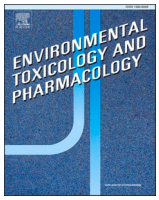
Environmental Toxicology and Pharmacology 104 (2023) 104299

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap



Targets for pollutants in rat and human pancreatic beta-cells: The effect of prolonged exposure to sub-lethal concentrations of hexachlorocyclohexane isomers on the expression of function- and survival-related proteins

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ARTICLE INFO

Edited by Dr. M.D. Coleman

Keywords:
Diabetes
Beta-cells
Hexachlorocyclohexane
Insulin
ATP citrate lyase
Perilipin-2

ABSTRACT

Decades after most countries banned hexachlorocyclohexane, HCH isomers still pollute the environment. Many studies described HCH as a pro-diabetic factor; nevertheless, the effect of HCH isomers on pancreatic beta-cells remains unexplored. This study investigated the effects of a one-month exposure to α-HCH, β-HCH, and γ-HCH on protein expression in human (NES2Y) and rat (INS1E) pancreatic beta-cell lines. α-HCH and γ-HCH increased proinsulin and insulin levels in INS1E cells, while β-HCH showed the opposite trend. α-HCH altered the expression of PKA, ATF3, and PLIN2. β-HCH affected the expression of GLUT1, GLUT2, PKA, ATF3, p-eIF2α, ATP-CL, and PLIN2. γ-HCH altered the expression of PKA, ATF3, PLIN2, PLIN5, and IDH1. From the tested proteins, PKA, ATF3, and PLIN-2 were the most sensitive to HCH exposure and have the potential to be used as biomarkers.

1. Introduction

Most countries have banned hexachlorocyclohexane (HCH) mixtures and their-HCH isomers, such as lindane, decades ago. Nevertheless, even without fresh input, HCH isomers remain a burden to the environment due to their persistence. The number of scientific papers recently published with HCH as a keyword shows that HCH pollution of the environment remains a current problem (Capparelli et al., 2023; Gandla et al., 2023; Iwasaki et al., 2023; Rafeeinia et al., 2023; Reina-Perez et al., 2023; Xu et al., 2023).

Three HCH isomers dominate the HCH pollution of the environment: α-HCH, β-HCH, and γ-HCH. In the last two years (2021–2022), their presence has been detected worldwide, including lake sediments in Northeast China (Zhao et al., 2022), bovine milk (Sharma et al., 2021), drinking water in Mexico (Rodriguez et al., 2022), and Brazil (Panis et al., 2022), Nigerian food crops (Omeje et al., 2021), sediments in Indonesia (Oginawati et al., 2022), agricultural soils in Tanzania (Nyihirani et al., 2022), surface sediments in Antarctica (Ma et al., 2021), a coal mining subsidence area in China (Chen et al., 2022), river waters in Iran (Fard et al., 2022), the breast milk of women in China (Dong et al., 2022), vegetables from India (Sharma et al., 2022), fish from the Nile river, Egypt (Saleh et al., 2021) and adipose tissue of people from Southern Spain (Salcedo-Bellido et al., 2022).

HCH isomers can enter humans through food, water, air, or through the skin (Chen, 2005). Our fat tissue stores a large portion of HCH isomers that enter our body; in rats, isomers also accumulate in the brain, liver, or kidney (Chen, 2005). The toxicity of the three HCH isomers differs. γ-HCH is neurotoxic: acute exposure to lindane causes ataxia, disorientation, tremors, seizures, and death, while chronic exposure inhibits liver function, causes cardiac arrhythmias, and affects the menstruation cycle (Nolan et al., 2012). The acute effects of α-HCH, which consists of two optical isomers, are quite similar to those of γ-HCH: it works as a neurostimulant and convulsant (Chen, 2014). However, unlike γ-HCH, which interferes with estrogen signaling (Briz et al., 2011), both enantiomers of α-HCH inhibit androgen receptor activity (Pavlikova et al., 2012). The fate of α-HCH enantiomers differs depending on the environment: e.g., in polar mammals, (+)-α-HCH is more abundant than (-)-α-HCH due to its higher ability to biomagnify (Wiberg et al., 2000). β-HCH is primarily a neuro depressant (Chen, 2014) and has xenoestrogen activity (Imazaki et al., 2015; Steinmetz et al., 1996).

Concerning the connection between the presence of

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https://doi.org/10.1016/j.etap.2023.104299
Received 15 June 2023; Received in revised form 12 October 2023; Accepted 17 October 2023
Available online 20 October 2023

hexachlorocyclohexane isomers in the human body and diabetes, the epidemiological studies are persuasive: higher β-HCH levels in organisms correlate with the incidence of diabetes (Berg et al., 2021; Gasull et al., 2018; Han et al., 2020; Ryander et al., 2015; Schwarz et al., 2021; Tyagi et al., 2021; Zong et al., 2018). Also, β-HCH was one of four organochlorine pollutants whose presence correlated with decreased insulin secretion in humans (Lee et al., 2017). However, data concerning the effects of α- and γ- isomers are scarce (Al-Othman et al., 2014).

Only a few published studies describe the effects of β-HCH on pancreatic beta cells: β-HCH exposure increased ROS production and decreased insulin secretion by rat pancreatic beta cells RIN-m5F (Park et al., 2020) and significantly decreased insulin secretion and increased insulin content in the rat pancreatic beta cells INS1E (Lee et al., 2017). However, published studies on pancreatic beta-cells are limited to detecting oxidative stress and insulin production. Indeed, there is no extant data about the effects of α-HCH and γ-HCH on pancreatic beta-cells. This study aimed to test how a one-month exposure to α-, β-, or γ-HCH affected the expression of proteins related to the survival and function of human and rat pancreatic beta-cells. The HCH isomers differ only in the position (axial or equatorial) of chlorines, so we wanted to evaluate how such differences affect their impact on pancreatic beta-cells. We were interested in finding changes in protein expression that might compromise cells' survival and function in the longer term. Another aim of this study was to detect proteins sensitive to exposure to pollutants in pancreatic beta-cell lines that could be potentially used as biomarkers.

2. Material and methods

2.1. Material

We obtained α-HCH (α-1,2,3,4,5,6-Hexachlorocyclohexane; 33856–50MG), β-HCH (β-1,2,3,4,5,6-Hexachlorocyclohexane; 33376–100MG), lindane (γ-1,2,3,4,5,6-Hexachlorocyclohexane; 45548–250MG), and RPMI medium from Sigma-Aldrich (Merck Life Science, Prague, Czech Republic). The primary antibodies to ATP citrate lyase, aconitase 2, isocitrate dehydrogenase 1, glutathione reductase, superoxide dismutase 1, binding immunoglobulin protein, phosphorylated inositol-requiring enzyme 1α (S724), and hypoxanthine-guanine phosphoribosyltransferase were purchased from Abcam (Abcam, Cambridge, UK); primary antibodies to biliverdin reductase B, glucose transporter 1, glucose transporter 2, perilipin-2, and perilipin-5 from Fisher Scientific (Thermo Fisher Scientific, Rockford, IL, USA); primary antibody to activating transcription factor 3, and actin from Sigma-Aldrich (Merck Life Science, Prague, Czech Republic); and primary antibody to phosphorylated protein kinase A (Thr197) from Cell Signaling (https://www.cellsignal.com). To determine protein concentrations, Pierce™ B.C.A. Protein Assay Kit was used (Thermo Fisher Scientific, Rockford, IL, USA).

2.2. Cell culture

For our experiments, we used the human pancreatic beta-cell line NES2Y with constitutive insulin secretion (kindly provided by Dr. Roger F. James, Department of Infection, Immunity and Inflammation, University of Leicester) and the rat pancreatic beta-cell line INS1E with glucose-stimulated insulin secretion (kindly provided by Dr. Claes B. Wollheim at the Centre Medical Universitaire de Genève, Geneva, Switzerland). The cells were cultured in a medium based on RPMI 1640, containing phenol red, L-glutamine, sodium pyruvate, HEPES, penicillin, streptomycin, and supplemented with 10% fetal bovine serum (FBS), as previously described (Pavlikova et al., 2015). The cells were maintained in a humidified atmosphere of 5% CO2 in air at 37 °C.

