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A dual mixture of persistent organic pollutants modifies carbohydrate metabolism in the human hepatic cell line HepaRG

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Running title: a POP mixture decreases hepatic carbohydrate metabolism

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Abstract

Individuals as well as entire ecosystems are exposed to mixtures of Persistent Organic Pollutants (POPs). Previously, we showed, by a non-targeted approach, that the expression of several genes involved in carbohydrate metabolism was almost completely inhibited in the human hepatic cell line HepaRG following exposure to a mixture of the organochlorine insecticide alpha-endosulfan and 2,3,7,8 tetrachlorodibenzo-p-dioxin. In this European HEALS project, which studies the effects of the exposome on human health, we used a Physiologically Based BioKinetic model to compare the concentrations previously used *in vitro* with *in vivo* exposures for humans. We investigated the effects of these POPs on the levels of proteins, on glycogen content, glucose production and the oxidation of glucose into CO₂ and correlated them to the expression of genes involved in carbohydrate metabolism as measured by RT-qPCR. Exposure to individual POPs and the mixture decreased the expression of the proteins investigated as well as glucose output (up to 82%), glucose oxidation (up to 29%) and glycogen content (up to 48%). siRNAs that specifically inhibit the expression of several xenobiotic receptors were used to assess receptor involvement in the effects of the POPs. In the HepaRG model, we demonstrate that the effects are mediated by the Aryl Hydrocarbon Receptor and the estrogen receptor alpha, but not the pregnane X receptor or the constitutive androstane receptor. These results provide evidence that exposure to combinations of POPs, acting through different signalling pathways, may affect, more profoundly than single pollutants alone, metabolic pathways such as carbohydrate/energy metabolism and play a potential role in pollutant associated metabolic disorders.

Key words: pesticide, carbohydrate metabolism, dioxin, alpha-endosulfan, pollutant mixture, liver

1. Introduction

Persistent organic pollutants (POPs) raise specific concerns since they resist degradation, persist in the environment for long periods of time, even after being banned, and are found in isolated regions or areas distant from their sources. They accumulate throughout the food chain because of their lipophilicity and are stored mainly in adipose tissue and liver. Moreover, several of the POPs act as endocrine disruptors and, thus, may modify the normal physiology of the organism. Increasing epidemiological and toxicological evidence suggests that POPs can contribute (along with other obvious factors such as a sedentary lifestyle and diet) to the increase in incidence of several pathologies, including diabetes and obesity, both in western and emerging countries (Lee et al., 2011; Magliano et al., 2014; Taylor et al., 2013). More importantly, in animal models, long term multi-exposure to low levels of endocrine disruptors triggers energy and metabolic disorders, sometimes in a sex-dependent manner (for review, (Le Magueresse-Battistoni et al., 2017, 2018)).

Whereas the effects of members of each family of contaminants have been studied extensively alone, little is known about the effects of combinations, with the notable exception of those sharing the same mechanism of action, such as dioxin-like compounds (Desaulniers et al., 2005; Howard et al., 2010; Thayer et al., 2012), or pesticides (Rizzati et al., 2016). Since the general population is exposed to low levels of complex mixtures of pollutants, acting through different pathways, more attention needs to be paid to the effects of such combinations on human health (Kortenkamp et al., 2007). Determination of the toxicity of complex combinations of pollutants, which contribute to the exposome (*i.e.* the totality of human environmental exposures from conception onwards), has become a challenge for modern toxicology (Cui et al., 2016; Wild, 2005) and it is one of the aims of the European project HEALS. Nevertheless, only a few recent studies have focused on the effects of

complex mixtures of POPs in various models (Gill et al., 2013; Lyche et al., 2013; Merhi et al., 2010).

In humans, the liver is one of the primary targets as well as a storage site of POPs (Watanabe et al., 2013). Most studies use animal models, which imperfectly reflect the human species (Okey, 2007; Seok et al., 2013). In the present study, we used the human liver-derived cell line, HepaRG, a recognized model for the human hepatocyte for the study of both drug metabolism (Anthérieu et al., 2010) and intermediary metabolism (Rogue et al., 2012; Samanez et al., 2012). This cell line expresses high levels of xenobiotic metabolizing enzymes and xenosensors (Guillouzo et al., 2007) the expression of which remains stable for several weeks (Jossé et al., 2008; Kanebratt and Andersson, 2008). It expresses also genes which encode important proteins involved in carbohydrate, lipid and lipoprotein metabolisms (Tascher et al., 2019).

We studied two model POPs which are also endocrine disruptors, 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and α -endosulfan, the most toxic isomer of this organochlorine (OC) insecticide. OC pesticides are among the most widespread contaminants in the environment and endosulfan, in spite of its restriction and ban in the European Union, is still used in China and India and residues are detected in foods (European Union Report on Pesticide Residues in Food, 2011; Lozowicka et al., 2015) and in humans (Monteagudo et al., 2016). It is known also that dioxins may contaminate chlorinated industrial products such as pesticides, fungicides or herbicides (Schechter et al., 2006) and, thus, that TCDD and endosulfan can be found as a mixture.

These two POPs have distinct mechanisms of action. TCDD is a well known activator of the Aryl Hydrocarbon Receptor (AHR) signaling pathway (Guyot et al., 2013). α -endosulfan has been described as an activator of either the pregnane X receptor (PXR), the

estrogen receptor (ER) and to a lesser extent the constitutive androstane receptor (CAR) (Casabar et al., 2006; Coumoul et al., 2002; Lemaire et al., 2006; Savary et al., 2014). Moreover, in a previous study in human mammary cell lines expressing the AHR and the ER, we showed that the combination of TCDD and α -endosulfan led to a decrease of ethoxyresorufin-*O*-deethylase activity (CYP1 activity) and of the level of CYP1A1 mRNA as compared to TCDD alone without any modification of the mRNA level of the other AHR target CYP1B1 (Coumoul et al., 2001). Therefore, the same combination of POPs may have different effects, as compared to each pollutant alone, on the same endpoints.

Using a non-targeted whole genome transcriptome analysis, we showed that the expression of several genes (particularly the bi-directional glucose transporter GLUT2 (SLC2A2) and the Glucose-6-Phosphatase catalytic subunit (G6PC)) which encode proteins involved in carbohydrate metabolism was drastically decreased in HepaRG cells following exposure for 30 h to a combination of 25 nM TCDD and 10 μ M α -endosulfan (Ambolet-Camoit et al., 2015). Neither the metabolic consequences of these effects on intermediary metabolism nor the contribution of each contaminant in this deregulation is known. We first compared the concentrations of POPs used *in vitro* with environmental and accidental doses found in humans. We then demonstrated that the dual mixture decreases glucose production and oxidation as well as cellular glycogen content. We showed that these endpoints are modified by TCDD through the AHR pathway and that the ER α is involved in the α -endosulfan effect.

2. Materials and methods

2.1. Internal dose modeling and dose selection

Selection of doses to be used for treatment of the cell line was based on translating external intakes of TCDD and α -endosulfan into liver tissue dosimetry. We developed a

generic Physiologically Based BioKinetic (PBBK) model, which incorporates life stage changes and physiological and metabolic efficiency changes over an individual's lifetime (from conception till 80 years of age), to associate external intake estimates or plasma biomonitored levels into target tissue (liver cells) dosimetry. Relevant external intakes of TCDD and α -endosulfan were determined through the use of the PBBK model (Supplementary Methods) and the respective internal doses in hepatic cells were estimated based on an extensive review of exposure conditions, so as to identify the ranges of exposure levels. The review process included representative environmental, accidental and occupational conditions, so as to estimate the upper bounds of the plausible intake levels, which in turn were translated into internal doses in the hepatic cells.

2.2. Compounds and cell culture

TCDD and α -endosulfan were obtained from LGC Standards (France). HepaRG cells (Dr Guguen-Guillouzo, Rennes, France) were cultured in Williams' E medium supplemented as described (Aninat et al., 2006) and differentiated with 1.5% DMSO (Attignon et al., 2017a), designated differentiation medium. The differentiated cells were treated with 25 nM TCDD, 10 μ M α -endosulfan or the mixture for 24 or 30 h and 72 h, respectively, for mRNA and protein assays. In some instances, studies of gene expression and metabolic flux were performed under "gluconeogenic" conditions (Supplementary Methods). Time course experiments are described in the Supplementary Methods.

2.3. Glucose production assay

Differentiated HepaRG cells were seeded into 6-well plates. After 66 h of exposure to the POPs in differentiation medium, the cells were washed with PBS and incubated in the "gluconeogenic" medium (Supplementary Methods). After 6 h of incubation in this medium in the presence of the pollutants, the supernatant was collected and glucose production was

measured with the Glucose (GO) Assay kit (Gago-2) from Sigma. Proteins were collected from the same wells using RIPA buffer and their concentrations measured as previously described (Blouin et al., 2011).

2.4. Measurement of glucose oxidation and lactate production

Differentiated HepaRG cells, seeded into 6-well plates, were exposed 72 h to the POPs, washed with PBS, trypsinized, resuspended in differentiation medium and counted. They were centrifuged and resuspended in Krebs-Ringer phosphate buffer to obtain 2 million cells in 950µL. They were then incubated for 90 min at 37°C in a total volume of 1 mL of Krebs-Ringer phosphate buffer, containing 5mM U-¹⁴C-glucose (250µCi/µmol) and the reaction was performed as described previously (Blouin et al., 2011). The amount of protein, for each condition, was measured as described (Blouin et al., 2011). Lactate production was quantified as described in the Supplementary Methods.

2.5. Measurement of intracellular glycogen content

Differentiated HepaRG cells, seeded into 12-well plates for 5 days, were exposed for 66 h to the POPs. The cells were scraped into 150µL water after 3 washings in ice-cold PBS, heated at 100°C for 5 min and centrifuged at 12,000 g for 5 min. The supernatant was frozen at -80°C until measurement of the glycogen content using the glycogen assay kit (MAK016 Sigma) as described by the manufacturer. The glycogen content was normalized to the amount of protein assayed as described above.

2.6. siRNA transfection, RNA isolation, reverse transcription, qPCR and immunoblotting.

siRNAs and the transfection protocol, preparation of RNA samples, reverse transcription, PCR and western blot experiments are described in the Supplementary Methods.

Oligonucleotides (Supplementary Table 1) were obtained from the literature or designed using the OLIGO Explorer software and their efficiency was assessed.

2.7. Statistical analysis

The data are expressed as mean \pm SD of at least three different experiments. Statistical analysis was carried out in R (*agricolae* package) using the Kruskal–Wallis test (non parametric comparison of k independent series) followed by a *post hoc* test (Dunn). The p values are indicated as: *, \$, # p < 0.05; **, \$\$, ## p < 0.01; ***, \$\$\$, ### p < 0.001.

3. Results

3.1. TCDD and α -endosulfan doses are based on realistic exposure estimates

In order to estimate how the concentrations used in this study and in our previous transcriptome analysis (25 nM TCDD and 10 μ M α -endosulfan) (Ambolet-Camoit et al., 2015) compared with human exposure, a Physiologically Based BioKinetic (PBBK) model was developed (Figure 1).

