e); Deferoxamine+BE vs Deferoxamine alone (Figure 6)] with $p<0.05$; § indicates a statistically significant effect of inhibitors/antioxidants *vs* control counterpart [CH223191 vs control and CH223191+BE vs BE (Figure 4a-d,f,g; Figure 7); Quercetin vs control, Quercetin+BE vs BE, Vitamin E vs control, Vitamin E+BE vs BE (Figure 5d,e); Deferoxamine vs control and Deferoxamine+BE vs BE (Figure 6)]with $p<0.05$; # indicates a statistically significant interaction between co-exposure and inhibitors/antioxidants (Figure 4a-d, f, g, 5d,e, 6, 7) or between co-exposure and AhR knock-out (Figure 2e) with $p<0.05$.

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