2.3. Neutral red assay

The cells were seeded into a 96-well microplate using the medium described above: the NES2Y cells at a density of 10 000 cells per well and INS1E cells at a density of 45 000 per well. After 24 h (for NES2Y cells) or 48 h (for INS1E cells), the cells were exposed to DMSO (dimethyl sulfoxide, solvent control) or the tested concentrations of α-HCH, β-HCH, or γ-HCH. The concentration of DMSO (in which HCH isomers were dissolved) in the medium did not exceed 0.5%. We tested the following HCH isomers concentrations: 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM. After 24 h and 48 h, the viability of cells was measured using a protocol developed by (Repetto et al., 2008) with modifications according to (Pavlikova et al., 2012). The medium with HCH isomers was removed, and cells were washed twice with PBS. Then, 100 μl of medium containing neutral red, prepared according to (Repetto et al., 2008), was added to each well with cells and three empty wells (later used as blank), and the microplate was incubated at 37 °C for two hours. After incubation, the medium was removed, the cells were washed carefully with PBS and lysed using 200 μl of lysing buffer per well (1% acetic acid in 50% ethanol). The absorbance was measured at 570 nm.

2.4. Exposure to pollutants

We maintained NES2Y and INS1E cells in a usual way (NES2Y were passaged twice per week; INS1E once per week and, after four days, the medium was replaced with a fresh one) in a medium containing 10 μM α-HCH, β-HCH, γ-HCH, and DMSO as solvent control. The concentration of DMSO (the stock solutions of hexachlorocyclohexane isomers were dissolved in DMSO) was 0.5%. After one month, cells were subjected to further analysis.

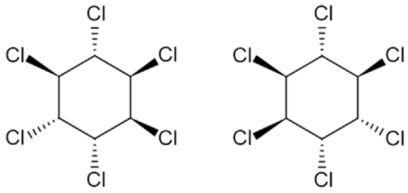
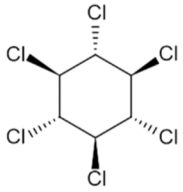
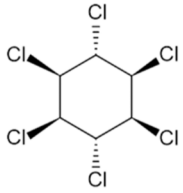
2.5. Western blot

After the one-month exposure to HCH isomers, the cells were trypsinized, washed three times with PBS, and the cell pellet was frozen at −80°C for at least twelve hours. The next day, the pellet was dissolved in RIPA lysis buffer and incubated for at least one hour at 4°C. The samples were centrifuged to separate the protein solution from cell debris, and the supernatants were transferred into a new Eppendorf tube. The protein concentrations were quantified employing the BCA commercial kit.

We performed western blotting as described previously (Nemcova-Furstova et al., 2019; Sramek et al., 2019) with minor modifications. 20 μg samples of total protein (whole cell lysates) and 10% or 12% polyacrylamide were used for separation. After the electrophoresis, proteins were blotted to the nitrocellulose membrane (70 min, 100 V) employing a Bio-Rad device. The membrane was blocked using 5% low-fat milk in TBST for 60 min, and then it was changed for the primary antibody.

We applied the following dilutions of primary antibodies: 1:1000 for the rabbit monoclonal antibody to ATP citrate lyase (ATP-CL; ab40793), 1:1000 for the rabbit monoclonal antibody to aconitase 2 (ACO2; ab129069), 1:1000 for the rabbit monoclonal antibody to isocitrate dehydrogenase 1 (IDH1; ab172964), 1:1000 for the rabbit polyclonal antibody to glutathione reductase (Glu-Red; ab16801), 1:1000 for the rabbit monoclonal antibody to superoxide dismutase 1 (SOD1; ab51254), 1:1000 for the rabbit polyclonal antibody to biliverdin reductase B (BLVRB; 17729–1-AP), 1:5000 for the rabbit polyclonal antibody to phosphorylated protein kinase A (Thr197) (p-PKA; #4781), 1:300 for the rabbit polyclonal antibody to cyclic AMP-dependent transcription factor (ATF3; HPA001562), 1:10 000 for the rabbit polyclonal antibody to binding immunoglobulin protein (BiP; ab21685), 1:500 for the rabbit polyclonal antibody to the phosphorylated inositol-requiring enzyme 1α (S724) (p-IRE; ab48187), 1:5000 for the rabbit polyclonal antibody to the phosphorylated eukaryotic translation initiation factor 2 A (Ser51) (p-eIF2α; #9721); 1:1000 for the rabbit polyclonal antibody to glucose transporter 1 (GLUT1; 21829–1-AP); 1:1000

Table 1
The chemical structure and log $K_{O/W}$ of hexachlorocyclohexane isomers. The log $K_{O/W}$ values were taken from (Xiao et al., 2004).

Name	Chemical structure	Log $K_{octanol/water}$ (25°C)
α-HCH	 (+)-α-HCH (-)-α-HCH	3.81
β-HCH		3.80
γ-HCH		3.72

for the rabbit polyclonal antibody to glucose transporter 2 (GLUT2; 20436–1-AP); 1:1000 for the rabbit polyclonal antibody to perilipin 2 (PLIN2; 15294–1-AP), and 1:1000 for the rabbit polyclonal antibody to perilipin 5 (PLIN5; 26951–1AP). As a loading control, we used dilution 1:1000 for the mouse polyclonal antibody to beta-actin (act; a3853) or 1:10 000 for the rabbit polyclonal antibody to hypoxanthine-guanine phosphoribosyltransferase (HPRT; ab109021). After washing three times with PBS, the corresponding horseradish peroxidase-conjugated secondary antibodies were used (Proteintech: SA00001–1, SA00001–2). The chemiluminescent signal was detected using the Supersignal reagent from Pierce (Rockford, IL, USA) and the ChemiDoc device (Bio-Rad).

2.6. ELISA

The intracellular insulin and proinsulin level was determined using commercial ELISA kits (Mercodia, Uppsala, Sweden; 10–1232–01, 10–1250–01) following the manufacturer's instructions. The protein samples were diluted to stock solutions of 1 μg/ μl. Then, the stock solutions were diluted 1:2000 with the Mercodia Diabetes Sample Buffer (Mercodia, Uppsala, Sweden, 10–1195–01) and used for experiments. After the ELISA experiment, the protein concentrations of the 1 μg / μl sample solutions were measured using the BCA kit (Pierce™ BCA Protein Assay Kit, #23227). The results from ELISA were adjusted to the actual concentrations of the stock solutions.

2.7. Statistical analysis

We determined statistical significance by one-way ANOVA – Dunnet's test employing SigmaPlot 14.0.

3. Results and discussion

Persistent pollutants are stored in human fat tissue and, from there, slowly released into the blood. Therefore, human beta-cells are exposed to potentially growing concentrations for their entire lifespan. If it were possible to mimic this exposure in lab conditions, measuring insulin secretion after exposure would represent the only information needed to evaluate the toxic potential of the measured pollutants. Unfortunately, in lab conditions, the exposure time is limited. Most in vitro experiments measured insulin secretion after one or two days of exposure (Lee et al., 2017; Park et al., 2021); in the case of in vivo experiments, the exposure time usually reached several weeks (Bahadar et al., 2015; Cetkovic-Cvrlje et al., 2016). If such experiments showed no alterations in insulin secretion, the question is whether it follows that a one-year

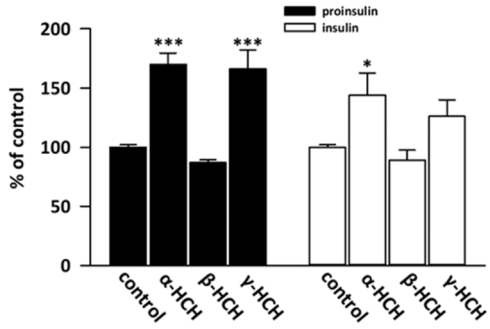


Fig. 2. The levels of intracellular insulin and proinsulin in rat pancreatic beta-cells INS1E exposed to 10 μM concentrations of α-HCH, β-HCH, and γ-HCH for one month detected by a commercial ELISA kit. The graph shows the average of four independent experiments ± SEM. * ** means statistical significance ($p < 0.001$), * means statistical significance ($p < 0.05$) determined by ONE-WAY ANOVA (Dunnet's test).