For environmental exposure to TCDD, estimates of daily intake of polychlorodibenzo-dioxins/furanes (PCDDs/PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) TEQ (Toxic Equivalent concentration) were used, assuming that the sum of these compounds exerts a similar toxicological response (mode of action) to TCDD. Hence, treating the cells with a given concentration, A, of TCDD, is similar to treating the cells with a PCDDs/PCDFs mixture of the TEQ concentration equal to A. The detailed reconstructed exposure is described in the Supplementary Results. By reconstructing human intake from these data and accounting for inter-individual variability in biokinetics (Bois et al., 2010), intake levels up to 15 pg/kg_bw/d TEQ were estimated. Additional biomonitoring data were obtained from studies related to the accidental exposure to PCDD/Fs/dl-PCBs in Seveso (Italy) in 1976

(Kerger et al., 2006; Warner et al., 2014). In this case, the highest serum levels observed correspond to an intake of up to 15 ng/kg_bw/d TEQ which, in turn, corresponds to an internal dose in the liver cells of up to 25 nM TCDD. Taking into account all the above, it was decided to select this as a relevant dose for the *in vitro* studies, although lower concentrations (0.2-5 nM) also were used.

For endosulfan, a review of the respective intake levels for the general population and pesticide sprayers was carried out. For the general population, the intake of endosulfan is close to 1 ng/kg_bw/d. However, for upper bound scenarios of crop contamination and diets rich in crops sprayed with endosulfan, intake levels are up to 800 ng/kg_bw/d (Nougadère et al., 2012). To estimate the highest feasible (yet realistic) intake of endosulfan, studies involving exposure to applicators also were reviewed. Exposure to applicators was up to 150 µg/kg_bw/d (Dalvie et al., 2009; Skrbić and Predojević, 2008) which corresponds to an internal dose in hepatic cells close to 10 µM. This was the highest dose chosen for *in vitro* testing, although concentrations of 1 and 3 µM also were used. The correspondence between the equivalent external dose used for testing in the hepatic cells and environmental, occupational and accidental exposures is illustrated in Figure 1.

3.2. TCDD and α -endosulfan disrupt the expression of genes involved in glucose oxidation and storage

Exposure of HepaRG cells for 30 h to the POPs alone or as a mixture altered the mRNA levels of several genes involved in the glycolysis/gluconeogenesis pathway (Ambolet-Camoit et al., 2015). When HepaRG cells were cultivated in the presence of 11mM glucose (differentiation medium) and exposed for 30 h to the mixture of POPs, the glucose transporter GLUT2 mRNA expression decreased up to 90% (Figure 2A), and the protein level decreased

up to 55% (Figure 2B and C). Exposure to the POPs altered the mRNA expression of several other genes involved in glycolysis, glucose storage and the tricarboxylic acid cycle (hexokinase 2 (HK2), liver pyruvate kinase (PKLR), pyruvate dehydrogenase kinase 2 (PDK2) and glycogen synthase 2 (GYS2)) (right part of Supplementary Table 2). Thus, we assessed POP effects on the oxidative capacities of HepaRG cells by measuring the release of radioactive $^{14}\text{CO}_2$ from radioactive U- ^{14}C -glucose *via* the Krebs cycle. Glucose oxidation was not affected by TCDD. In contrast, exposure of the cells to α -endosulfan or to the mixture decreased CO_2 production by 20 and 29%, respectively, compared to the unexposed cells (Figure 2D). Concomitantly, there was no modification in the level of lactate following the treatments (Figure 2E). Since the level of GYS2 mRNA was decreased after exposure to the pollutants, we measured the level of glycogen in the cells after exposure to the POPs. The glycogen content of the cells was significantly reduced following exposure to TCDD, α -endosulfan or the mixture (by 33, 39 and 48% respectively), as compared to untreated cells (Figure 2F).

3.3. Effects of pollutants are time and concentration-dependent

HepaRG cells were then exposed to 25 nM TCDD, 10 μM α -endosulfan or to their combination for various times. Significant decreases in the expression of the most down-regulated genes in the microarray experiment, G6PC and GLUT2, were observed as early as 8h after exposure to the POPs with no modification at 4h (Figure 3).

The PBBK model showed that the concentrations used in this study correspond to the highest doses found during occupational and accidental exposures (Figure 1). In order to employ experimental conditions closer to environmental levels, HepaRG cells were exposed for 8 days to lower concentrations of the POPs (0.2 to 5 nM TCDD and 1, 3 or 10 μM for α -endosulfan alone and to mixtures), with no observed toxicity (Ambolet-Camoit et al., 2015).

Remarkably, G6PC and GLUT2 mRNA expression decreased up to 50% for the lowest concentrations of TCDD (0.2 nM) and to 37% (although not statistically significant) for α -endosulfan, (3 μ M) (Supplementary Figure 1). Exposure of cells to 1 μ M of α -endosulfan led to a 35% decrease (although not statistically significant) in G6PC expression with no modification in GLUT2 expression (data not shown). Moreover, the mixture has more significant effects than each pollutant alone for several concentrations (Supplementary Figure 1).

3.4. Exposure to TCDD and α -endosulfan decreases both glucose production and the expression of genes coding for enzymes involved in gluconeogenesis

In the transcriptomic study, the level of G6PC mRNA, the last enzyme in gluconeogenesis, was decreased up to 90% by the mixture of the POPs. We studied the molecular and metabolic effects of 25 nM TCDD, 10 μ M α -endosulfan or the mixture on HepaRG cells exposed to the pollutants under gluconeogenic conditions (no glucose medium, Supplementary Methods). The expression of G6PC mRNA decreased drastically with the mixture (90%) as compared to TCDD or α -endosulfan alone (55% and 75% decreases, respectively) (Figure 4A). GLUT2 mRNA expression also decreased with all the treatments (up to 70% with the mixture, Figure 4B). We measured the mRNA level of other enzymes involved in gluconeogenesis (left part of Supplementary Table 2). The mRNA expression of the cytosolic phosphoenolpyruvate carboxykinase (PCK1) and of the mitochondrial form (PCK2), recently described as playing a role in gluconeogenesis (Stark et al., 2014), was decreased by all the treatments (up to 80% by the mixture). There were also decreases, albeit less pronounced, in the amount of PCK2 protein for all treatments (Supplementary Figure 2, A, B). The amounts of PCK1 and G6PC proteins decreased only for treatment with TCDD or the mixture (Supplementary Figure 2, C, D and E, F).

We then measured the production of glucose, which was decreased by 76%, 74% and 82%, respectively, following exposure for 72 h to TCDD, α -endosulfan or the mixture, as compared to cells cultivated in the absence of the pollutants (Figure 4C).

3.5. The TCDD-induced repression of G6PC, GLUT2 and glucose production is mediated through the AHR

TCDD is known to activate the AHR/ARNT pathway as well as the non-genomic SRC pathway (Guyot et al., 2013). Upon transfection with siRNA directed against AHR and ARNT the decreases of the amounts of AHR and ARNT proteins were 80% and 60%, respectively, compared to the levels observed with the control siRNA as described previously (Attignon et al., 2017b). These decreases were accompanied by a decrease in the effect of TCDD on the expression of both the G6PC and GLUT2 genes as compared to the siRNA control (siCtl) (Figure 5A and B and supplementary Figure 3A, B). The non-genomic SRC pathway was not involved in the regulation of these two genes (Supplementary Figure 3A, B). Thus, the effect of TCDD is mediated by the AHR/ARNT pathway. Transfection with the siAHR, as compared to the siCtl, did not modify the production of glucose in untreated cells (2.57 μ g/mL/mg protein) whereas it partially reversed the decrease in glucose production after TCDD treatment (78% inhibition with siCtl (0.57 μ g/mL/mg protein) and 43% inhibition with siAHR (1.44 μ g/mL/mg protein)) (Figure 5C). Since the role of TCDD-inducible poly(ADP-ribose) polymerase, PARP7 (TiPARP) in the TCDD-induced inhibition of gluconeogenesis *via* G6PC seems to be opposite in the mouse and chick embryo models (Diani-Moore et al., 2010, 2013; MacPherson et al., 2013) we tested its role in the human HepaRG model. The siRNA-mediated loss of TiPARP further decreased the expression of G6PC and GLUT2 as compared to the siCtl, in the presence of TCDD (Supplementary Figure 4).

3.6. The α -endosulfan-induced repression of G6PC, GLUT2 and glucose production is partially mediated through the ER α

α -endosulfan has been described to act through several receptors. Although PXR mRNA and protein expression and the level of its target gene CYP3A4 were decreased effectively by siPXR transfection (Attignon et al., 2017b), there was no modification of the inhibition of the expression of G6PC and GLUT2 following exposure of cells to α -endosulfan (Supplementary Figure 5, A, B). Then, we investigated whether the ER α was implicated in the α -endosulfan effect. We previously showed that siER α , decreases ER α mRNA content by 75% (Attignon et al., 2017b). ER α silencing reversed partially the effect of α -endosulfan on G6PC in the presence of the pesticide (Figure 5D) although there was a 66% increase of the basal level of expression. For GLUT2, there was no change in the basal level of expression following transfection with the siER α compared to the control siRNA and the siER α abolished the α -endosulfan effect on this gene (Figure 5E).

The role of ER α on glucose production was then assessed. Treatment with α -endosulfan of HepaRG cells transfected with the siCtl decreased glucose production by 78% (0.43 μ g/mL/mg protein compared to 1.95 μ g/mL/mg protein). When the siER α was transfected, the basal glucose production was decreased by 45% (1.06 μ g/mL/mg protein) compared to the siCtl. Further treatment with α -endosulfan decreased the glucose production to the same level as the siCtl (0.43 μ g/mL/mg protein) (Figure 5F). Since it is difficult to draw a definitive conclusion from these results, we investigated whether another xenosensor, CAR, had a role in the α -endosulfan effect. siCAR transfection did not change G6PC and GLUT2 expression (Supplementary Figure 5C, D) even though its siRNA abolished CYP2B6 mRNA expression as shown previously (Attignon et al., 2017b).

Discussion

The liver is the main organ involved in detoxification of xenobiotics and a major target of these compounds. In the present study, we investigated the effects of a combination of two persistent organic pollutants also acting as endocrine disruptors on carbohydrate metabolism in the human liver-derived cell line, HepaRG. We used an organochlorine pesticide, α -endosulfan, and the most toxic dioxin, TCDD. Previously, we reported drastic reductions of the levels of mRNAs encoding GLUT2 (SLC2A2) and key enzymes of glycolysis (PKLR) and gluconeogenesis (G6PC) in response to the combination of these pollutants in this hepatic cell line (Ambolet-Camoit et al., 2015). Therefore, we studied the effects of this mixture on glucose metabolism and measured metabolic fluxes. Glucose oxidation and glucose production as well as the cellular glycogen content were decreased by the mixture. Other pollutants have similar effects on carbohydrate metabolism. For example, bisphenol S and AF also decrease liver glycogen content in mice (Meng et al., 2018) and chlorpyrifos decreases it in fish (Majumder and Kaviraj, 2018). Bisphenol A and a phthalate (DEHP) also decrease glucose oxidation in rat liver (Jayashree et al., 2013; Rajagopal et al., 2019). In our model, the expression of key genes for either glycolysis or gluconeogenesis is affected similarly by the POPs, whatever the amount of glucose in the culture medium. These results highlight the predominant role of POPs on carbohydrate metabolism compared to the nutritional status of the cells.

