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Research Article

A Human Relevant Defined Mixture of Persistent Organic Pollutants (POPs) Affects *In Vitro* Secretion of Glucagon-Like Peptide 1 (GLP-1), but Does Not Affect Translocation of Its Receptor

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ABSTRACT

Environmental exposure to persistent organic pollutants (POPs) has been suggested as a contributing factor for the increased rate of type 2 diabetes and obesity. A complex mixture of 29 POPs (Total mixture), based on human blood concentrations, was used to expose a glucagon-like peptide 1 (GLP-1) secreting enteroendocrine cell line (pGIP/neo: STC-1) *in vitro* for 3 and 24 h. Significant increases of GLP-1 occurred when cells were exposed to the Total mixture at ×500 blood levels. Six sub-mixtures representing chlorinated (Cl), brominated (Br), and perfluorinated chemicals (PFAA), and their combinations (Cl + Br, Cl + PFAA, Br + PFAA) were also tested at ×500. Secretion levels seen for these remained lower than the Total mixture, and the Br mixture had no effect. After 24 h, increased secretion was seen with all mixtures at ×1 blood levels. Cytotoxicity was present for ×100 and ×500 blood levels. When tested in a GLP-1 receptor translocation assay (U2OS-GLP1R-EGFP), neither agonistic nor antagonist effects on receptor internalization were seen for any of the mixtures. We conclude individual classes of POPs, alone or in combination, can affect GLP-1 secretion and may contribute as a molecular mechanism linking environmental toxicants and diabetes.

Key words: metabolic disruption; gut hormones; mixture; enteroendocrine cell; high content analysis; ELISA.

The incidence of metabolic diseases such as metabolic syndrome, obesity, and type 2 diabetes (T2D) is increasing globally. Environmental chemicals have been proposed as additional risk factors, in addition to main contributors like genetic predisposition, excess calorie intake, or lack of exercise (Jeon *et al.*, 2015). Experimental and epidemiological evidence suggests a role for early and chronic exposure to low doses of chemical pollutants with endocrine and metabolic disrupting activity, including persistent organic pollutants (POPs) (Chevalier and Fénichel, 2015). In humans, the main exposure to POPs in non-occupational

settings occurs through ingestion (Darnerud *et al.*, 2006; Vestergren *et al.*, 2012). This makes the gastrointestinal tract the first organ of exposure, and thus it is conceivable that enteroendocrine (EE) cells are a potential target for endocrine disruption.

POPs constitute a diverse group of chemicals that are resistant to environmental degradation, bioaccumulate within living organisms, and biomagnify through the food web. Due to their mixing in the environment, food web, and the long-term accumulation in fatty tissues, exposure to POPs always occurs as a mixture (WHO/UNEP, 2013).

Human and experimental evidence linking chlorinated POPs with obesity and/or T2D has been extensively reviewed (Lee *et al.*, 2014). Associations with brominated POPs have been reported by Lim *et al.* (2008). Background exposure of particular brominated POPs (PBB-153 and PBDE-153) may be closely linked to disturbance of glucose and lipid metabolism in the general population (Lim *et al.*, 2008). Perfluorinated alkylated acids (PFAAs) have been associated with disturbed lipid metabolism and shown to bind to peroxisome proliferator-activated receptors (PPARs), which are of major importance for lipid metabolism and fat storage (Fang *et al.*, 2015; Zhang *et al.*, 2014). A study by Lind *et al.* (2014) assessed the relationship between circulating levels of PFAAs and markers of insulin secretion and resistance. The results revealed that perfluorononanoic acid (PFNA) is positively correlated to prevalent diabetes.

In addition to the mechanisms outlined above, it is possible that POP exposure leads to an endocrine disrupting action on gut hormones involved in controlling satiety responses and insulin release. Glucagon-like peptide 1 (GLP-1) is one such hormonal system involved and vital in maintaining glucose and weight homeostasis.

GLP-1 is a peripheral humoral factor secreted by EE L-type cells (Lim and Brubaker, 2006). After food ingestion, early phase secretion starts within 10–15 min as triggered by the mechano- and chemo-sensitive afferent neurons. Direct nutrient contact with the cells induces GLP-1 secretion within 30 min to 3 h of meal intake. Exocytosis of GLP-1 secretory granules requires activation of intracellular signaling pathways such as PKA, PKC, and ion channels-dependent membrane depolarization (Nadkarni *et al.*, 2014). Secreted GLP-1 either acts locally in the gut with EE cells, vagal afferents, or is drained into the circulation via the hepatic portal vein or lymph to bind to specific receptors (Steinert and Begliger, 2011).

The GLP-1 receptor (GLP-1R), a class B G protein-coupled receptor, is distributed in multiple tissues and has been known to mediate GLP-1 actions (Graaf *et al.*, 2016). The pancreas is one of the major sites for GLP-1R expression. GLP-1 binding promotes the exocytosis of insulin vesicles in beta cells and supresses the secretion of the hyperglycemic hormone, glucagon, in alpha cells. In addition, GLP-1 increases cell proliferation and inhibits beta cell apoptosis.

In this study, we investigated the potential for POP mixtures to modify GLP-1 secretion, and to interact with the GLP-1R, providing insight into alternative mechanisms by which POPs may be involved in the pathogenesis of diabetes and obesity.