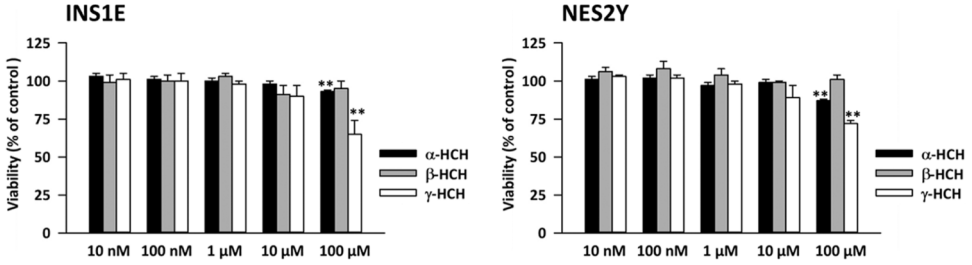


Fig. 1. The viability of rat pancreatic beta-cells INS1E and human pancreatic beta-cells NES2Y when exposed to 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM of α-HCH, β-HCH, and γ-HCH for 48 h detected by a Neutral red assay. The graph shows the average of three independent experiments ± SEM. * * means statistical significance ($p < 0.01$) determined by ONE-WAY ANOVA (Dunnet's test).

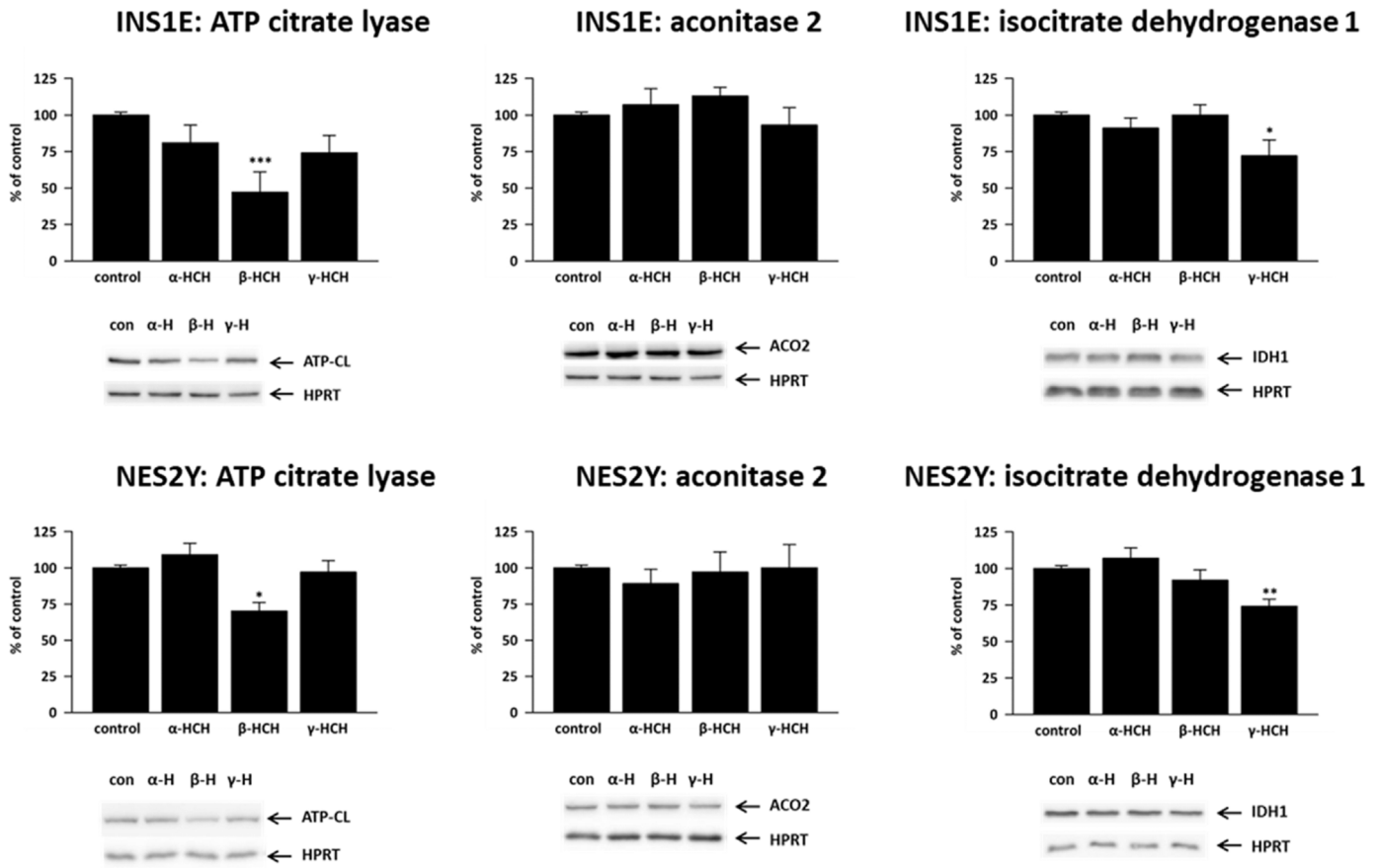


Fig. 3. The protein expression of ATP citrate lyase (ATP-CL), aconitase 2 (ACO2), and isocitrate dehydrogenase 1 (IDH1) in the rat (INS1E) pancreatic beta-cells and human (NES2Y) pancreatic beta-cells exposed to 10 μM concentrations of α-HCH, β-HCH, and γ-HCH for one month detected by western blot. Actin or HPRT was used as a loading control. The graphs represent an average of densitometric analyses of at least four western blots made of four independent sets of samples ± SEM. Below each graph, a representative western blot is shown. * means statistical significance ($p < 0.05$), * * means statistical significance ($p < 0.01$), * ** means statistical significance ($p < 0.001$) determined by ONE-WAY ANOVA (Dunnet's test).

exposure to the same concentration of the same chemical would have the same effect. Currently this is not possible to predict and long-term exposure to pollutants may gradually affect the pancreatic beta-cells homeostasis. Subtle protein expression or function changes can precede an actual alteration of beta-cell core function. The changes can build up over time and only then alter insulin secretion.

Our project focused on how exposure to HCH isomers altered protein expression in pancreatic beta-cells, including proinsulin and insulin expression. We searched for small changes in the expression of proteins participating in insulin production regulation and beta-cell survival. Such proteins could be indicators of future beta-cell dysfunction and used for toxicity monitoring. Because one-month exposure is still much shorter than the typical human exposure, we decided to use higher concentrations than are usually detected in humans. Most detected HCH concentrations in human organisms are detected as ng/g of lipids (Helou et al., 2019; Charles et al., 2022; Muller et al., 2019; Ploteau et al., 2016) which could not be used for the present study. In those few studies that detected HCH per volume, the highest serum concentrations reached 0.5 μM (La Merrill et al., 2019; Lino and da Silveira, 2006). The concentration used in our study – 10 μM – is consistent with other similar projects published (Park et al., 2020, 2021; Rubini et al., 2020). Also, the beta-cell lines were exposed to individual HCH isomers rather than their mixture. No universal ratio of HCH isomers in human organisms exists; therefore, this study focused instead on the individual isomers' effects.