As expected, TCDD acted through the AHR/ARNT pathway, since inhibition of expression of the AHR interfered with the action of TCDD on mRNA levels and glucose production. Then, we tested the role of TiPARP in the regulation of G6PC and GLUT2 after treatment of HepaRG cells with TCDD, since opposing results were obtained in the chicken and mouse models concerning the AHR signaling (Diani-Moore et al., 2010; MacPherson et al., 2013). We observed that decreased expression of TiPARP by siRNA almost abolished the

expression of G6PC and GLUT2 in the presence of TCDD. This is in line with the observation that the loss of TipARP in a mouse KO model increased the sensitivity of mice to the TCDD treatment due to the loss of mono-ADP-ribosylation of AHR (which leads to AHR degradation) (Ahmed et al., 2015). The stabilization of AHR in the KO animals thus increased steatosis and hepatotoxicity and the inhibition of PCK1 mRNA expression by TCDD.

Concerning the mode of action of α -endosulfan, our results showed that the ER α is partly responsible of the inhibition of gene expression with no implication of PXR or CAR. However, the measures of glucose production did not provide a definitive conclusion since the siER α further decreased (by 50%) the basal glucose production found in the presence of the control siRNA. Despite this result, the involvement of the ER α , at least partially, in the effect of α -endosulfan in the HepaRG model, seems likely, on the basis of the mRNA results and of a comparison of our results with a transcriptome analysis (Jennen et al., 2010) in which the effect of 30 μ M 17 β -estradiol (E2) on HepaRG cells was studied. Pathways down-regulated in that study (bile acid biosynthesis, glycolysis and gluconeogenesis, regulation of lipid metabolism-FXR dependent) were also down-regulated in our analysis (Ambolet-Camoit et al., 2015). We selected 43 genes differentially expressed in our study (Supplementary Table 3). The expression of these genes from both studies was positively correlated ($R^2=0.64$) (Supplementary Figure 6). After exposure of cells to E2 or α -endosulfan, expression of G6PC decreased to 19 and 25%, respectively, and GLUT2 expression decreased to 31 and 58%, respectively, of the control values. Thus, E2 and α -endosulfan would appear to affect expression of these genes in the HepaRG cells in a similar fashion.

Complex cross-talk with opposite effects between the AHR and the ER have been described, i.e. the recruitment of ER to AHR activated genes to the detriment of ER target genes which lead to ER degradation by the proteasome and either an inhibition or a slight activation of AHR targets after estradiol treatment (Matthews and Gustafsson, 2006; Tarnow

et al., 2019). In contrast, in our study, we observed an effect of the mixture in the same direction as those of each pollutant alone. Since the crosstalk studies were performed with E2, it is difficult to extrapolate how AHR activation by TCDD could influence the ER activation by α -endosulfan in our cell model. Indeed, E2 and α -endosulfan have different metabolisms which could explain the absence of the negative crosstalk described in the literature. In our previous transcriptomic study (Ambolet-Camoit et al., 2015), we also observed effects on mRNA expression mainly in the same direction by each pollutant alone and by the mixture. However, of 32 genes for which selective inhibitory crosstalk between these pollutants was found, the addition of TCDD inhibited the pesticide effect for 10 genes and α -endosulfan inhibited the effect of TCDD in 22 genes (see figure 2 and discussion in (Ambolet-Camoit et al., 2015)).

A metabolomic study of mice exposed to a low dose of endosulfan in the diet revealed gender-specific modifications of energy metabolism. Increased plasma glucose levels were found in both sexes but the relationship to the liver glucose content was different. For the females, the increase in plasma glucose was linked to a decreased hepatic glucose level whereas no such decrease was observed in the males. In the latter case, there was a decreased hepatic lactate level and the authors suggested a decrease in the hepatic glycolytic pathway (Canlet et al., 2013). This study and another one on the effect of a mixture of pesticides (Merhi et al., 2010) also showed that female and male mice respond differently to a contaminant exposure. Concerning the AHR activation, a comparative analysis of both the hepatic metabolome and transcriptome was performed in mice and rats treated with TCDD (Forgacs et al., 2012). It showed that lipid metabolism was altered in mice, consistent with increased hepatic triglyceride and fatty acid accumulation. In contrast, rats did not accumulate lipids at the same level whereas choline metabolism was modified with an increase in phosphocholine and a decrease of betaine. This decrease could be linked to type 2 diabetes as

betaine supplementation improves glucose homeostasis in mice with diet-induced obesity (Ejaz et al., 2016). Betaine and choline supplementation also improves the placental response to gestational diabetes mellitus induced in mice fed a high-fat diet (Nanobashvili et al., 2018). This treatment also significantly reduced glucose accumulation in human placenta-derived BeWo cells under high or normal glucose culture conditions (Nanobashvili et al., 2018). In addition, exposure of mice with a diet-induced liver injury to PCB126 (a dioxin-like compound) worsens hepatic fibrosis and induces metabolic disturbances due to modifications in hepatic gene expression (including the neoglucogenic genes G6PC and PCK1) leading to dysfunctional energy homeostasis (Wahlang et al., 2017). However, the authors did not validate these effects in an AHR KO model.

Other metabolomic/transcriptomic studies of the effects of TCDD on mice revealed changes in carbohydrate metabolism, with modifications of gene expression similar to our results (this study and M Aggerbeck, personal results) for a selection of genes (Nault et al., 2016b, 2016a). However, there are also differences in the expression of other genes between mice and HepaRG cells and it is known that the animal models (mainly rodents) extrapolate imperfectly to humans (Forgacs et al., 2012, 2013; Seok et al., 2013). Although the HepaRG cell culture model does not reproduce the complexity of the whole organism, it is still a useful tool for studies in a human model and comparison with *in vivo* experiments.

4. Conclusion

Our study is one of the first to address the question of the effect of a dual exposure to contaminants acting *via* different molecular pathways on carbohydrate/energy metabolism in a human cellular model. Indeed, most previous studies on the effect of a cocktail of pollutants in liver have used diverse non human species (Le Magueresse-Battistoni et al., 2018).

The simultaneous use of human and animal models, in conjunction with epidemiological studies and PBBK models, such as the one described in this study, are necessary to grasp the real risks of exposure to a variety of chemicals for humans throughout their lives. Many epidemiological studies have attempted to assess the link between human exposure to contaminants, which usually occurs at low levels throughout life, and the increased epidemic levels of the metabolic syndrome and type 2 diabetes (Cranmer et al., 2000; Lee et al., 2011; Magliano et al., 2014; Son et al., 2010). As recently reviewed, no obvious dependent relationship between exposure to POPs and diabetes in the general population is observed whereas most studies on occupational or non-occupational high risk cohorts suggest an increased risk of diabetes with past exposure to dioxins/PCBs and/or herbicides (Magliano et al., 2014). However, the authors also point out that, because of the high prevalence of diabetes and of the inevitable exposure to POPs, even a very small increased risk of disease in susceptible populations could have major public health significance. It is clear that more epidemiological studies are necessary together with the study of various biological models to decide if a causal link does or does not exist between life-long multiple-exposures to POPs and the epidemic increase in metabolic pathologies in humans, especially in the context of alcohol or diet-induced fatty liver disease.

The Supplementary Data contains:

- 1) a file which includes a Methods section, a Result section describing the PBBK model in more details, a Supplementary Reference section and the Legends to the Supplementary Figures
- 2) a file with 6 Supplementary Figures
- 3) a file with 3 Supplementary Tables

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References

- Ahmed, S., Bott, D., Gomez, A., Tamblyn, L., Rasheed, A., Cho, T., MacPherson, L., Sugamori, K.S., Yang, Y., Grant, D.M., et al. (2015). Loss of the Mono-ADP-ribosyltransferase, Tiparp, Increases Sensitivity to Dioxin-induced Steatohepatitis and Lethality. *J. Biol. Chem.* 290, 16824–16840.
- Ambolet-Camoit, A., Ottolenghi, C., Leblanc, A., Kim, M.J., Letourneur, F., Jacques, S., Cagnard, N., Guguen-Guillouzo, C., Barouki, R., and Aggerbeck, M. (2015). Two persistent organic pollutants which act through different xenosensors (alpha-endosulfan and 2,3,7,8 tetrachlorodibenzo-p-dioxin) interact in a mixture and downregulate multiple genes involved in human hepatocyte lipid and glucose metabolism. *Biochimie* 116, 79–91.
- Aninat, C., Piton, A., Glaise, D., Le Charpentier, T., Langouët, S., Morel, F., Guguen-Guillouzo, C., and Guillouzo, A. (2006). Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab. Dispos. Biol. Fate Chem.* 34, 75–83.
- Anthérieu, S., Chesné, C., Li, R., Camus, S., Lahoz, A., Picazo, L., Turpeinen, M., Tolonen, A., Uusitalo, J., Guguen-Guillouzo, C., et al. (2010). Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. *Drug Metab. Dispos. Biol. Fate Chem.* 38, 516–525.
- Attignon, E.A., Leblanc, A.F., Le-Grand, B., Duval, C., Aggerbeck, M., Rouach, H., and Blanc, E.B. (2017a). Novel roles for AhR and ARNT in the regulation of alcohol dehydrogenases in human hepatic cells. *Arch. Toxicol.* 91, 313–324.
- Attignon, E.A., Distel, E., Le-Grand, B., Leblanc, A.F., Barouki, R., de Oliveira, E., Aggerbeck, M., and Blanc, E.B. (2017b). Down-regulation of the expression of alcohol dehydrogenase 4 and CYP2E1 by the combination of α -endosulfan and dioxin in HepaRG human cells. *Toxicol. Vitro Int. J. Publ. Assoc. BIBRA*.
- Blouin, J.-M., Penot, G., Collinet, M., Nacfer, M., Forest, C., Laurent-Puig, P., Coumoul, X., Barouki, R., Benelli, C., and Bortoli, S. (2011). Butyrate elicits a metabolic switch in human colon cancer cells by targeting the pyruvate dehydrogenase complex. *Int. J. Cancer J. Int. Cancer* 128, 2591–2601.
- Bois, F.Y., Jamei, M., and Clewell, H.J. (2010). PBPK modelling of inter-individual variability in the pharmacokinetics of environmental chemicals. *Toxicology* 278, 256–267.
- Canlet, C., Tremblay-Franco, M., Gautier, R., Molina, J., Métais, B., Blas-Y Estrada, F., and Gamet-Payraastre, L. (2013). Specific metabolic fingerprint of a dietary exposure to a very low dose of endosulfan. *J. Toxicol.* 2013, 545802.
- Casabar, R.C.T., Wallace, A.D., Hodgson, E., and Rose, R.L. (2006). Metabolism of endosulfan-alpha by human liver microsomes and its utility as a simultaneous in vitro probe for CYP2B6 and CYP3A4. *Drug Metab. Dispos. Biol. Fate Chem.* 34, 1779–1785.