In addition, to simulate real-life exposure scenarios relevant to humans, we utilized a synthetic mixture of 29 different POPs (Table 1) designed as relevant to the Scandinavian population, with the individual concentration of the different POPs based on mean human blood levels (Supplementary Table 3, Shannon et al., 2019). In addition to the Total mixture, six sub-mixtures containing the same concentration of chlorinated + brominated (Cl + Br), chlorinated + perfluorinated (Cl + PFAA), brominated + perfluorinated (Br + PFAA), or chlorinated (Cl), brominated (Br), or perfluorinated compounds (PFAA) only were constructed. Using these sub-mixtures enables investigating the effect of adding or removing one or more chemical groups (Berntsen *et al.*, 2017). The concentration of the 29 POPs within the mixtures was based on the blood levels found in the Scandinavian population. However, a wider range of concentrations (from below and up to 500× blood levels) were tested to reflect human body burden levels in different exposure scenarios, including populations exposed to much higher POP concentrations. For example, Chinese occupational exposure to PFAAs is 100–500 times higher than that reported in the Scandinavian population (Fu *et al.*, 2016). In addition, the US exposure levels to some POPs are ten to hundreds times higher than the European population (Schecter *et al.*, 2005). The body burden of POPs is higher also in Inuit populations (Krüger *et al.*, 2007; Laird *et al.*, 2013).

Table 1. Overview of the 29 Different POPs Constituting the Total Mixture for *In Vitro* Evaluation

Chlorinated		Brominated	Perfluorinated
PCB	**OCP**	**BFR**	**PFAAs**
PCB 28	p,p′-DDE	PBDE 47	PFHxS
PCB 52	HCB	PBDE 99	PFOS
PCB 101	α-Chlordane	PBDE 100	PFOA
PCB 118	Oxychlordane	PBDE 153	PFNA
PCB 138	Trans-Nonachlor	PBDE 154	PFDA
PCB 153	α-HCH	PBDE 209	PFUnDA
PCB 180	β-HCH	HBCD	
	γ-HCH (Lindane)		
	Dieldrin		

For a complete description of selection of compounds and their individual concentration, see Berntsen *et al.* (2017).

In summary, this study investigates seven complex POP mixtures relevant to real life human exposure scenarios (Berntsen *et al.*, 2017) for effects on:

1. Cytotoxicity in GLP-1 secreting EE gut cells (pGIP/neo: a subclone of the STC-1 cell line).
2. Glucagon-like peptide 1 hormone secretion by EE gut cells.
3. Endocrine disrupting potential via (ant)agonism of the GLP-1R internalization in the U2OS-GLP1R-EGFP cell line.

MATERIALS AND METHODS

Chemicals. All brominated and chlorinated chemicals were originally purchased from Chiron As (Trondheim, Norway). All PFAAs were obtained from Sigma-Aldrich (St. Louis, MO) except perfluorohexanesulfonic acid (PFHxS) which was from Santa Cruz (Dallas). Hexabromocyclododecane (HBCD) was obtained from Sigma-Aldrich (Dorset, UK). Mitochondrial membrane potential dye (MMPD) and Hoechst nuclear stain 33342 were purchased from Perbio (Northumberland, England). Cell culture reagents were supplied by Life Technologies (Paisley, UK). All other reagents were standard laboratory grade.

Mixtures of POPs. The POP mixtures used in the study were based on concentrations of relevant POPs measured in human blood of Scandinavian population. The compounds were mixed in concentration ratios relevant to average human blood levels. An extensive description and information of chemical composition and concentrations can be found in Berntsen *et al.* (2017). Seven mixtures were used in the study: (1) Total mixture, containing

all the test compounds, (2) chlorinated mixture (Cl), (3) brominated mixture (Br), (4) perfluorinated mixture (PFAA), (5) chlorinated and brominated mixture (Cl + Br), (6) chlorinated and perfluorinated mixture (Cl + PFAA), and (7) brominated and perfluorinated mixture (Br + PFAA).

Cell culture. pGIP/neo: STC-1 cells are an EE cell model and its GLP-1 secretory ability has been extensively investigated (Gillespie *et al*., 2015; Hand *et al*., 2012; Jafri *et al*., 2016). These were a gift from Dr B. Wice (Washington University of St. Louis) with permission from Dr D. Hanahan (University of California, San Francisco, CA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax, 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, and 0.5 mg/ml G418. Cells were used between passages 15 and 50.

The U2OS-GLP1R-EGFP cell line was obtained from Thermo Scientific (UK), and stably expresses the human GLP-1R with a C-terminal enhanced green fluorescent protein tag. The cells were cultured routinely in DMEM with Glutamax, 10% FBS, 1% penicillin and streptomycin, and 0.5 mg/ml G418 at 37°C with 5% CO_2. The assay has been validated with cells up to a passage of 30 (Thermo Scientific, UK).