For our experiments, we chose the human pancreatic beta-cell line NES2Y with constitutive insulin secretion and the rat pancreatic beta-cell line INS1E with glucose-stimulated insulin secretion. The human beta-cell lines with glucose-stimulated insulin secretion include mainly EndoβC cells, which are difficult to maintain and would probably fail to survive one-month exposure to pollutants; therefore, we chose the NES2Y human beta-cell line. The human and rodent beta-cell differ in several aspects, including GLUT transporter expression, ability to form lipid droplets, and others (Eizirik et al., 1994; Klemen et al., 2017; McCulloch et al., 2011). Therefore using a human cell line, albeit imperfect, can bring information that using, e.g., two rodent cell lines cannot. The in vitro system of our study reflects current trends in environmental toxicology, which prefer in vitro studies over in vivo due to sustainability and ethical reasons.

As tested compounds, we used three stable hexachlorocyclohexane isomers: alpha, beta, and gamma (Table 1). Isomer alpha consists of two enantiomers; a racemic mixture 1:1 was used. Isomers differ in the positions of chlorines (axial and equatorial), which causes different tension and a different shape of the molecule.

3.1. Cell viability

We determined the viability of NES2Y and INS1E cells after exposure to various concentrations of hexachlorocyclohexane (HCH) isomers for 48 h by employing a Neutral Red Assay. From tested concentrations (10 nM, 100 nM, 1 μM, 10 μM, 100 μM), only the highest concentration - 100 μM – affected cell viability (Fig. 1). 100 μM α-HCH significantly decreased the human beta-cell viability to 87% of the control and the rat beta-cell viability to 93% of the control. The exposure to β-HCH failed to affect the cell lines' viability. 100 μM γ-HCH significantly decreased the

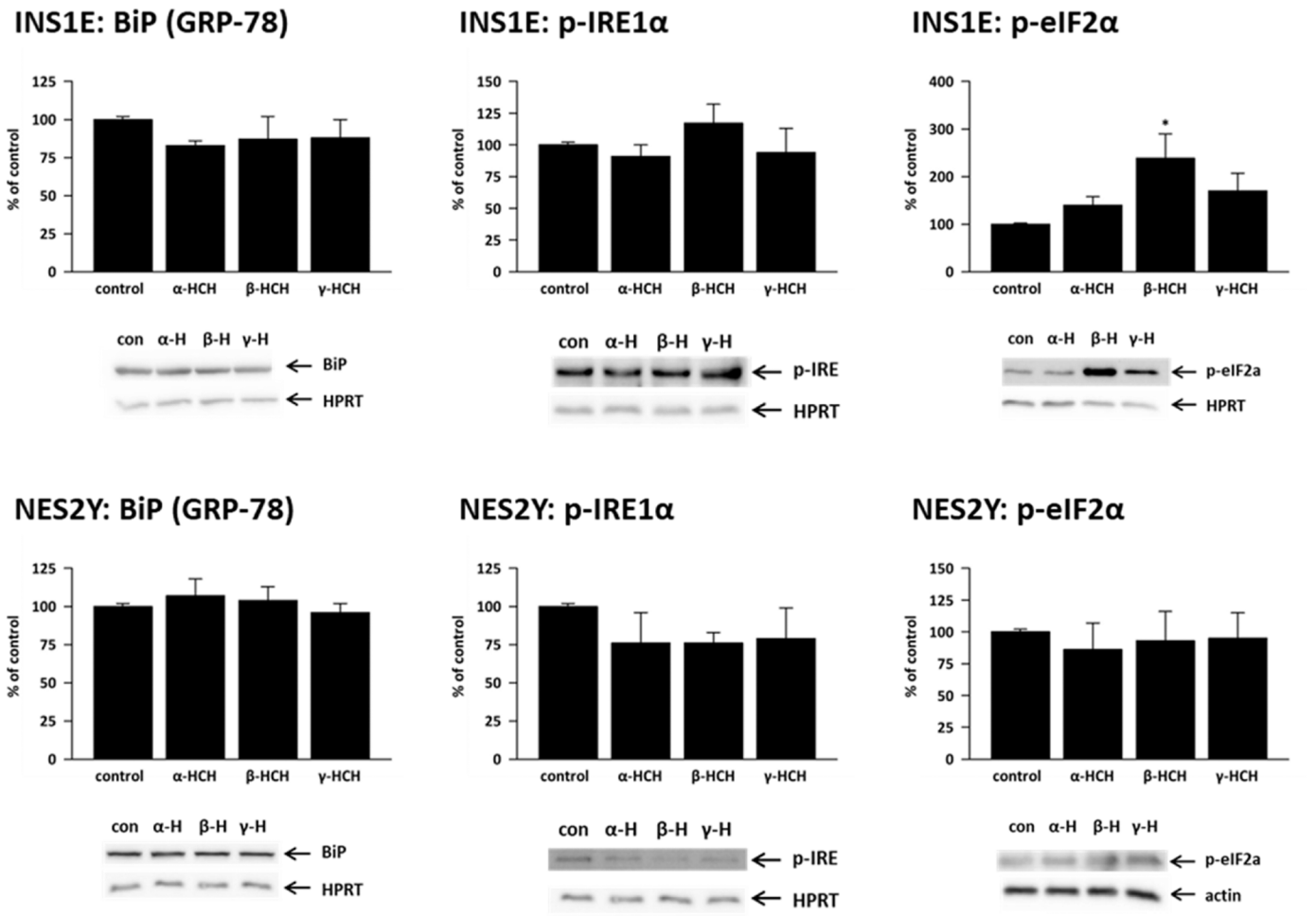


Fig. 4. The protein expression of binding immunoglobulin protein (BiP/GRP-78), inositol-requiring enzyme 1α (p-IRE1α), and phosphorylated eukaryotic initiation factor 2 (p-eIF2α) in the rat (INS1E) pancreatic beta-cells and human (NES2Y) pancreatic beta-cells exposed to 10 μM concentrations of α-HCH, β-HCH, and γ-HCH for one month detected by western blot. Actin or HPRT was used as a loading control. The graphs represent an average of densitometric analyses of at least four western blots made of three independent sets of samples ± SEM. Below each graph, a representative western blot is shown. * means statistical significance ($p < 0.05$) determined by ONE-WAY ANOVA (Dunnet's test).

viability of the human NES2Y cells to 72% of the control and the viability of the rat INS1E cells to 65% of the control (Fig. 1).

The acute toxicity of HCH isomers at 100 μM correlated with their solubility in DMSO: β-HCH (the least soluble) showed no toxicity even at this high concentration, while γ-HCH (the best soluble) significantly decreased the cell viability. α-HCH was moderately soluble and showed medium toxicity. β-HCH has the most stable structure of the three isomers and biomagnifies (Porta et al., 2013). We expected it to affect the pancreatic beta-cell lines most in the long term due to correlations revealed by published epidemiological studies (see Introduction). Nevertheless, after 24 and 48 h, beta-HCH failed to affect cell viability.

For all α-HCH and γ-HCH, the 10 μM concentration was the lowest concentration from our scale that did not affect the cell viability. Therefore, we used it for the one-month exposure for all HCH isomers. This concentration is higher than concentrations found in humans (the highest concentrations ranged from 1 to 2 μM) (Lino and da Silveira, 2006), but our project was planned as a mechanistic study. Also, we have experience with this type of exposure from our previous work with DDT and DDE (Pavlikova et al., 2019; Pavlikova et al., 2015). (See Supplementary data Fig. S1 for changes in HCH isomer levels in cell media during exposure.).

3.2. *Intracellular insulin and proinsulin level in rat beta-cells*

In rat beta-cells, α-HCH increased the proinsulin level to 177% of the control and the intracellular insulin level to 140% of the control; both changes were statistically significant (Fig. 2). β-HCH decreased the proinsulin level to 87% of the control; the change was not significant when analyzed by Dunnet's test (one-way ANOVA) but was significant when analyzed by the Student's t-test. The intracellular insulin level also dropped, but only insignificantly. γ-HCH increased the proinsulin level to 166% of the control and the intracellular insulin level to 126% of the control; only the increase in proinsulin level was statistically significant (Fig. 2).