- Coumoul, X., Diry, M., Robillot, C., and Barouki, R. (2001). Differential regulation of cytochrome P450 1A1 and 1B1 by a combination of dioxin and pesticides in the breast tumor cell line MCF-7. *Cancer Res.* *61*, 3942–3948.
- Coumoul, X., Diry, M., and Barouki, R. (2002). PXR-dependent induction of human CYP3A4 gene expression by organochlorine pesticides. *Biochem. Pharmacol.* *64*, 1513–1519.
- Cranmer, M., Louie, S., Kennedy, R.H., Kern, P.A., and Fonseca, V.A. (2000). Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is associated with hyperinsulinemia and insulin resistance. *Toxicol. Sci. Off. J. Soc. Toxicol.* *56*, 431–436.
- Cui, Y., Balshaw, D.M., Kwok, R.K., Thompson, C.L., Collman, G.W., and Birnbaum, L.S. (2016). The Exposome: Embracing the Complexity for Discovery in Environmental Health. *Environ. Health Perspect.* *124*, A137-140.
- Dalvie, M.A., Africa, A., Solomons, A., London, L., Brouwer, D., and Kromhout, H. (2009). Pesticide exposure and blood endosulfan levels after first season spray amongst farm workers in the Western Cape, South Africa. *J. Environ. Sci. Health B* *44*, 271–277.
- Desaulniers, D., Cooke, G.M., Leingartner, K., Soumano, K., Cole, J., Yang, J., Wade, M., and Yagminas, A. (2005). Effects of postnatal exposure to a mixture of polychlorinated biphenyls, p,p'-dichlorodiphenyltrichloroethane, and p-p'-dichlorodiphenyldichloroethene in prepubertal and adult female Sprague-Dawley rats. *Int. J. Toxicol.* *24*, 111–127.
- Diani-Moore, S., Ram, P., Li, X., Mondal, P., Youn, D.Y., Sauve, A.A., and Rifkind, A.B. (2010). Identification of the aryl hydrocarbon receptor target gene TiPARP as a mediator of suppression of hepatic gluconeogenesis by 2,3,7,8-tetrachlorodibenzo-p-dioxin and of nicotinamide as a corrective agent for this effect. *J. Biol. Chem.* *285*, 38801–38810.
- Diani-Moore, S., Zhang, S., Ram, P., and Rifkind, A.B. (2013). Aryl hydrocarbon receptor activation by dioxin targets phosphoenolpyruvate carboxykinase (PEPCK) for ADP-ribosylation via 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible poly(ADP-ribose) polymerase (TiPARP). *J. Biol. Chem.* *288*, 21514–21525.
- Ejaz, A., Martinez-Guino, L., Goldfine, A.B., Ribas-Aulinas, F., De Nigris, V., Ribó, S., Gonzalez-Franquesa, A., Garcia-Roves, P.M., Li, E., Dreyfuss, J.M., et al. (2016). Dietary Betaine Supplementation Increases Fgf21 Levels to Improve Glucose Homeostasis and Reduce Hepatic Lipid Accumulation in Mice. *Diabetes* *65*, 902–912.
- European Union Report on Pesticide Residues in Food (2011). The 2009 European Union Report on Pesticide Residues in Food - - 2011 - EFSA Journal - Wiley Online Library.
- Forgacs, A.L., Kent, M.N., Makley, M.K., Mets, B., DelRaso, N., Jahns, G.L., Burgoon, L.D., Zacharewski, T.R., and Reo, N.V. (2012). Comparative metabolomic and genomic analyses of TCDD-elicited metabolic disruption in mouse and rat liver. *Toxicol. Sci. Off. J. Soc. Toxicol.* *125*, 41–55.
- Forgacs, A.L., Dere, E., Angrish, M.M., and Zacharewski, T.R. (2013). Comparative analysis

of temporal and dose-dependent TCDD-elicited gene expression in human, mouse, and rat primary hepatocytes. *Toxicol. Sci. Off. J. Soc. Toxicol.* *133*, 54–66.

Gill, S., Bowers, W.J., Nakai, J.S., Yagminas, A., Mueller, R., and Pulido, O. (2013). Effects of environmentally relevant mixtures of persistent organic pollutants on the developmental neurobiology in rats. *Toxicol. Pathol.* *41*, 38–47.

Guillouzo, A., Corlu, A., Aninat, C., Glaise, D., Morel, F., and Guguen-Guillouzo, C. (2007). The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem. Biol. Interact.* *168*, 66–73.

Guyot, E., Chevallier, A., Barouki, R., and Coumoul, X. (2013). The AhR twist: ligand-dependent AhR signaling and pharmaco-toxicological implications. *Drug Discov. Today* *18*, 479–486.

Howard, G.J., Schlezinger, J.J., Hahn, M.E., and Webster, T.F. (2010). Generalized concentration addition predicts joint effects of aryl hydrocarbon receptor agonists with partial agonists and competitive antagonists. *Environ. Health Perspect.* *118*, 666–672.

Jayashree, S., Indumathi, D., Akilavalli, N., Sathish, S., Selvaraj, J., and Balasubramanian, K. (2013). Effect of Bisphenol-A on insulin signal transduction and glucose oxidation in liver of adult male albino rat. *Environ. Toxicol. Pharmacol.* *35*, 300–310.

Jennen, D.G.J., Magkoufopoulou, C., Ketelslegers, H.B., van Herwijnen, M.H.M., Kleinjans, J.C.S., and van Delft, J.H.M. (2010). Comparison of HepG2 and HepaRG by whole-genome gene expression analysis for the purpose of chemical hazard identification. *Toxicol. Sci. Off. J. Soc. Toxicol.* *115*, 66–79.

Jossé, R., Aninat, C., Glaise, D., Dumont, J., Fessard, V., Morel, F., Poul, J.-M., Guguen-Guillouzo, C., and Guillouzo, A. (2008). Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. *Drug Metab. Dispos. Biol. Fate Chem.* *36*, 1111–1118.

Kanebratt, K.P., and Andersson, T.B. (2008). Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. *Drug Metab. Dispos. Biol. Fate Chem.* *36*, 1444–1452.

Kerger, B.D., Leung, H.-W., Scott, P., Paustenbach, D.J., Needham, L.L., Patterson, D.G., Gerthoux, P.M., and Mocarelli, P. (2006). Age- and concentration-dependent elimination half-life of 2,3,7,8-tetrachlorodibenzo-p-dioxin in Seveso children. *Environ. Health Perspect.* *114*, 1596–1602.

Kortenkamp, A., Faust, M., Scholze, M., and Backhaus, T. (2007). Low-level exposure to multiple chemicals: reason for human health concerns? *Environ. Health Perspect.* *115 Suppl 1*, 106–114.

Le Magueresse-Battistoni, B., Labaronne, E., Vidal, H., and Naville, D. (2017). Endocrine disrupting chemicals in mixture and obesity, diabetes and related metabolic disorders. *World*

J. Biol. Chem. 8, 108–119.

Le Magueresse-Battistoni, B., Vidal, H., and Naville, D. (2018). Environmental Pollutants and Metabolic Disorders: The Multi-Exposure Scenario of Life. *Front. Endocrinol.* 9, 582.

Lee, D.-H., Steffes, M.W., Sjödin, A., Jones, R.S., Needham, L.L., and Jacobs, D.R. (2011). Low dose organochlorine pesticides and polychlorinated biphenyls predict obesity, dyslipidemia, and insulin resistance among people free of diabetes. *PloS One* 6, e15977.

Lemaire, G., Mnif, W., Mauvais, P., Balaguer, P., and Rahmani, R. (2006). Activation of alpha- and beta-estrogen receptors by persistent pesticides in reporter cell lines. *Life Sci.* 79, 1160–1169.

Lozowicka, B., Abzeitova, E., Sagitov, A., Kaczynski, P., Toleubayev, K., and Li, A. (2015). Studies of pesticide residues in tomatoes and cucumbers from Kazakhstan and the associated health risks. *Environ. Monit. Assess.* 187, 609.

Lyche, J.L., Grześ, I.M., Karlsson, C., Nourizadeh-Lillabadi, R., Berg, V., Kristoffersen, A.B., Skåre, J.U., Alestrøm, P., and Ropstad, E. (2013). Parental exposure to natural mixtures of POPs reduced embryo production and altered gene transcription in zebrafish embryos. *Aquat. Toxicol. Amst. Neth.* 126, 424–434.

MacPherson, L., Tamblyn, L., Rajendra, S., Bralha, F., McPherson, J.P., and Matthews, J. (2013). 2,3,7,8-Tetrachlorodibenzo-p-dioxin poly(ADP-ribose) polymerase (TiPARP, ARTD14) is a mono-ADP-ribosyltransferase and repressor of aryl hydrocarbon receptor transactivation. *Nucleic Acids Res.* 41, 1604–1621.

Magliano, D.J., Loh, V.H.Y., Harding, J.L., Botton, J., and Shaw, J.E. (2014). Persistent organic pollutants and diabetes: a review of the epidemiological evidence. *Diabetes Metab.* 40, 1–14.

Majumder, R., and Kaviraj, A. (2018). Acute and sublethal effects of organophosphate insecticide chlorpyrifos on freshwater fish *Oreochromis niloticus*. *Drug Chem. Toxicol.* 1–9.

Matthews, J., and Gustafsson, J.-A. (2006). Estrogen receptor and aryl hydrocarbon receptor signaling pathways. *Nucl. Recept. Signal.* 4, e016.

Meng, Z., Wang, D., Yan, S., Li, R., Yan, J., Teng, M., Zhou, Z., and Zhu, W. (2018). Effects of perinatal exposure to BPA and its alternatives (BPS, BPF and BPAF) on hepatic lipid and glucose homeostasis in female mice adolescent offspring. *Chemosphere* 212, 297–306.

Merhi, M., Demur, C., Racaud-Sultan, C., Bertrand, J., Canlet, C., Estrada, F.B.Y., and Gamet-Payraastre, L. (2010). Gender-linked haematopoietic and metabolic disturbances induced by a pesticide mixture administered at low dose to mice. *Toxicology* 267, 80–90.

Monteagudo, C., Mariscal-Arcas, M., Heras-Gonzalez, L., Ibañez-Peinado, D., Rivas, A., and Olea-Serrano, F. (2016). Effects of maternal diet and environmental exposure to organochlorine pesticides on newborn weight in Southern Spain. *Chemosphere* 156, 135–142.