Analysis of cytotoxicity parameters by high content analysis. Briefly, pGIP/neo: STC-1 cells were seeded 6×10^4 in 96-well plates 24 h prior to the assay. Mitochondrial membrane potential dye was prepared by adding 117 µl of anhydrous dimethyl sulfoxide (DMSO) to make a 1 mM stock. Following incubation (3 and 24 h), 50 µl of dye was added to each well for 30 min at 37°C and protected from light. The dye was removed and cells were then fixed with a 10% formalin solution for 20 min at RT, and washed with PBS. Hoechst 33342 dye at a final concentration of 1.6 µM was added to each well and incubated for 10 min at RT; after which cells were washed with PBS four times and evaluated on a CellInsight™ NXT high content screening (HCS) Platform (Thermo Fisher Scientific, UK). Data were captured at ×20 objective magnification with selected filters: Hoechst dye (Ex/Em 350/461 nm) and MMPD (Ex/Em 554/576 nm).

GLP-1 secretion studies. The pGIP/neo: STC-1 cells were seeded into 24-well plates (1×10^6 per well) and cultured overnight at 37°C in a humidified atmosphere of 5% CO_2. Medium was removed and cells were washed once with HBSS buffer and equilibrated for 1 h in 500 µl Hank's balanced salt solution (HBSS) buffer. Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were used as a combined positive control (PC) at a concentration of 10 µM each. POP mixtures, PC, and solvent control (SC) were made at a final DMSO concentration of 0.2% in HBSS buffer. A 1 ml volume of each working concentration of the POP mixtures containing HBSS buffer, or 500 µl of DMSO SC, was added to the relevant wells of the plate. For the 24 h study, POP mixtures and controls were added in serum containing DMEM. Supernatant was removed after 3 and 24 h and stored at −20°C prior to GLP-1 hormone analysis by ELISA.

Hormone analysis. GLP-1 was measured using a GLP-1 (Active) ELISA kit (EGLP-35K); in accordance with the manufacturer's instructions (Millipore, Watford, UK). All experiments were performed in triplicate for each experimental point and repeated in three independent experiments. For 3 h exposure, the supernatants were diluted by HBSS buffer 1 in 4 prior to the measurement of GLP-1 hormone levels by ELISA. For 24 h exposure, the supernatants were diluted by 1 in 40 in media. Absorbance was measured using a Sunrise spectrophotometer (TECAN, Switzerland).

GLP-1 receptor redistribution assay. U2OS-GLP1R-EGFP cells were seeded (100 µl of 6×10^4 cells/well) in 96-well black plates with clear, flat bottoms in seeding media (DMEM with Glutamax, 1% FBS, 1% penicillin and streptomycin [50 U/ml]). After 24 h, 100 µl of the POP mixtures and the GLP-1 (7-37) standards at a final DMSO concentration of 0.2% were added. Test mixtures and standards were diluted in serum free media. The ability of 150 nM GLP-1 to induce internalization of the receptor was assessed in the presence and absence of the POP mixtures. Cells were incubated for 1 h then fixed with formalin (approximately 4% formaldehyde) for 20 min. Cells were washed prior to the addition of 100 µl of 1 µM Hoechst staining solution. After 30 min, the plate was imaged on a HCS Platform with the filters: Hoechst dye (350/461 nm) and GFP/FITC (488/509 nm) (Thermo Fisher Scientific, UK). The assay was performed in triplicate for each experimental point and repeated in three independent exposures.

Statistical analysis. All values shown are expressed as mean ± standard error of the mean (SEM) of the three independent exposures for the POP mixtures. Data from the high content analysis (HCA) cytotoxicity, GLP-1 secretion, and GLP-1R redistribution assays were analyzed using Microsoft Excel and GraphPad PRISM 5 software (GraphPad Software Inc, San Diego, CA). A one-way analysis of variance followed by Dunnett's multiple comparison test was used to determine significant differences between treatments and the corresponding control. A p-value of $\leq .05$ was considered as significant ($^{*}p \leq .05$, $^{**}p \leq .01$ and $^{***}p \leq .001$).

RESULTS

Effects of POP Mixture Exposure on Cellular Health and GLP-1 Secretion in pGIP/Neo: STC-1 Cells

Cellular health evaluated by HCA analysis. The general health of the exposed cell populations was evaluated by a multiparameter approach on the HCA platform. Hoechst-staining of nuclei provided information on cell number (object count) and possible morphological changes were monitored using nuclear area (NA) and nuclear intensity (NI). A mitochondrial specific fluorescent label was used to quantitate mitochondrial mass (MM) and the mitochondrial membrane potential (MMP). Mean values, standard error of mean, and p-values for the Total mixture and six sub-mixtures are listed in Supplementary Table 1 (Shannon *et al*., 2019).