We tested intracellular insulin and proinsulin levels only in INS1E rat pancreatic beta-cells; human NES2Y cells secrete insulin constitutively, and their insulin level is below the detection level. β-HCH slightly decreased the proinsulin level in rat INS1E cells; this change could participate in a pro-diabetic effect reported by epidemiological studies (see Introduction). In a short-term study (48-hour exposure), β-HCH (1 μM) increased the intracellular insulin level in rat INS1E cells and decreased insulin secretion (Lee et al., 2017). We hypothesize that the short-term and prolonged effects of β-HCH exposure may differ.

Interestingly, α-HCH and γ-HCH had the opposite effect than β-HCH: they increased intracellular proinsulin and insulin levels. No published data exist about the effects of these two HCH isomers on insulin

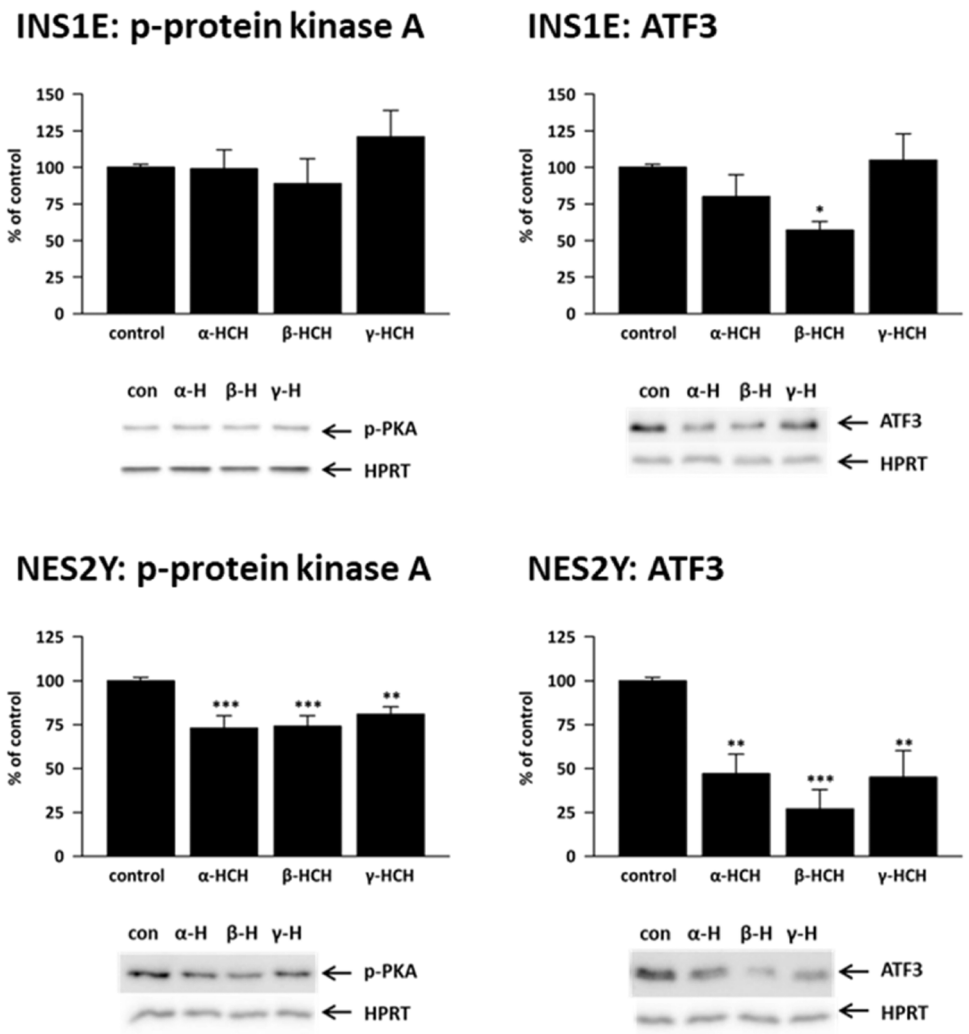


Fig. 5. The protein expression of phosphorylated protein kinase A (p-PKA) and activating transcription factor 3 (ATF3) in the rat (INS1E) pancreatic beta-cells and human (NES2Y) pancreatic beta-cells exposed to 10 μM concentrations of α-HCH, β-HCH, and γ-HCH for one month detected by western blot. Actin or HPRT was used as a loading control. The graphs represent an average of densitometric analyses of at least four western blots made of a minimum of three independent sets of samples ± SEM. Below each graph, a representative western blot is shown. * means statistical significance ($p < 0.05$), * * means statistical significance ($p < 0.01$), * ** means statistical significance ($p < 0.001$) determined by ONE-WAY ANOVA (Dunnet's test).

production. Nevertheless, overproduction of insulin, if it is accompanied by insulin oversecretion, could lead to increased body weight (more secreted insulin means faster removal of glucose from the blood into tissues and becoming hungry again). Hoyeck and coworkers (Hoyeck et al., 2022) described the oversecretion of beta-cells as a result of exposure to some non-dioxin-like polychlorinated biphenyls; hypothetically, α-HCH and γ-HCH can induce a similar effect. In human organisms, studies usually detect more than one HCH isomer (Al-Othman et al., 2014; La Merrill et al., 2019; Muller et al., 2019; Tyagi et al., 2021); therefore, the actual effect would depend on the ratio of the isomers present and the presence of other pollutants.

3.3. Enzymes involved in metabolic pathways

β-HCH significantly decreased the protein expression of ATP citrate lyase (ATP-CL) to 47% of the control in rat INS1E cells and 70% of the control in human NES2Y cells (Fig. 3). ATP-CL connects glucose and fatty acid metabolism by cleaving citrate into oxaloacetate and acetyl-CoA, a precursor of fatty acids. Some studies showed that ATP-CL inhibitors - hydroxycitrate and radicicol - inhibited glucose-stimulated insulin secretion in beta-cells (Flamez et al., 2002; Guay et al., 2007). Moreover, one of the ATP-CL downstream products is malonyl-CoA, which affects insulin secretion in the presence of fatty acids (Roduit et al., 2004). These data show that ATP-CL products participate in insulin secretion. β-HCH decreased ATP-CL expression in both beta-cell lines. We hypothesize that this effect can participate in the predicted pro-diabetic activity of β-HCH. Interestingly, palmitate (a saturated fatty acid) also decreased the expression of ATP-CL in beta-cells (Chu et al., 2010), besides causing other adverse effects on beta-cells viability and function (Chu et al., 2010). Hypothetically, exposure to β-HCH can potentiate adverse effects of saturated fatty acids on pancreatic beta-cells. Also, acetyl-CoA produced by ATP-CL is used for histone acetylation. Therefore, ATP-CL expression often correlates with acetylated histone levels (Bradshaw, 2021; Dominguez et al., 2021; Icard et al., 2020). Nevertheless, we have not found that correlation in our samples (see Supplementary data, Fig. S2).