- Nanobashvili, K., Jack-Roberts, C., Bretter, R., Jones, N., Axen, K., Saxena, A., Blain, K., and Jiang, X. (2018). Maternal Choline and Betaine Supplementation Modifies the Placental Response to Hyperglycemia in Mice and Human Trophoblasts. *Nutrients* 10.
- Nault, R., Fader, K.A., Kirby, M.P., Ahmed, S., Matthews, J., Jones, A.D., Lunt, S.Y., and Zacharewski, T.R. (2016a). Pyruvate Kinase Isoform Switching and Hepatic Metabolic Reprogramming by the Environmental Contaminant 2,3,7,8-Tetrachlorodibenzo-p-Dioxin. *Toxicol. Sci. Off. J. Soc. Toxicol.* 149, 358–371.
- Nault, R., Fader, K.A., Ammendolia, D.A., Dornbos, P., Potter, D., Sharratt, B., Kumagai, K., Harkema, J.R., Lunt, S.Y., Matthews, J., et al. (2016b). Dose-Dependent Metabolic Reprogramming and Differential Gene Expression in TCDD-Elicited Hepatic Fibrosis. *Toxicol. Sci. Off. J. Soc. Toxicol.* 154, 253–266.
- Nougadère, A., Sirot, V., Kadar, A., Fastier, A., Truchot, E., Vergnet, C., Hommet, F., Baylé, J., Gros, P., and Leblanc, J.-C. (2012). Total diet study on pesticide residues in France: levels in food as consumed and chronic dietary risk to consumers. *Environ. Int.* 45, 135–150.
- Okey, A.B. (2007). An aryl hydrocarbon receptor odyssey to the shores of toxicology: the Deichmann Lecture, International Congress of Toxicology-XI. *Toxicol. Sci. Off. J. Soc. Toxicol.* 98, 5–38.
- Rajagopal, G., Bhaskaran, R.S., and Karundevi, B. (2019). Developmental exposure to DEHP alters hepatic glucose uptake and transcriptional regulation of GLUT2 in rat male offspring. *Toxicology* 413, 56–64.
- Rizzati, V., Briand, O., Guillou, H., and Gamet-Payraastre, L. (2016). Effects of pesticide mixtures in human and animal models: An update of the recent literature. *Chem. Biol. Interact.* 254, 231–246.
- Rogue, A., Lambert, C., Spire, C., Claude, N., and Guillouzo, A. (2012). Interindividual variability in gene expression profiles in human hepatocytes and comparison with HepaRG cells. *Drug Metab. Dispos. Biol. Fate Chem.* 40, 151–158.
- Samanez, C.H., Caron, S., Briand, O., Dehondt, H., Duplan, I., Kuipers, F., Hennuyer, N., Clavey, V., and Staels, B. (2012). The human hepatocyte cell lines IHH and HepaRG: models to study glucose, lipid and lipoprotein metabolism. *Arch. Physiol. Biochem.* 118, 102–111.
- Savary, C.C., Jossé, R., Bruyère, A., Guillet, F., Robin, M.-A., and Guillouzo, A. (2014). Interactions of endosulfan and methoxychlor involving CYP3A4 and CYP2B6 in human HepaRG cells. *Drug Metab. Dispos. Biol. Fate Chem.* 42, 1235–1240.
- Schechter, A., Birnbaum, L., Ryan, J.J., and Constable, J.D. (2006). Dioxins: an overview. *Environ. Res.* 101, 419–428.
- Seok, J., Warren, H.S., Cuenca, A.G., Mindrinos, M.N., Baker, H.V., Xu, W., Richards, D.R., McDonald-Smith, G.P., Gao, H., Hennessy, L., et al. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc. Natl. Acad. Sci.* 110, 3507–3512.

- Skrbić, B., and Predojević, Z. (2008). Levels of organochlorine pesticides in crops and related products from Vojvodina, Serbia: estimated dietary intake. *Arch. Environ. Contam. Toxicol.* *54*, 628–636.
- Son, H.-K., Kim, S.-A., Kang, J.-H., Chang, Y.-S., Park, S.-K., Lee, S.-K., Jacobs, D.R., and Lee, D.-H. (2010). Strong associations between low-dose organochlorine pesticides and type 2 diabetes in Korea. *Environ. Int.* *36*, 410–414.
- Stark, R., Guebre-Egziabher, F., Zhao, X., Feriod, C., Dong, J., Alves, T.C., Ioja, S., Pongratz, R.L., Bhanot, S., Roden, M., et al. (2014). A role for mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) in the regulation of hepatic gluconeogenesis. *J. Biol. Chem.* *289*, 7257–7263.
- Tarnow, P., Tralau, T., and Luch, A. (2019). Chemical activation of estrogen and aryl hydrocarbon receptor signaling pathways and their interaction in toxicology and metabolism. *Expert Opin. Drug Metab. Toxicol.* *15*, 219–229.
- Tascher, G., Burban, A., Camus, S., Plumel, M., Chanon, S., Le Guevel, R., Shevchenko, V., Van Dorsselaer, A., Lefai, E., Guguen-Guillouzo, C., et al. (2019). In-Depth Proteome Analysis Highlights HepaRG Cells as a Versatile Cell System Surrogate for Primary Human Hepatocytes. *Cells* *8*.
- Taylor, K.W., Novak, R.F., Anderson, H.A., Birnbaum, L.S., Blystone, C., Devito, M., Jacobs, D., Köhrle, J., Lee, D.-H., Rylander, L., et al. (2013). Evaluation of the association between persistent organic pollutants (POPs) and diabetes in epidemiological studies: a national toxicology program workshop review. *Environ. Health Perspect.* *121*, 774–783.
- Thayer, K.A., Heindel, J.J., Bucher, J.R., and Gallo, M.A. (2012). Role of environmental chemicals in diabetes and obesity: a National Toxicology Program workshop review. *Environ. Health Perspect.* *120*, 779–789.
- Wahlang, B., Perkins, J.T., Petriello, M.C., Hoffman, J.B., Stromberg, A.J., and Hennig, B. (2017). A compromised liver alters polychlorinated biphenyl-mediated toxicity. *Toxicology* *380*, 11–22.
- Warner, M., Mocarelli, P., Brambilla, P., Wesselink, A., Patterson, D.G., Turner, W.E., and Eskenazi, B. (2014). Serum TCDD and TEQ concentrations among Seveso women, 20 years after the explosion. *J. Expo. Sci. Environ. Epidemiol.* *24*, 588–594.
- Watanabe, M.X., Kunisue, T., Ueda, N., Nose, M., Tanabe, S., and Iwata, H. (2013). Toxicokinetics of dioxins and other organochlorine compounds in Japanese people: association with hepatic CYP1A2 expression levels. *Environ. Int.* *53*, 53–61.
- Wild, C.P. (2005). Complementing the genome with an “exposome”: the outstanding challenge of environmental exposure measurement in molecular epidemiology. *Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol.* *14*, 1847–1850.

Legends to the figures

Figure 1. Corresponding levels of external intake of TCDD and α -endosulfan with *in vitro* concentrations. The levels of external intake are illustrated in this plot, covering environmental and accidental exposure to TCDD, as well as environmental and occupational exposure to α -endosulfan. The dose used for treating the cell cultures corresponded to the upper bounds of the external intake estimates for both compounds.

Figure 2. Effect of the exposure of HepaRG cells to 25 nM TCDD, 10 μ M α -endosulfan or the mixture on GLUT2 expression, glucose oxidation, lactate production and glycogen content. The levels of GLUT2 mRNA (A) and protein (B, C) were measured after 30 h and 72 h, respectively, of exposure to POPs. Quantification of radioactive glucose oxidation into radioactive CO₂ (D) and lactate production (E) was performed after 72 h of POP exposure. Glycogen cellular content was measured after 66 h of exposure to the POPs (F). The values are the means of 3-6 independent experiments \pm the standard deviation. Stars represent the significance of the fold-change as compared to the control (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). \$ represents the significance of the fold-change between the corresponding bars (\$\$, $p < 0.01$, \$\$\$, $p < 0.001$).

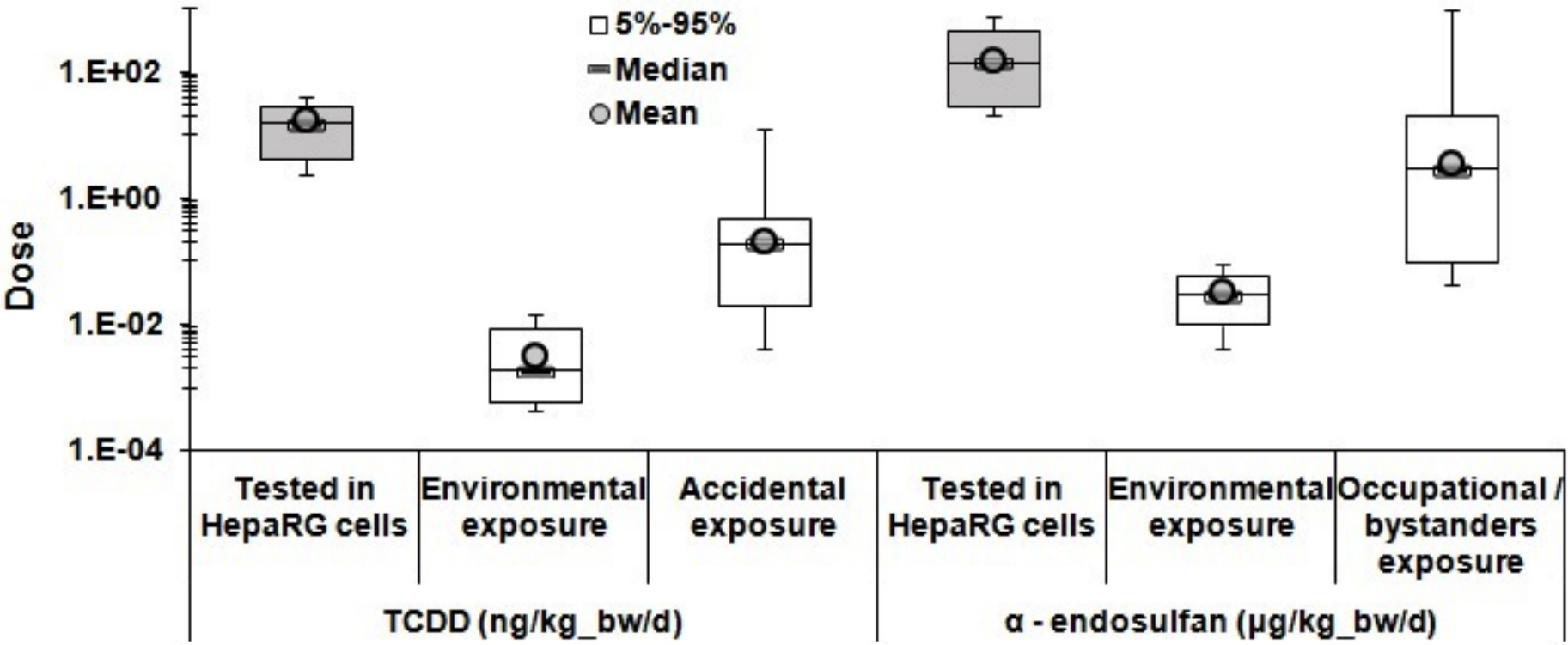
Figure 3. Time course of gene expression following exposure of HepaRG cells to pollutants. The amount of G6PC (A) and GLUT2 (B) mRNA was measured 4, 8, 16 and 30 h following exposure of cells to 25 nM TCDD, 10 μ M α -endosulfan or the mixture. The values are the means \pm standard deviation of 4 independent experiments in duplicate. Stars represent the significance of the fold-change as compared to the control (***, $p < 0.001$). \$ represents the significance of the fold-change between the corresponding bars (\$, $p < 0.05$, \$\$, $p < 0.01$, \$\$\$, $p < 0.001$).