In summary, exposure to the Total mixture (1:10 to ×500 blood level) for 3 h did not significantly alter the cell number. For 24 h exposure, a reduction in cell number compared with SC (0.2% DMSO) was seen at the two highest concentrations tested (×100 and ×500) (Figure 1A). No significant changes were seen for the Cl mixture after 3 h exposure (Figure 1A), however after 24 h, all concentrations tested led to a significant reduction (Figure 1B). For the Br mixture at 3 h exposure, only ×500 led to a significant decrease (Figure 1A), whereas the 24 h exposure induced significant decreases at ×50 and higher (Fig. 1B). PFAA exposure had no effect at 3 h, but reduced cell number from ×1 blood level upwards after 24 h (Figure 1B). Cl +Br mixture did not induce any significant decrease after 3 h exposure, whereas 24 h treatment resulted in the significant decrease for ×50, ×100, and ×500 blood level (Figure 1B). For the PFAA + Cl mixture, a significant decrease in cell number was seen for ×100 and ×500 after 3 h exposure (Figure 1A). For 24 h, a decrease was already seen from ×50 upwards (Figure 1B). Br + PFAAs mixture exposure at 3 h only caused significant decrease at ×500 blood

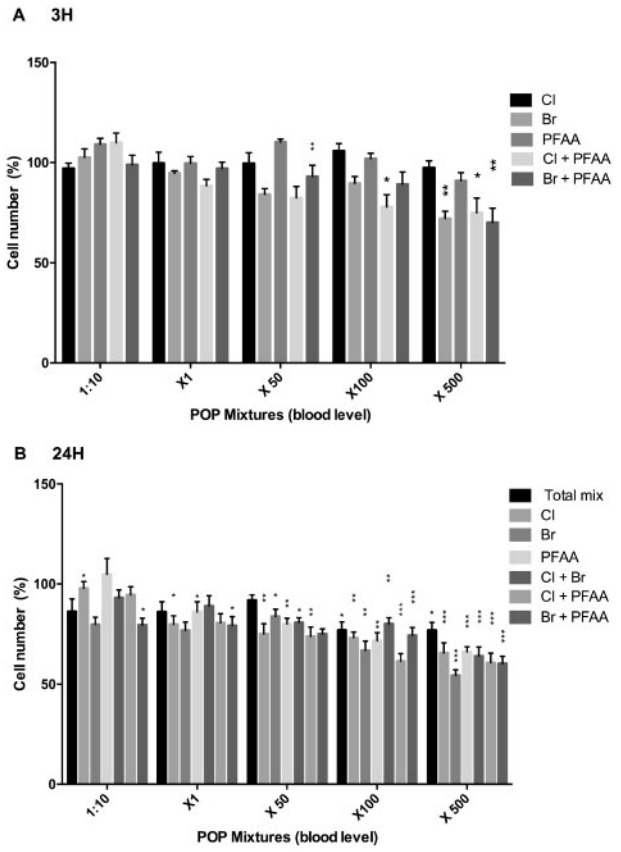

Figure 1. Cell number changes in pGIP/neo: STC-1 cells after (A) 3 h and (B) 24 h exposure to POP mixtures. Cells were exposed to five concentrations of POPs (×1:10, ×1, ×50, ×100 and ×500 blood levels) and cytotoxicity was measured by cell number parameter. Data are expressed as a percentage of untreated control for each parameter; mean ± SEM, $n = 3$. $^{*}p \leq .05$, $^{**}p \leq .01$, and $^{***}p \leq .001$ represent significant cytotoxic effects. Abbreviations: POP, persistent organic pollutants; SEM, standard error of the mean.

level (Figure 1A), however, the 24 h exposure induced significant decrease at all tested concentrations (Figure 1B).

Apart from cell number, cellular health was further evaluated using the area and fluorescent intensity of selected nuclei as pre-lethal markers. Only the Br, PFAA, and Br + PFAA mixtures induced significant NA decrease at ×500 after 24 h exposure (Figure 2). Other mixtures did not change NA significantly at all tested levels for 3 or 24 h (Supplementary Table 1A and Supplementary Table 1B) Exposure to any of the seven mixtures tested did not induce any significant change of NI (Supplementary Table 1A and Supplementary Table 1B).

Finally, markers for mitochondrial health, MM, and MMP were evaluated. For MM, no significant changes were observed for any of the seven mixtures at the five concentrations tested. A summary of the results is shown in Supplementary Table 1A (3 h exposure) and Supplementary Table 1B (24 h exposure) (Shannon *et al.*, 2019). Cytotoxicity results in fewer cells and a reduction in MMP. The uptake of MMPD is dependent on MMP levels. Therefore, lower MMP levels are reflected in a decrease of MMPD intensity as quantified by HCA measurement. Such changes are illustrated in Figure 3 whereby the PFAAs + Br mix at ×500 blood levels induced nuclear and mitochondrial changes as shown by a decrease in cell number and decrease in fluorescence intensity when compared with the DMSO SC. Visual changes in decreased MMPD dye intensity corresponding to MMP levels after 24 h exposure to the POP mixtures is illustrated in Figure 4.

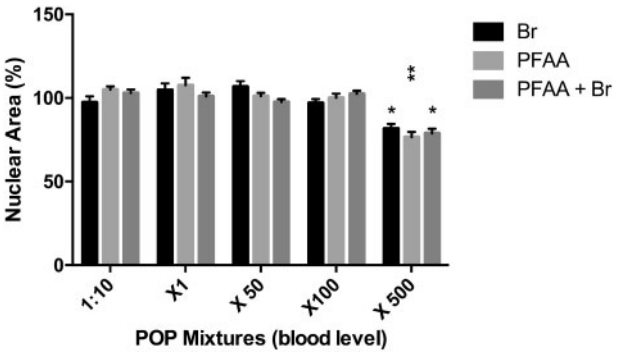

Figure 2. Nuclear area changes in pGIP/neo: STC-1 cells after exposure to POP mixtures. Cells were exposed to concentrations of POPs (1:10, ×1, ×50, ×100 and ×500 blood levels) for 24 h and cytotoxicity measured by NA parameter. Data are expressed as a percentage of untreated control for each parameter; mean ± SEM, $n = 3$. $^{*}p \leq .05$, $^{**}p \leq .01$, and $^{***}p \leq .001$ represent significant cytotoxic effects. Abbreviations: POP, persistent organic pollutants; SEM, standard error of the mean; NA, nuclear area.