γ-HCH significantly decreased the protein expression of isocitrate dehydrogenase 1 (IDH1) to 72% of the control in rat INS1Ecells and 74% of the control in human NES2Y cells (Fig. 3). Cytosolic isocitrate dehydrogenase 1 (IDH1) produces NADPH while turning isocitrate into α-ketoglutarate. The NADPH produced by isocitrate dehydrogenase 1 participates in insulin vesicle exocytosis (Campbell and Newgard, 2021). γ-HCH decreased IDH1 expression in beta-cells, which could negatively affect insulin secretion. The increased intracellular insulin

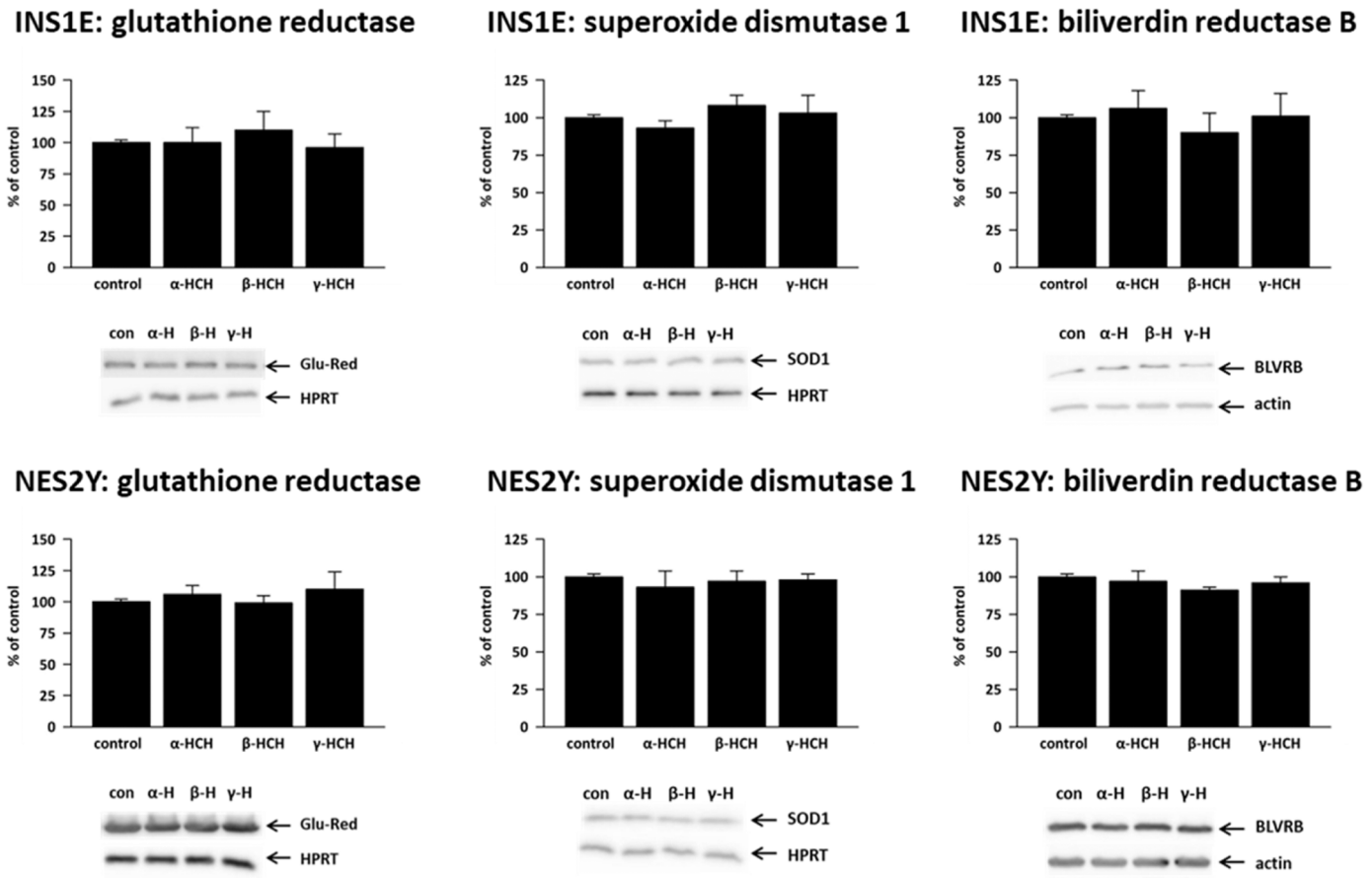


Fig. 6. The protein expression of glutathione reductase (Glu-Red), superoxide dismutase 1 (SOD1), and biliverdin reductase B (BLVRB) in the rat (INS1E) pancreatic beta-cells and human (NES2Y) pancreatic beta-cells exposed to 10 μM concentrations of α-HCH, β-HCH, and γ-HCH for one month detected by western blot. Actin or HPRT was used as a loading control. The graphs represent an average of densitometric analyses of at least four western blots made of four independent sets of samples ± SEM. Below each graph, a representative western blot is shown.

level in rat INS1E cells could result from these changes. Nevertheless, α-HCH exposure failed to change IDH1 expression in rat INS1E cells but still increased intracellular insulin levels; therefore, another mechanism can participate in this increase.

Mitochondrial aconitase 2 (ACO2) turns citrate into isocitrate and precedes IDH1 in the Krebs cycle. ACO2 remained unchanged after exposure to HCH isomers (Fig. 3).

All three proteins mentioned above are enzymes. Altered protein expression of an enzyme does not necessarily mean an increased or decreased level of the enzyme products; it can represent a compensation mechanism for altered protein functionality. Nevertheless, we believe that after exposure for as long as one month, altered enzyme expression more likely affected enzyme productivity than not.

3.4. Proteins involved in endoplasmic reticulum stress signaling

The protein expression of binding immunoglobulin protein (BiP) and the phosphorylated form of inositol-requiring enzyme 1α (S724) (p-IRE1α) remained unchanged after exposure to HCH isomers (Fig. 4).

The ER heat shock protein 70 family member BiP is an ATP-dependent chaperone that plays a critical role in unfolded protein response (UPR) during ER stress (Pobre et al., 2019). Inositol-requiring enzyme 1α (IRE1α) is an ER stress sensor (Marcu et al., 2002). The absence of increased BiP and p-IRE1α expression suggests that HCH isomers do not induce endoplasmic reticulum stress (ER stress) in beta-cells, at least not under the conditions used in our experiments. The ER stress represents a dangerous condition for beta-cells; it results in diminished insulin secretion and, if not contained, it leads to apoptosis.

β-HCH significantly increased the expression of phosphorylated eukaryotic initiation factor 2α (p-eIF2α) to 239% of the control in rat beta-cells; in human beta-cells, the expression remained unchanged (Fig. 4). Eukaryotic initiation factor 2α (eIF2α) is a stress-sensitive repressor of translation when phosphorylated; it saves the cell energy by decreasing general protein biosynthesis while increasing the translation of specific proteins involved in dealing with stress, e.g., ATF4 (Humeau et al., 2020; Marks et al., 2017). Increased p-eIF2α levels in rat INS1E cells exposed to β-HCH showed that β-HCH did induce stress in rat INS1E cells but not ER stress. The absence of the same effect in human NES2Y cells can reflect that human beta-cell line NES2Y with constitutive insulin secretion lack the robustness of the protein synthesis apparatus necessary in cells with glucose-induced insulin secretion.

3.5. Proteins involved in cAMP signaling

In human beta-cells, α-HCH decreased the protein expression of a phosphorylated protein kinase A (p-PKA) to 73% of the control, β-HCH to 74% of the control, and γ-HCH to 81% of the control; all changes were statistically significant (Fig. 5). In rat beta-cells, the p-PKA protein expression remained unchanged after exposure to HCH isomers. Protein kinase A (PKA) is a cAMP-binding enzyme that undergoes autophosphorylation to become fully active. cAMP signaling in beta-cells participates in the amplification of insulin secretion. All three HCH isomers decreased the level of phosphorylated PKA in human beta-cells, possibly because of decreased cAMP level. We hypothesize that HCH isomers affect the activity of adenylyl cyclases that synthesize cAMP or cAMP-phosphodiesterases.