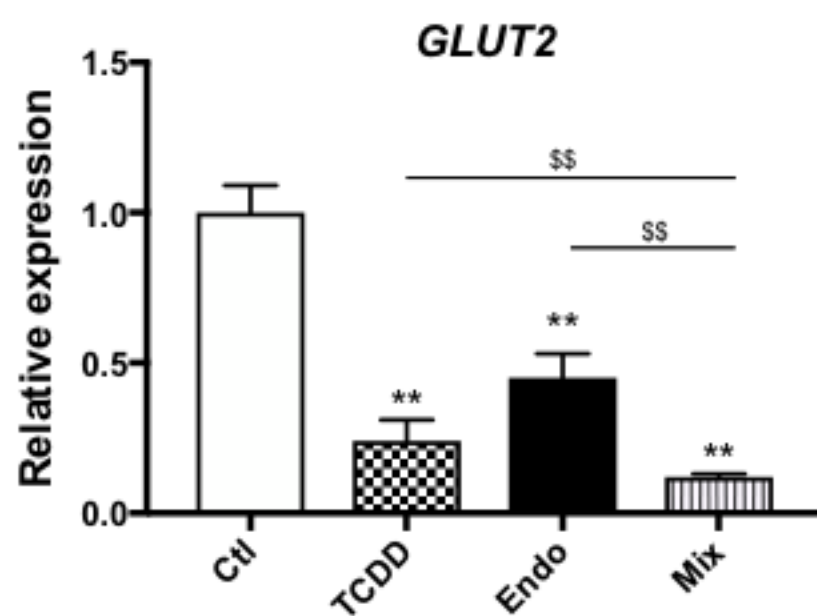
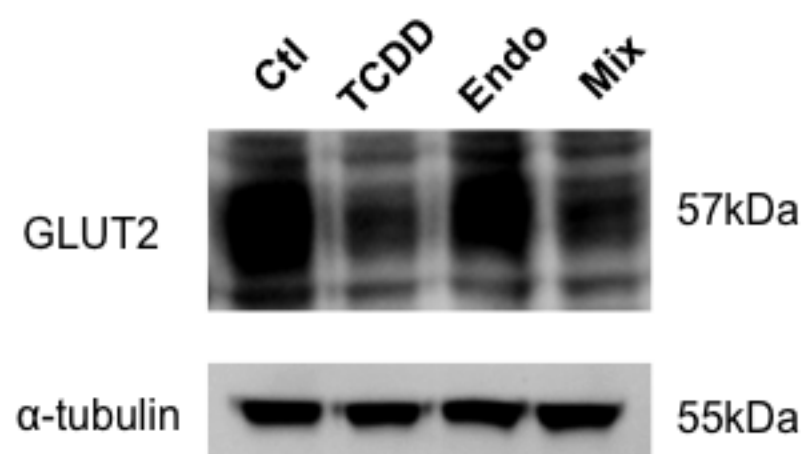
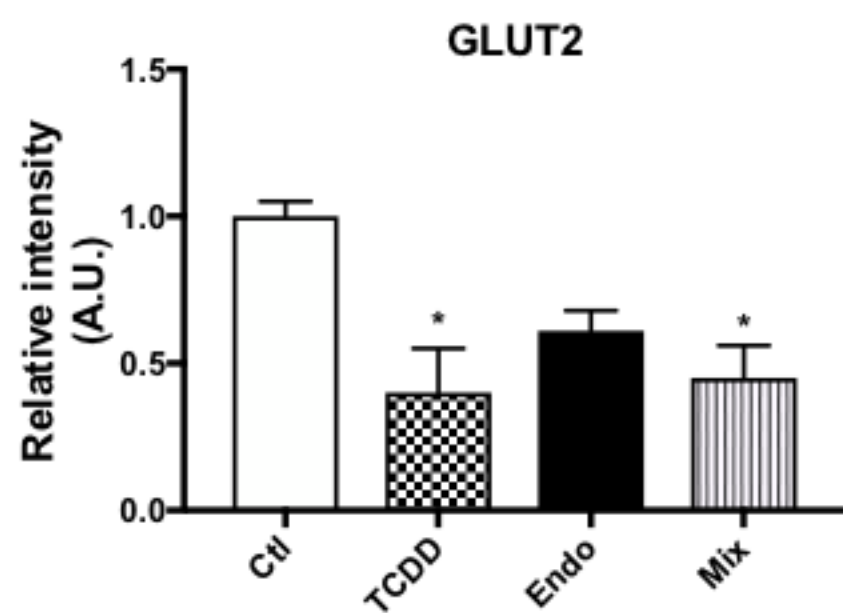
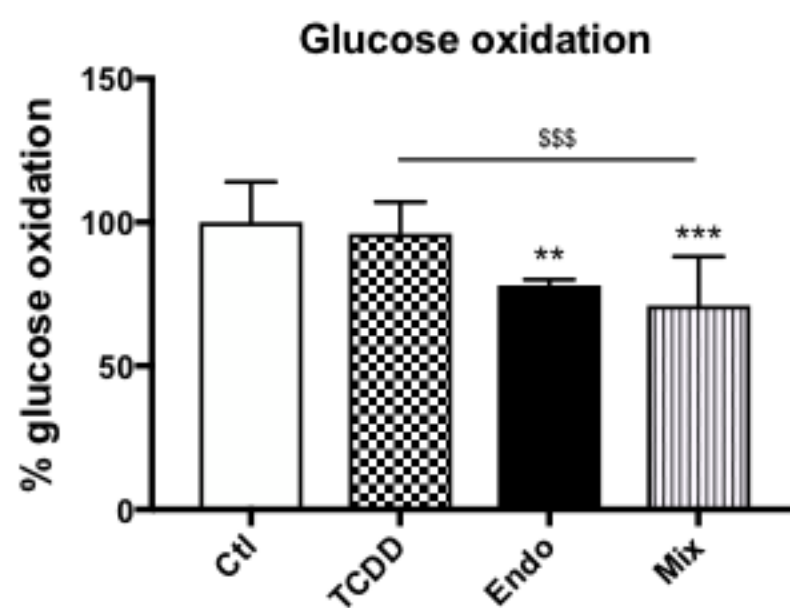
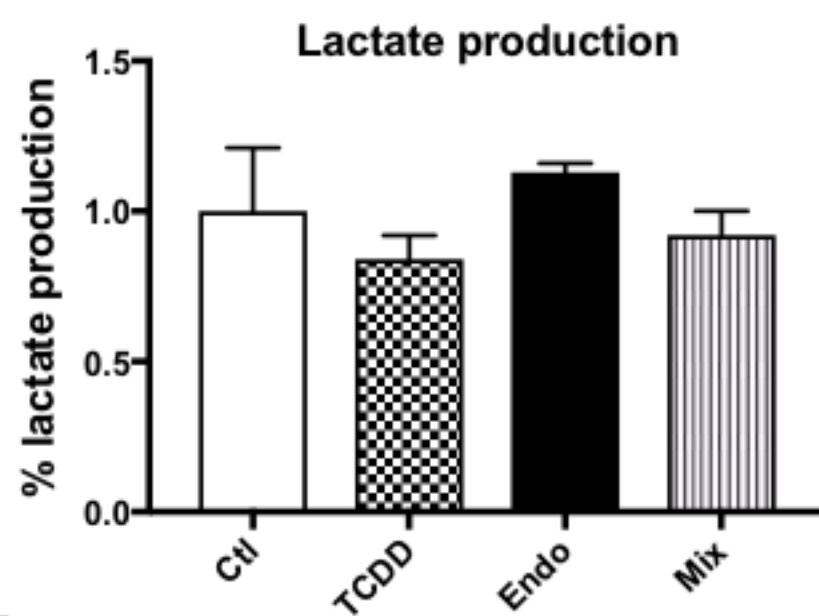
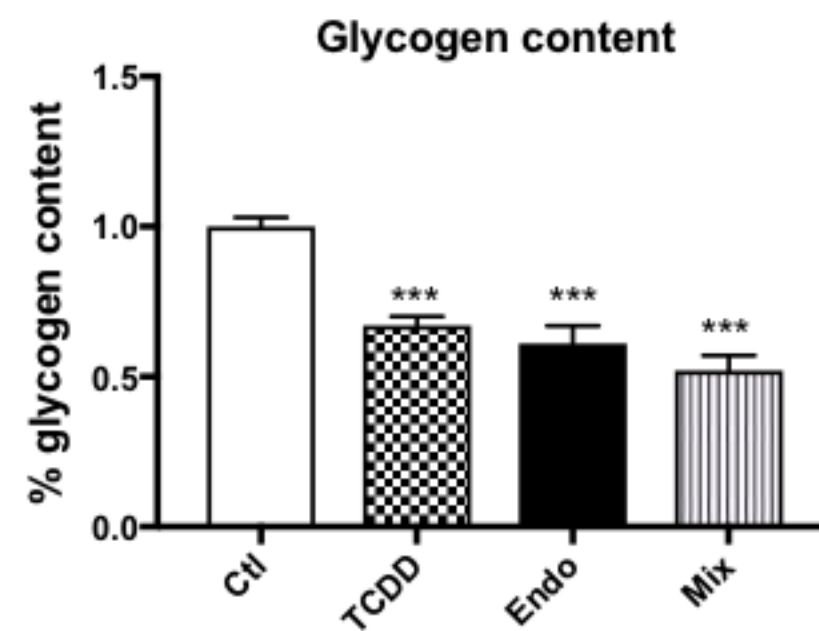
Figure 4. Effect of the exposure of HepaRG cells to 25 nM TCDD, 10 μ M α -endosulfan or the mixture on G6PC and GLUT2 expression and on glucose production. The levels of G6PC (A) and GLUT2 (B) mRNA were measured after 30 h of cultivation under gluconeogenic conditions in the presence of the POPs and glucose production was measured after 72 h of treatment (C). The values are the means of 3-6 independent experiments \pm the standard deviation. Stars represent the significance of the fold-change as compared to the