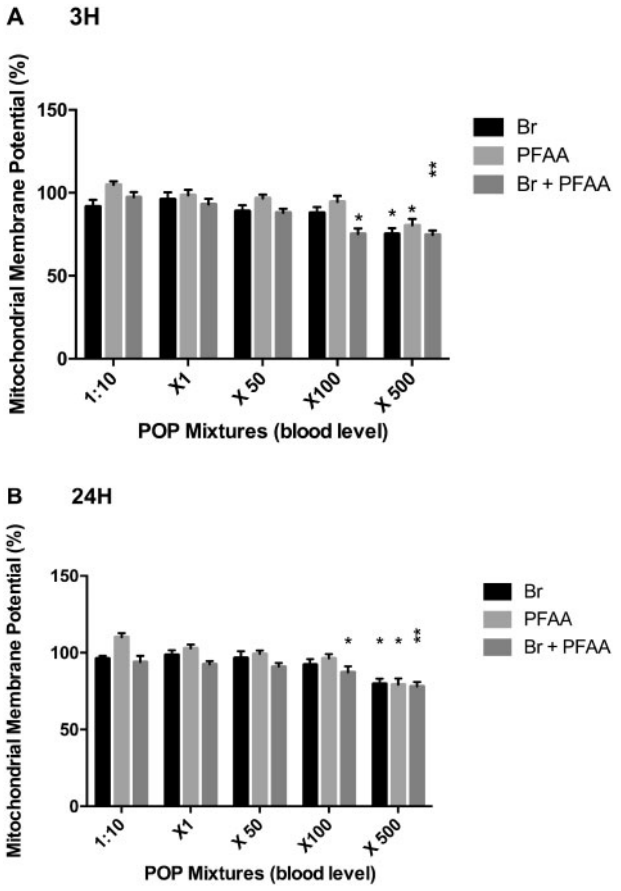

Figure 3. Mitochondrial changes after exposure to three POP mixtures, Br, PFAA, and Br + PFAA, in pGIP/neo: STC-1 cells. Cells were exposed to concentrations of POPs (1:10, ×1, ×50, ×100 and ×500 blood levels) for (A) 3 h and (B) 24 h, cytotoxicity measured by the HCA endpoint MMP. Data are expressed as a percentage of untreated control for each parameter; mean ± SEM, $n = 3$. $^{*}p \leq .05$, $^{**}p \leq .01$, and $^{***}p \leq .001$ represent significant cytotoxic effects. Abbreviations: POP, persistent organic pollutants; SEM, standard error of the mean; MMP, mitochondrial membrane potential.

GLP-1 secretion by pGIP/neo: STC-1 cells measured by ELISA analysis of culture medium. Exposure of the pGIP/neo: STC-1 cells were limited to non-cytotoxic ranges. Thus, for 3 h exposure,

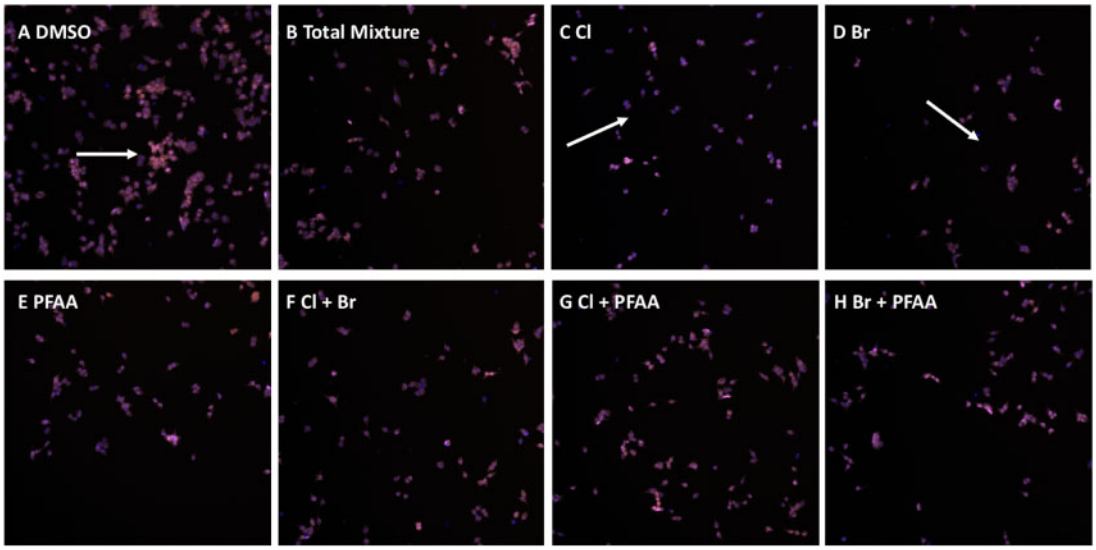

Figure 4. HCA images for (A) negative control (DMSO) and (B–H) POP mixtures after 24 h exposure. Each image was acquired at ×20 objective magnification using Hoechst dye (nuclear staining) and MMPD dye (mitochondrial staining). The arrow in (A) shows a higher cell number and more intense stained mitochondria, arrows in (C) and (D) show decreased cell number and decreased MMPD dye intensity. Abbreviations: POP, persistent organic pollutants; MMPD, mitochondrial membrane potential dye, HCA, high content analysis; DMSO, dimethyl sulfoxide.