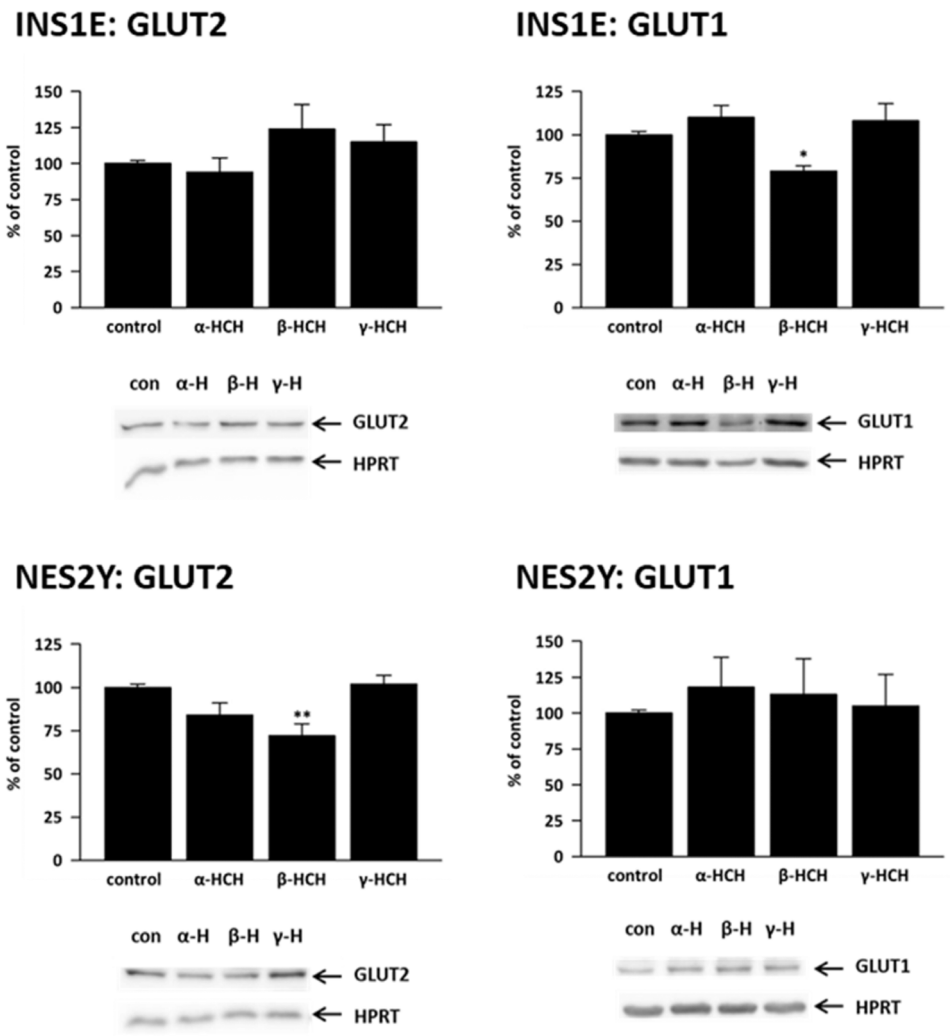


Fig. 7. The protein expression of glucose transporter 2 (GLUT2) and glucose transporter 1 (GLUT1) in the rat (INS1E) pancreatic beta-cells and human (NES2Y) pancreatic beta-cells exposed to 10 μM concentrations of α-HCH, β-HCH, and γ-HCH for one month detected by western blot. Actin or HPRT was used as a loading control. The graphs represent an average of densitometric analyses of at least four western blots made of four independent sets of samples. Below each graph, a representative western blot is shown ± SEM. * means statistical significance ($p < 0.05$), * * means statistical significance ($p < 0.01$) determined by ONE-WAY ANOVA (Dunnet's test).

In human beta-cells, α-HCH significantly decreased the protein expression of activating transcription factor 3 (ATF3) to 47% of the control, β-HCH to 27% of the control, and γ-HCH to 45% of the control (Fig. 5). In rat beta-cells, only β-HCH significantly decreased the ATF3 protein expression to 57% of the control (Fig. 5). A stress-inducible ATF3 is a member of the CREB (cAMP response element-binding) protein family of transcription factors; stress factors that induce its expression include high glucose, palmitate, oxidative stress, or ER stress (Ku and Cheng, 2020). Some studies identified overexpressed ATF3 as a negative factor for beta-cell function (Busch et al., 2002; Jang et al., 2011; Kim et al., 2017). Nevertheless, an experiment with knockout mice showed that the lack of ATF3 undermines beta-cell function because it works as a transcription factor for insulin genes (Zmuda et al., 2010) and enhances insulin secretion (Kim et al., 2018). Therefore, it seems that both ATF3 upregulation and downregulation can adversely affect beta-cell function. All three HCH isomers downregulated ATF3 expression in human beta-cells, while only β-HCH demonstrated this effect in rat beta-cells. The difference can result from the lower expression of ATF3 in human NES2Y cells compared to rat INS1E cells; the lower ATF3 levels can be more easily affected. The decreased ATF3 levels make beta-cells more vulnerable: besides lower insulin expression, it can also diminish cells' ability to handle various types of stress. We detected lower insulin expression in rat INS1E cells exposed to β-HCH, and β-HCH reduced ATF3 expression the most. Interestingly, ATF3 is more sensitive to exposure to HCH when compared to other tested proteins.

3.6. *Enzymes involved in defense against oxidative stress*

The protein expression of glutathione reductase (Glu-Red), superoxide dismutase 1 (SOD1), and biliverdin reductase B (BLVRB) remained unchanged after the exposure to HCH isomers in both beta-cell lines (Fig. 6).

Glutathione reductase and superoxide dismutase represent common antioxidant enzymes. Pancreatic beta-cells possess a relatively weak ability to handle oxidative stress compared to most other cell types due to low levels of antioxidant enzymes (Wang and Wang, 2017). Therefore, we have also determined the expression of biliverdin reductase B (BLVRB). Bilirubin can quench reactive oxygen species and become biliverdin; biliverdin reductase turns biliverdin back into bilirubin and keeps the system functional (Baranano et al., 2002; Sedlak and Snyder, 2004). The bilirubin/biliverdin switch represents additional antioxidant protection in pancreatic beta-cells (Ikeda et al., 2011; Mishra and Ndisang, 2014). Nevertheless, the exposure to HCH isomers failed to alter significantly the expression of any antioxidant enzymes tested. These