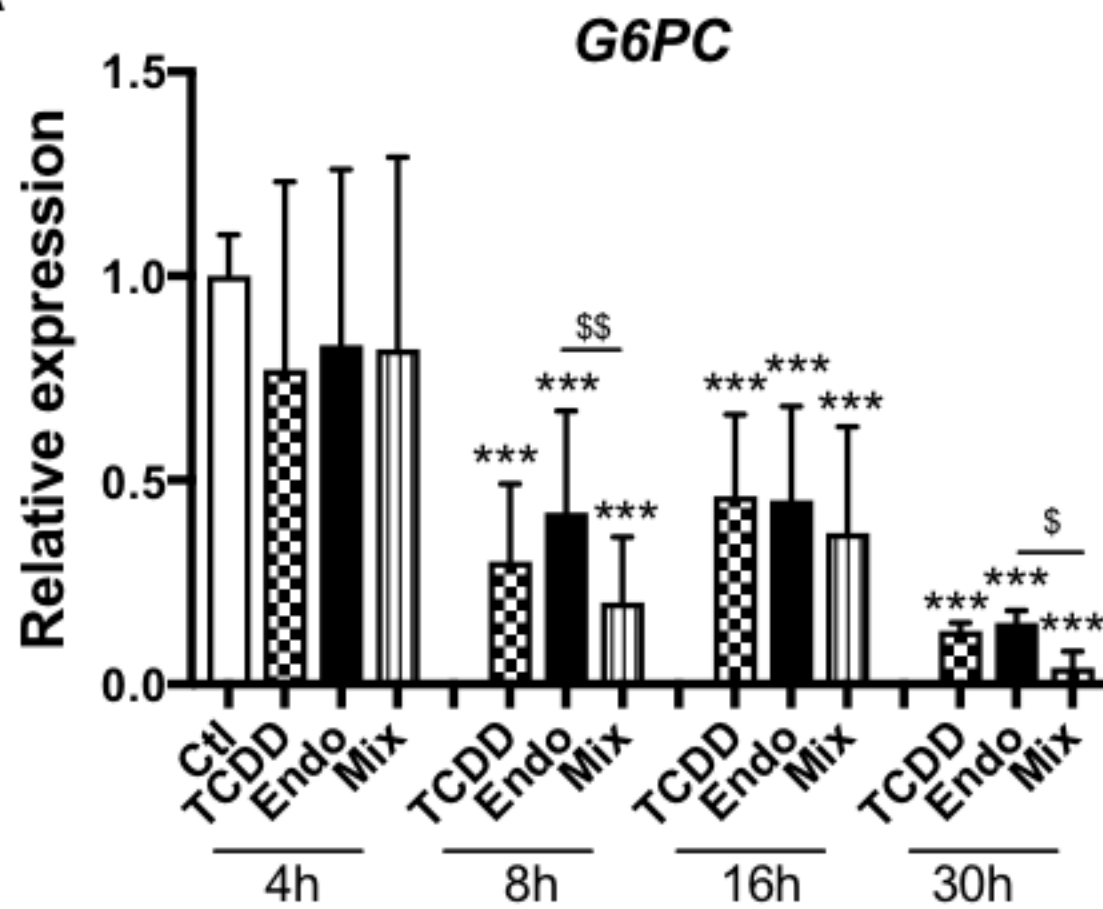
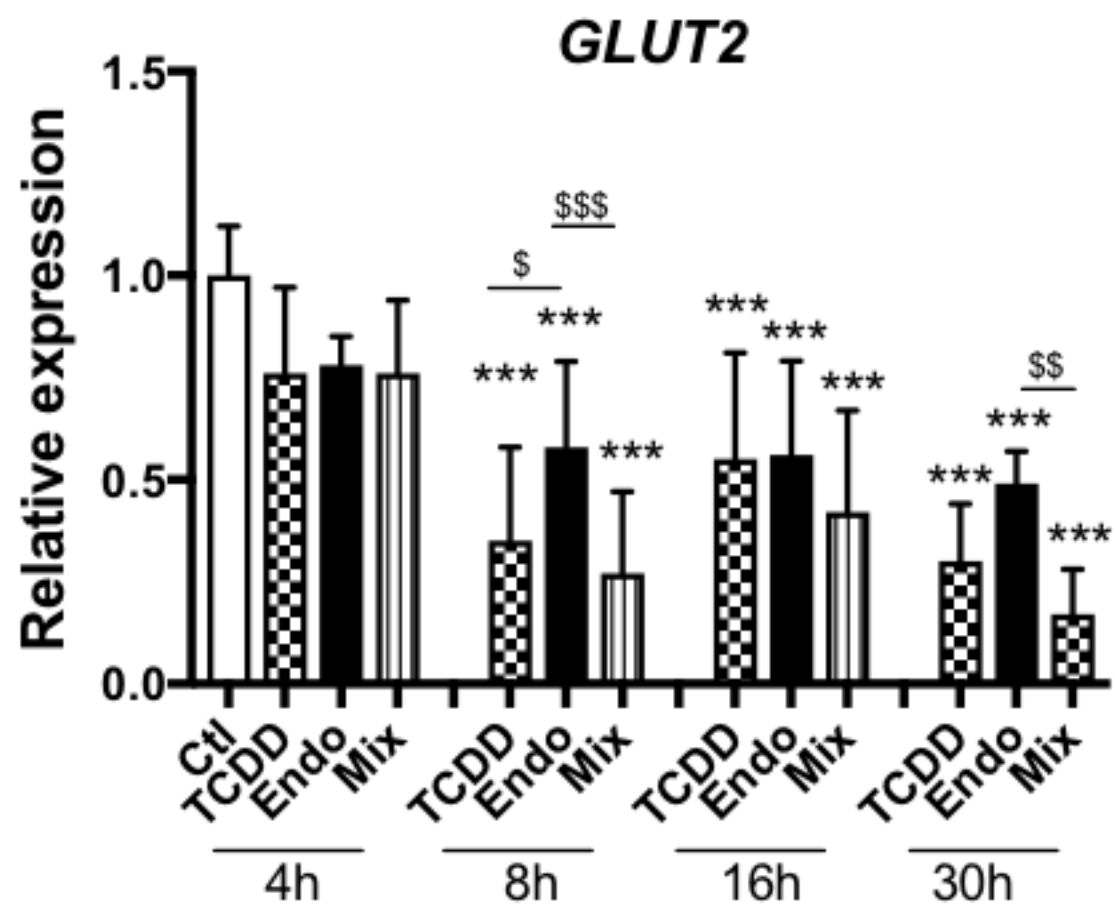
control (*, $p < 0.05$, **, $p < 0.01$). \$ represents the significance between the corresponding bars (\$, $p < 0.05$, \$\$, $p < 0.01$).

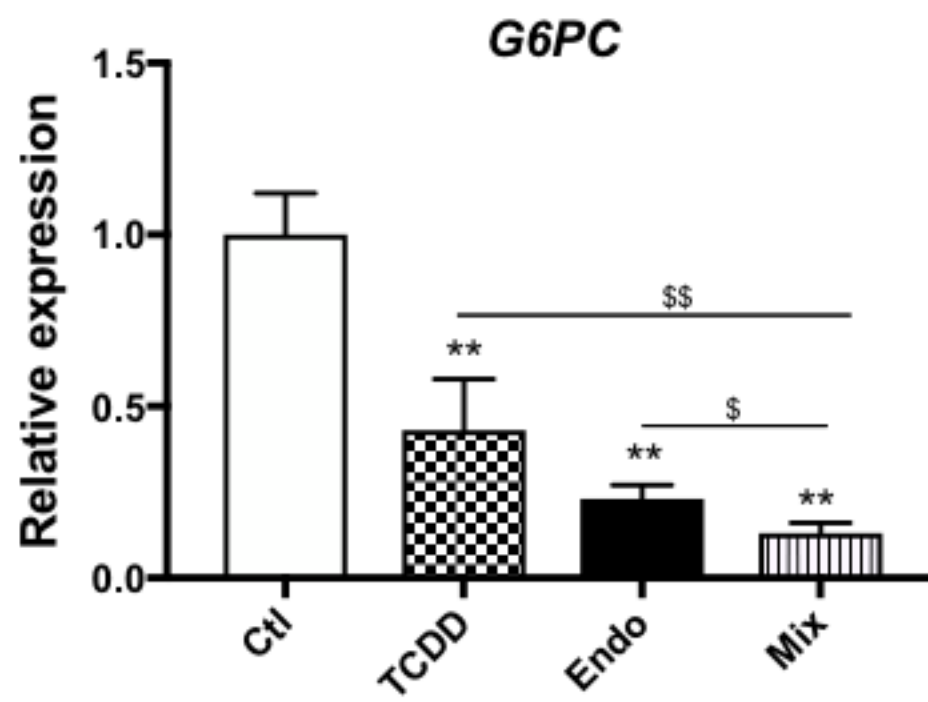
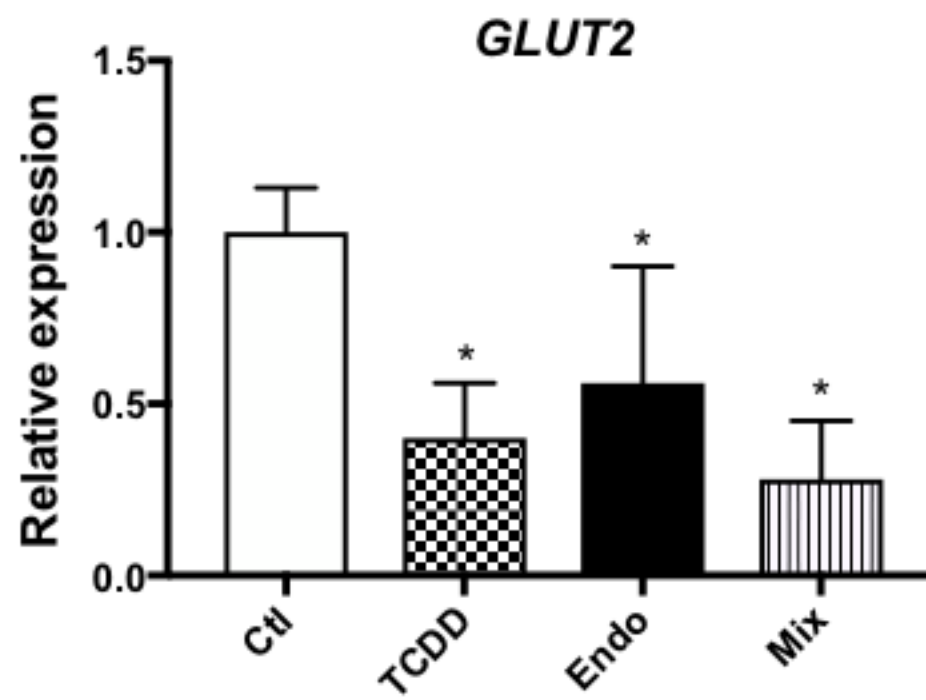
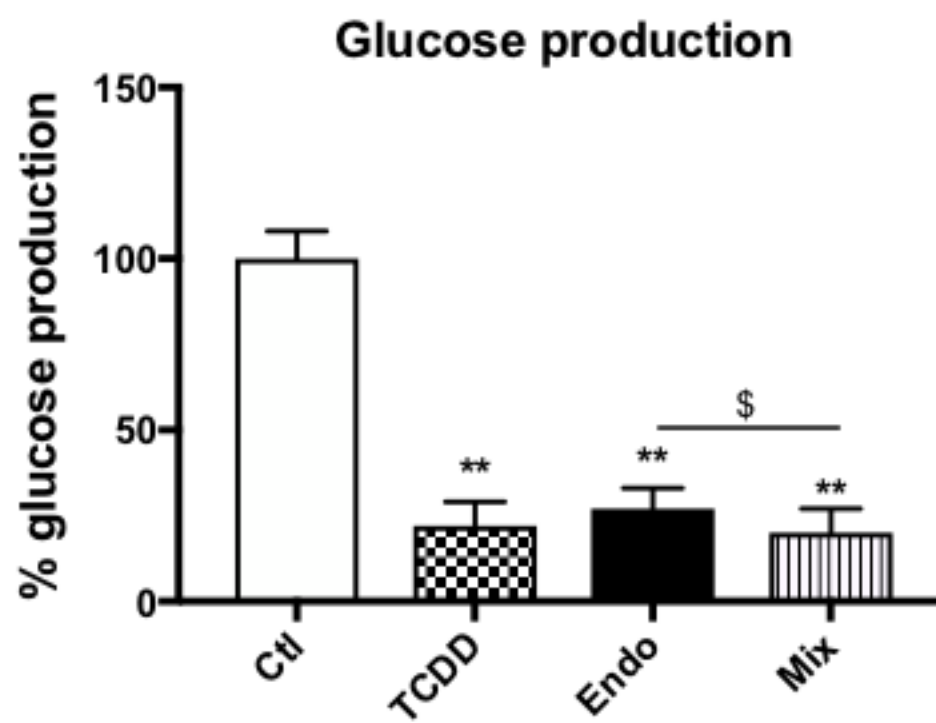
Figure 5. Effect of AHR or ER α silencing on gene expression and glucose production.