concentrations of the POP mixtures tested ranged from 1:10 to ×500 blood level. For 24 h exposure, the concentration range was limited from 1:10 to ×50. Basal levels of GLP-1 were measured in cells exposed to 0.2% DMSO only (SC). Forskolin combined with IBMX was used as a PC which triggers GLP-1 secretion via cAMP-elevating and intracellular calcium pathways (Simpson *et al.*, 2007). The collected media were analyzed by ELISA to quantify active GLP-1. Mean values, standard error of mean, and *p*-values for all mixtures tested, at all concentrations are summarized in Supplementary Table 2 for 3 h exposure (2A) and 24 h (2B) (Shannon *et al.*, 2019).

The Total mixture increased GLP-1 secretion significantly at ×500 blood levels after 3 h exposure (Figure 5A); whereas secretion was enhanced significantly at all tested levels (1:10, ×1, and ×50 blood level) after 24 h exposure (Figure 5B). For the Cl mixture, 3 h exposure significantly increased GLP-1 secretion at all concentrations tested (Figure 5B); however, 24 h exposure only promoted secretion at ×1 and ×50 blood level (Figure 6). The Br mixture failed to induce any significant change in GLP-1 secretion compared with the SC at 3 h (Figure 5B), whereas a significant increase in GLP-1 levels was detected after 24 h exposure at ×1 and ×50 blood level (Figure 6). The PFAA mixture led to a significant GLP-1 secretion increase at the highest concentration after 3 h (Figure 5B), and the pro-longed exposure resulted in a significant increase at ×1 and ×50 blood level (Figure 6). The Cl + Br and Cl + PFAAs mixtures induced significant GLP-1 increases at ×500 blood level after 3 h exposure (Figure 5B), and at all tested concentrations after 24 h exposure (Figure 6). For the Br + PFAAs mixture, both 3 and 24 h exposure resulted in significant increases at the two highest tested concentrations (Figures 5B and 6).

The effects of POP Mixture Exposure on GLP-1 Receptor Redistribution

A standard curve was generated using a concentration range of GLP-1 (1–300 nM) to drive receptor internalization. The HCA images show the location of the fluorescently tagged GLP-1R after 1 h treatment of SC and GLP-1 (at 300 nM) (Supplementary Figure 1). The EC_{50} value was calculated as 37 nM GLP-1. The seven POP mixtures at all concentrations tested did not change internalization of the GLP-1R in U2OS-GLP1R-EGFP cells with or without the presence of GLP-1 (Supplementary Figure 2).

DISCUSSION

Associations between POP exposure and obesity/diabetes risk have recently been highlighted without the underlying mechanisms being identified (Dirinck *et al.*, 2011; Evangelou *et al.*, 2016; Lee *et al.*, 2014, 2017a,b). The present study investigated the ability of human relevant POP mixtures (Berntsen *et al.*, 2017), to potentially exert their obesogenic/diabetogenic effects via disruption of gut cell health, GLP-1 secretion, and GLP-1R signaling, all of which are involved in weight and glucose homeostasis.

GLP-1 secretion from pGIP/neo: STC-1 cells was determined by ELISA. Five out of seven POP mixtures induced an increased GLP-1 secretion from the pGIP/neo STC-1 cells following 3 h exposure at higher concentrations (×100–×500 blood levels), whereas all mixtures promoted GLP-1 secretion in the 24 h study at ×1:10 to ×50. At this time point, higher concentrations (×100 and ×500 blood levels) induced significant cytotoxicity and thus were not tested here.

Interestingly, after 3 h exposure, the Total mixture induced a two times higher secretion of GLP-1 than any of its submixtures tested individually. The secretion level is also higher than the summed levels seen for the Cl, Br, and PFAA mixture suggestive of additive or synergistic interactions. But as summarized in Table 2A, interactions between two chemical groups are complex, with binary mixtures stimulating GLP-1 secretion to a lesser degree than would be expected by summing the two constituent mixtures.

Thus, the magnitude of GLP-1 induction by the different mixtures can be ranked as follows: Total mixture > Cl + PFAA > Br + PFAA > PFAA > Cl > Cl + Br > Br.