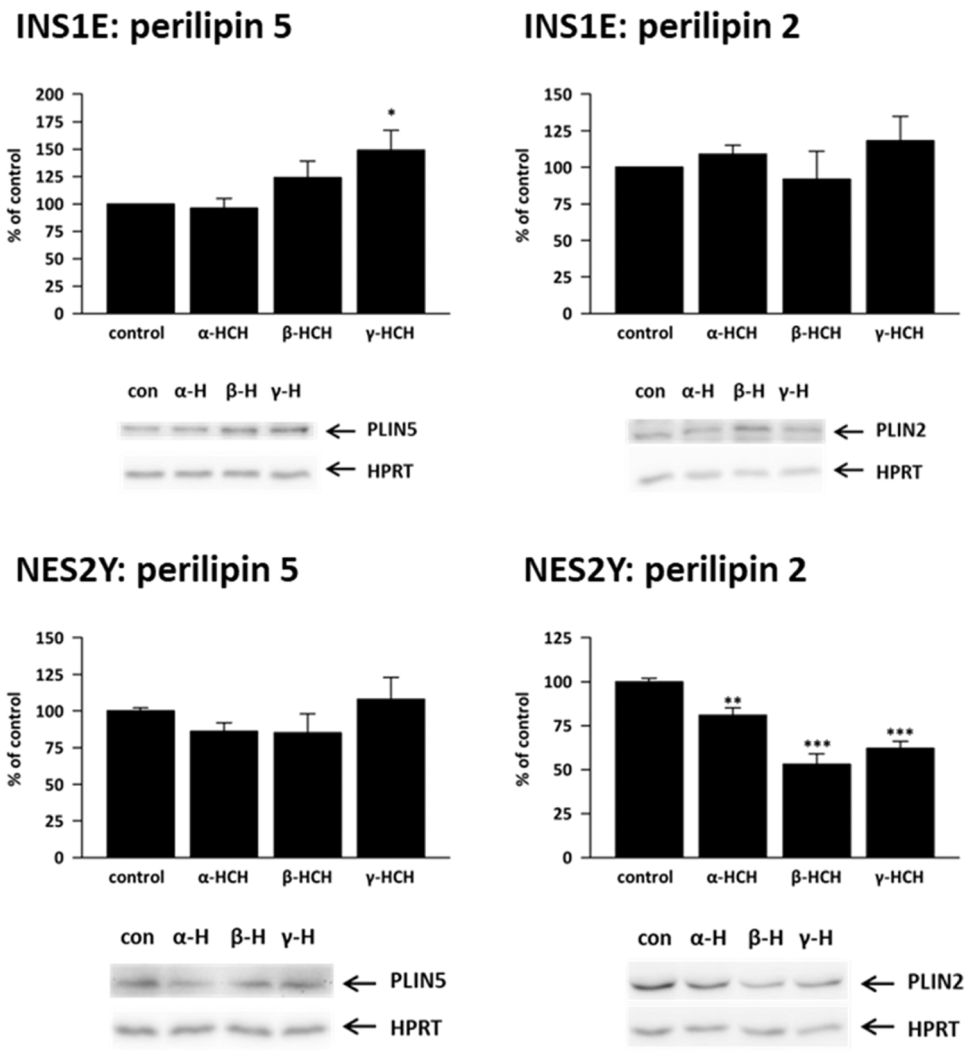


Fig. 8. The protein expression of perilipin-5 (PLIN5) and perilipin-2 (PLIN2) in the rat (INS1E) pancreatic beta-cells and human (NES2Y) pancreatic beta-cells exposed to 10 μM concentrations of α-HCH, β-HCH, and γ-HCH for one month detected by western blot. Actin or HPRT was used as a loading control. The graphs represent an average of densitometric analyses of at least four western blots made of four independent sets of samples ± SEM. Below each graph, a representative western blot is shown. * means statistical significance ($p < 0.05$), * * means statistical significance ($p < 0.01$), * ** means statistical significance ($p < 0.001$) determined by ONE-WAY ANOVA (Dunnet's test).

results show that oxidative stress (if induced by exposure to HCH isomers) is not strong enough to alter the protein expression of anti-oxidative stress enzymes.

3.7. Glucose transporters

β-HCH decreased the protein expression of glucose transporter 1 (GLUT1) in rat INS1E cells but not human NES2Y cells (Fig. 7). β-HCH decreased the protein expression of glucose transporter 2 (GLUT2) in human NES2Y cells but not rat INS1E cells (Fig. 7).

In rat pancreatic beta-cells, GLUT2 represents the dominant glucose transporter; in human beta-cells, GLUT1 dominates (Coppieters et al., 2011; Devos et al., 1995). In each cell line, β-HCH downregulated the less expressed glucose transporter. These results show that β-HCH possesses the potential to downregulate glucose transporters' expression. Nevertheless, under our experimental conditions, the effect was so small that it made a difference in the expression of low-expressed transporters but not those with a more robust expression.

3.8. Proteins involved in lipid droplets formation

In human NES2Y cells but not in rat INS1E cells, all three HCH isomers significantly decreased protein expression of perilipin-2 (Fig. 8). Lipid droplets consist of neutral lipids accompanied by enzymes and encircled by a membrane; these organelles typically occur in adipocytes. They can also be formed in pancreatic beta-cells due to nutrition overload; human beta-cells create them more readily than rodent beta-cells (Liu et al., 2020). Some studies showed that lipid droplets positively affected pancreatic beta-cell survival and protected beta-cells against lipotoxicity (Plotz et al., 2016; Sramek et al., 2021). The perilipins are lipid droplet-associated proteins that regulate lipid metabolism in lipid droplets. According to Chen and coworkers, perilipin-2 (PLIN2) participated in an unfolded protein response, a cell defense mechanism induced by ER stress (Chen et al., 2017). Also, PLIN2 downregulation impaired insulin secretion under nutritional stress and damaged mitochondria (Mishra et al., 2021). In human NES2Y cells, all three HCH isomers decreased the expression of PLIN2. The exposure to pollutants thus may compromise the human beta-cells ability to face lipotoxicity and stress.

In rat beta-cells, γ-HCH significantly increased the protein expression

of perilipin-5 (PLIN5) to 149% of the control (Fig. 8). According to Zhu and coworkers(Zhu et al., 2019), increased expression of perilipin-5 relieved palmitate-induced ER stress in INS1E cells and reduced oxidative damage associated with lipotoxicity (Zhu et al., 2020). Therefore, upregulated perilipin-5 in rat INS1E cells could represent increased protection against lipotoxicity-induced damage (γ-HCH is a hydrophobic,i.e., fat-like, chemical). Alternatively, γ-HCH (a relatively hydrophobic compound) could induce lipotoxicity in rat INS1E cells, and the cells increased the perilipin-5 expression to protect themselves. Interestingly, the other two HCH isomers failed to show a similar effect. A co-exposure of beta-cells to γ-HCH and a saturated fatty acid would show if γ-HCH induced lipotoxicity in rat beta-cells or rather protected them against it.

We found but three proteins with altered expression in both beta-cell lines. These results showed that INS1E rat pancreatic beta-cells and NES2Y human pancreatic beta-cells represented complementary models rather than identical ones. The differences between human and rodent beta-cells include, e.g., reaction to various stress factors (Eizirik et al., 1994; Law et al., 2010), the predominance of glucose transporters (McCulloch et al., 2011), or the presence of some ion channels and others (Klemen et al., 2017). Also, only the INS1E cells respond to increased glucose levels. Therefore, using two beta-cell lines give us a better idea about the effects of pollutants even when the results differ.

4. Conclusions

Of the tested HCH isomers, β-HCH impacted the expression of the tested proteins the most. It affected the expression of protein connecting glucose metabolism and fatty acid synthesis (decreased ATP-citrate lyase expression), a protein involved in defense against various types of stress (decreased ATF3 expression), and the expression of glucose transporters in both beta-cell lines. It also affected the translation (increased p-eIF2α expression) in the rat beta-cell line, cAMP signaling (decreased p-PKA expression), and lipid storage (decreased perilipin-2 expression) in the human beta-cell line. γ-HCH affected the cell's ability to produce NADPH (decreased IDH1 expression) in both beta-cell lines. Interestingly, the prolonged exposure to HCH isomers failed to induce the markers of the stress of the endoplasmic reticulum or oxidative stress in beta-cells.

Three proteins showed superior sensitivity toward exposure to pollutants: ATF3, p-PKA, and perilipin-2 decreased their expression in response to all three HCH isomers in the human beta-cells. Therefore, they represent potential biomarkers of the exposure of pancreatic beta-cells to pollutants, at least the more hydrophobic ones.To conclude, we have detected altered protein expression of several proteins in pancreatic beta-cells exposed to HCH isomers. We hypothesize that while those changes may not represent an immediate threat to beta-cell function or survival, they could negatively affect them in the long term. The changes could also compromise the beta-cells ability to face other adverse conditions caused by, e.g., unhealthy food.

CRediT authorship contribution statement

Nela Pavlikova: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Visualization, Resources, Original manuscript writing, Writing – review & editing, Project administration. **Jan Sramek:** Methodology, Writing – review & editing. **Martin Jacek:** Methodology, Formal analysis, Writing – review & editing. **Jan Kovar:** Writing – review & editing, Supervision, Project administration. **Vlasta Nemcova:** Methodology, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgments

The work has been supported from the project National Institute for Research of Metabolic and Cardiovascular Diseases (Programme EXCELES, ID Project No. LX22NPO5104) - Funded by the European Union—Next Generation EU., Charles University Cooperatio METD and Cooperatio Health Sciences and Czech Science Foundation (22–22398S).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2023.104299.

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