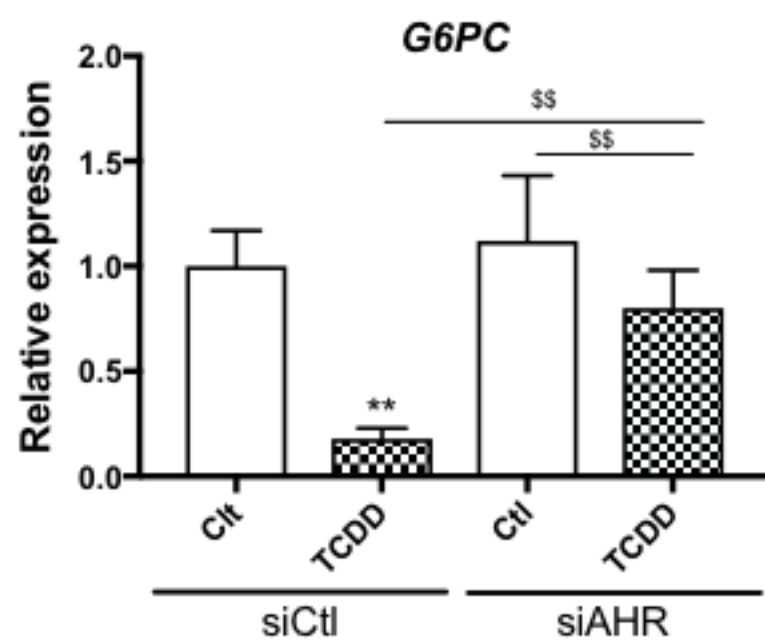
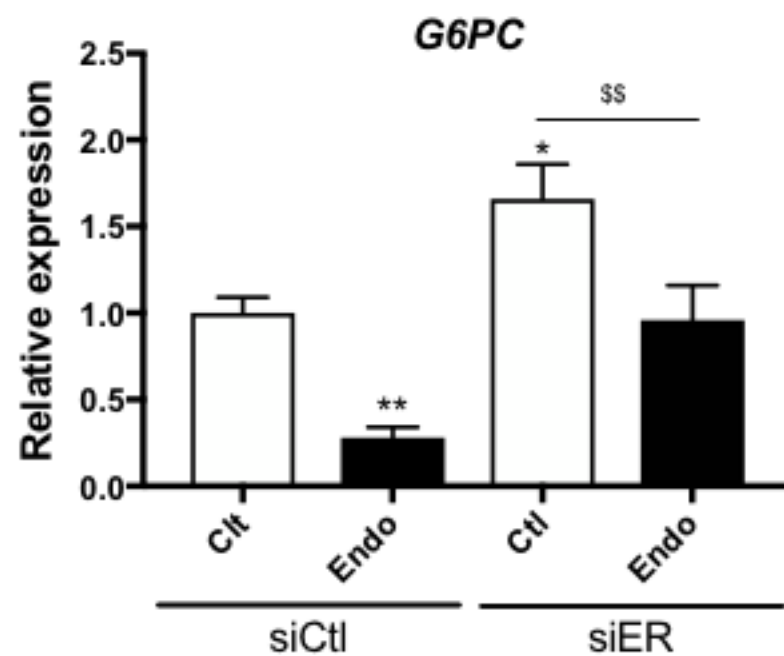
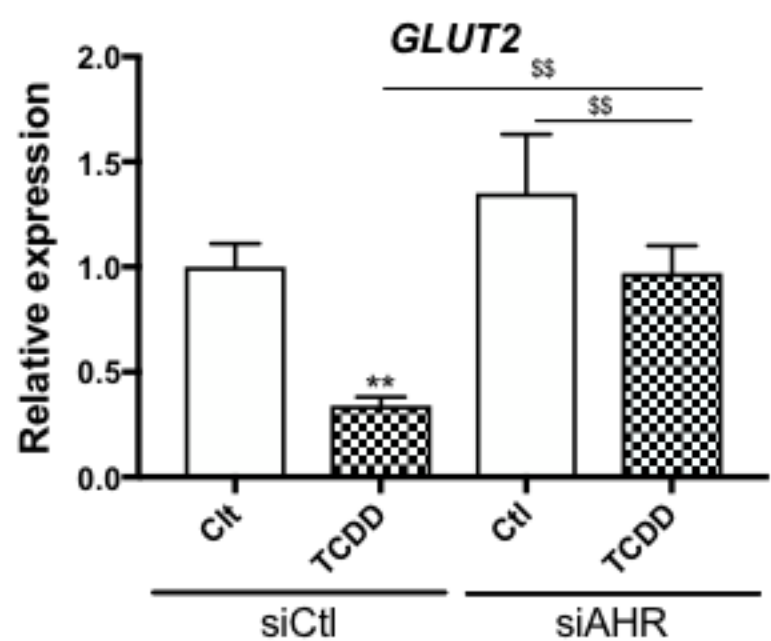
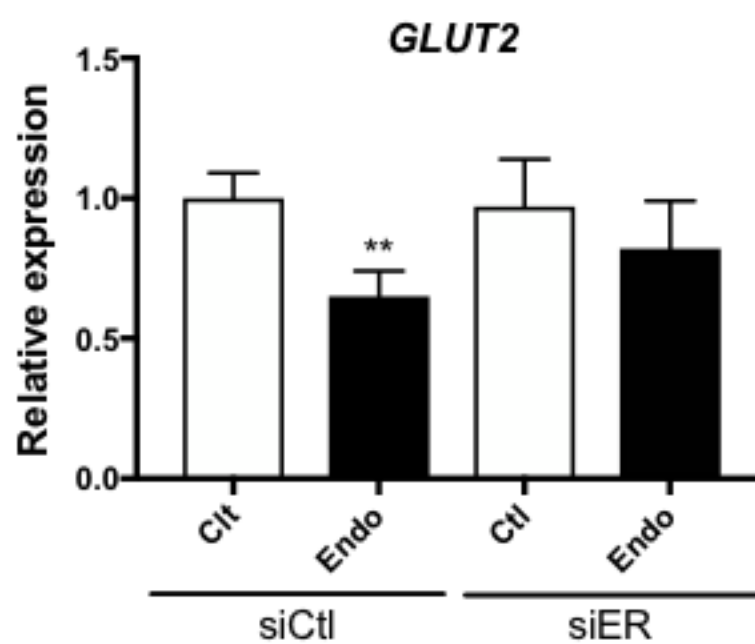
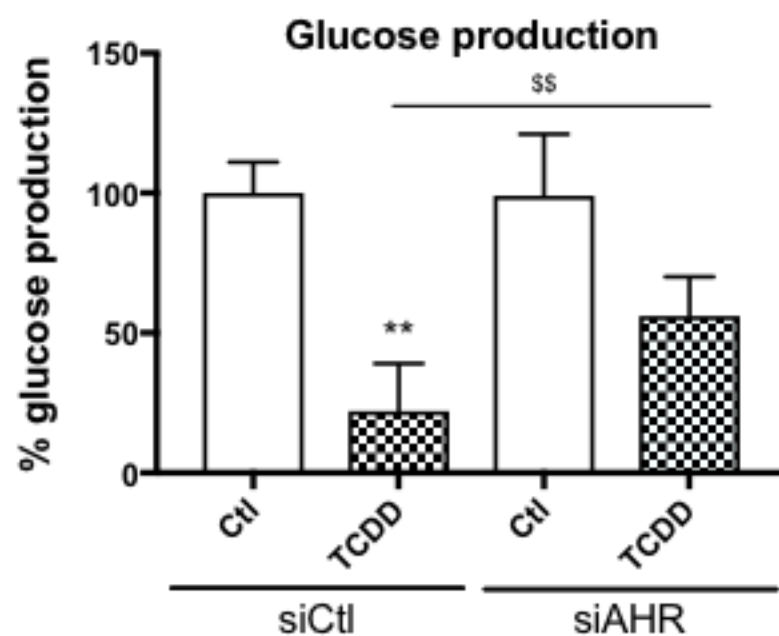
The expression of G6PC (A,D) and GLUT2 (B,E) was measured after transfection with either a control siRNA (siCtl) or siRNAs directed against AHR mRNA (siAHR) or against ER α mRNA (siER). The cells were exposed or not to 25 nM TCDD or 10 μ M α -endosulfan. Glucose production was measured after AHR silencing in the presence or absence of 25 nM TCDD (C) or after ER α silencing in the presence or not of 10 μ M α -endosulfan (F). The values are the means \pm the standard deviation of 3 or 4 experiments in duplicate. Stars represent the significance of the fold-change as compared to the control (*, $p < 0.05$, **, $p < 0.01$). \$ represents the significance between the corresponding bars (\$\$, $p < 0.01$).



A**B****C****D****E****F**

A**B**

A**B****C**

A**D****B****E****C****F**