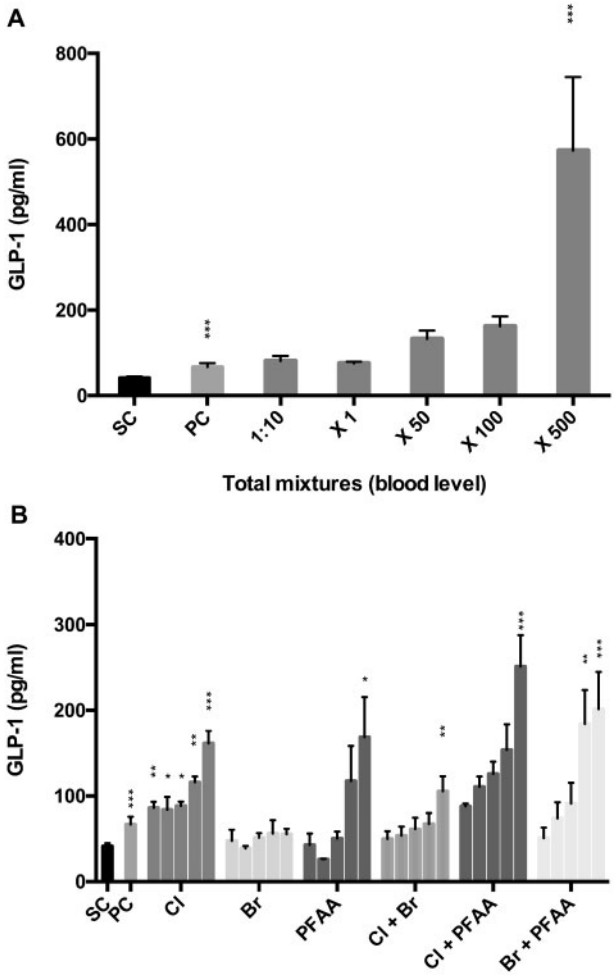

Figure 5. GLP-1 secretory responses of pGIP/neo: STC-1 cells during 3 h exposure to (A) Total mixture and (B) Cl, Br, PFAA, Cl + Br, Cl + PFAA, and Br + PFAA mixtures. Graph shows GLP-1 secretion from pGIP/neo: STC-1 cells (mean ± SEM, $n = 3$) following 3 h incubation with SC, PC, and each mixture at 1:10, ×1, ×50, ×100 and ×500 blood levels. $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$ represent significance. Abbreviations: PC, positive control; SEM, standard error of the mean; SC, solvent control; GLP-1, glucagon-like peptide 1.

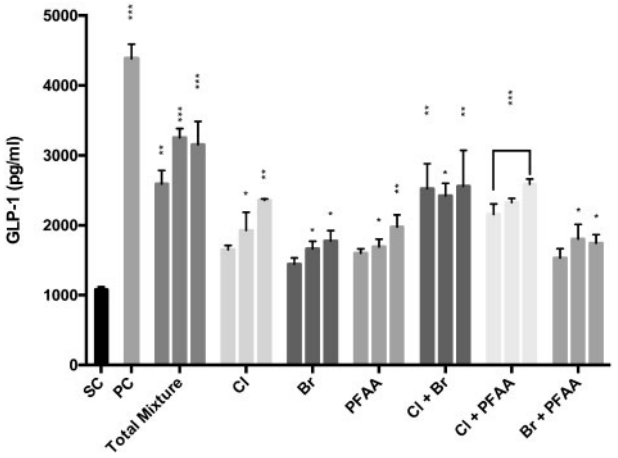

Figure 6. GLP-1 secretory responses of pGIP/neo: STC-1 cells during 24 h exposure to Total mixture, Cl, Br, PFAA, Cl + Br, Cl + PFAA, and Br + PFAA mixtures. Graph shows GLP-1 secretion from pGIP/neo: STC-1 cells (mean ± SEM, $n = 3$) following 3 h incubation with SC, PC, and (×1:10, ×1, and ×50 blood levels). $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$ represent significance. Abbreviations: PC, positive control; SEM, standard error of the mean; SC, solvent control; GLP-1, glucagon-like peptide 1.

The 24 h exposure induced considerable GLP-1 secretion even at concentrations as low as × 1:10 blood levels. At ×50 blood levels, the Total mixture still did not plateau out GLP-1 secretion as exposure of forskolin (10 μM), combined with IBMX (10 μM) still induced greater GLP-1 secretion. Secretion of GLP-1 peptide from pGIP/neo: STC-1 cells is triggered by several cellular pathways including cAMP/PKA, PKC, and intracellular calcium ions levels (Hand *et al.*, 2012). γ-HCH, PFOA, PFOS, and selective PCB congeners increased the levels of intracellular calcium in previous studies (Harada *et al.*, 2005; Liu *et al.*, 2011; Pessah *et al.*, 2006; Silvestroni *et al.*, 1997; Westerink, 2013). Only PBDE-47 increased the intracellular calcium levels at both 2 and 20 μM, but other more highly brominated congeners did not induce significant calcium fluctuation (Dingemans *et al.*, 2010, 2016). The different effects on GLP-1 secretion between the POP mixtures may be due to their various potential effects on intracellular calcium ion levels.

Taylor *et al.* (2013) reviewed epidemiological studies of POP exposure and diabetes and pointed out that organochlorine chemicals exposure were positively related to T2D, with nonorganochlorine POPs such as perfluoroalkyl acids and brominated compounds presenting less association. Our observations indicate however that perfluorinated, as well as brominated POPs should be evaluated in mechanistic assays related to T2D, as it is clear they may significantly contribute to the effect, or affect outcome through combinatorial effects.

HCA provides the means to investigate the cellular health of pGIP/neo: STC-1 cells at the level of pre-lethal cytotoxicity, whereby cells may still be alive but becoming unhealthy and jeopardizing the ability to function optimally (Clarke *et al.*, 2015; Wilson *et al.*, 2016). It has been proposed that both cell number and NA are among the most sensitive indicators of changes in cellular health (O'Brien *et al.*, 2006). The Total mixture only induced a decrease in cell number at the higher concentrations after 24 h exposure. Individual mixtures and sub-mixtures induced more remarkable changes (Figure 4). Cl mixtures decreased cell number after 24 h exposure at lower concentrations. Br induced MMP decrease and NA decrease for both 3 and 24 h exposure. The 3 h exposure to the PFAA mixture resulted in a significant reduction in cell number and effects on MMP at the higher concentrations. More profound toxic effects at lower concentrations were observed among these four mixtures following 24 h exposure. However, the Cl + Br mixture only led to a reduction of cell number for 24 h at high concentrations, which suggests that the Cl compounds may compensate for the Br compounds induced cellular damage. Cl + PFAA mixture led to cell number reduction after 3 h, but did not show in Cl or PFAA mixtures, which indicates an additive effect when Cl and PFAA chemicals are mixed together. However, the additive effect was not observed during 24 h exposure. The Br + PFAA mixture led to a MMP decrease at lower concentrations than individual Br or PFAA mixtures. Thus, the Br and PFAA compounds may lead to an additive effect on cellular damage via mitochondrial impairment. In summary, reduction in cell number can be ranked as: Br > Br + PFAA > Cl + PFAA > Cl + Br > Cl > PFAA > Total mixture.

The decrease in MMP along with a decrease in cell number is indicative of mitochondrial dysfunction, and in some cases this is considered as an early marker for cell death pathways (Bernardi *et al.*, 1992; Gottlieb *et al.*, 2003). In some cases, a

Table 2. The Comparison of Measured GLP-1 Peptide Levels Induced By Co-mixtures to Theoretical Sum (∑= *Sum*) of Corresponding Sub-mixtures

A. 3 h and × 500 blood levels				B. 24 h and × 50 blood levels			
Mixture				Mixture			
Mixture	Measured		∑= *Sum*	Mixture	Measured		∑= *Sum*
		Cl	161.1			Cl	2357
		Br	55.1			Br	1774
		PFAA	168.3			PFAA	1977
Total	**572.6*****	≫	∑=384.5	**Total**	**3154*****	≪	∑=6108
		Cl	161.1			Cl	2357
		Br	55.1			Br	1774
Cl + Br	**105.3****	≪	∑=216.2	**Cl + Br**	**2577****	≪	∑=4131
		Br	55.1			Br	1774
		PFAA	168.3			PFAA	1977
Br + PFAA	**201.4*****	≪	∑=223.4	**Br + PFAA**	**1741***	≪	∑=3751
		Cl	161.1			Cl	2357
		PFAA	168.3			PFAA	1977
Cl + PFAA	**250.8*****	≪	∑=329.4	**Cl + PFAA**	**2587*****	≪	∑=4334

$^{*}P \leq .05$,
$^{**}P \leq .01$,
$^{***}P \leq .001$

decrease in cell number was observed without changes in nuclear and mitochondrial parameters. This reduction is often the first consequence of toxic impact, even before cytotoxic parameters appear within the cell, therefore making it one of the most sensitive markers of cell health (Lucke and Mumtsidu, 2010). Abnormal mitochondrial function has been associated with diseases such as diabetes and metabolic syndrome in epidemiological studies, highlighting the significance of the results found in the present study in relation to mitochondrial effects (Park et al., 2013). The Br mixture presented higher cytotoxicity than the other POP mixtures and induced a decrease in secretion of GLP-1 by the pGIP/neo: STC-1 gut cells. In contrast, the Total mixture induced the greatest increase of any of the mixtures on GLP-1 secretion in the pGIP/neo: STC-1 gut cells. Thus, gut cell cytotoxicity may reduce function and secretion of GLP-1. On the other hand, the toxic impact may result in an altered gut microbiome which has been linked to increased obesity risk (Riva *et al.*, 2017).

No GLP-1R translocation was observed in the U2OS-GLP1R-EGFP cells after exposure to any of the POP mixtures at any tested concentration. This is the first study to investigate the interaction of POPs with GLP-1R signaling. Thus, the POP mixtures did not disrupt the GLP-1R.

CONCLUSIONS

To our knowledge, this is the first study to show that different classes of POPs can affect GLP-1 secretion. Interestingly, both "legacy" POPs such as chlorinated and brominated chemicals, as well as the emerging perfluorinated chemicals were found to be active inducers of GLP-1 secretion. Moreover, complex mixtures combining these three groups showed combinatorial effects. Strikingly, this effect was seen with concentrations of the chemicals at levels to those observed in human blood. No effect at the level of GLP-1R signaling was observed. These findings suggest POP exposure roles may contribute to the increasing rates of the pathogenesis of T2D and obesity. This study provides useful insight into the observations that POPs alter glucose homeostasis and should help guide future studies in this area.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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