EVALUATION OF A LIVER IN VITRO SYSTEM FOR INVESTIGATION OF THE ROLE OF ENVIRONMENTAL POLLUTANTS IN TYPE 2 DIABETES CASE STUDY: PFOA

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1) Introduction

Human metabolic diseases such as diabetes and obesity, are considered as one of the most important threats for human health in the 21st century. Current numbers on the prevalence of diabetes mellitus show that it is taking epidemic proportions and it is suggested that this will even increase the next decennia[1]. This global diabetes epidemic is chiefly due to type 2 diabetes as it makes up more than 90% of all diabetes cases. Although genetic predisposition and lifestyle choices (e.g. lack of physical exercise, diet) are commonly accepted causes for the development of type 2 diabetes, it is more and more argued that these factors alone can not fully explain the rapid rise in diabetes prevalence. The environment and more specifically environmental pollutants are mentioned as a major interfering candidate[2]. It has long been known that some environmental pollutants have the ability to interfere with hormonal pathways and these compounds were defined as endocrine disrupting compounds. Although initially endocrine disruptor research was focused on developmental and reproductive effects, it was recently suggested that the hormonal system that is responsible for regulation of the energy metabolism might also be an important target. In this framework, it was hypothesized that some EDCs, so called obesogens, could be related to adipogenesis and obesity, supported by laboratory and animal research as well as epidemiological studies[3]. However, as evidence is accumulating that this concept could be extended to other metabolic disorders as well, it was suggested that this subclass of EDCs would be better referred to as metabolic disruptors[4].

Although evidence that environmental pollutants might be an additional risk factor for diabetes development is accumulating, a systematic investigation of the possibility that pollutants could be key players in the etiology of type 2 diabetes has not yet been performed. Epidemiological studies have reported associations between increased diabetes prevalence and a broad amount of persistent organic pollutants (POPs) e.g. several polychlorinated biphenyls, dioxins, pesticides and pefluorinated and brominated compounds[5]. However if and how these compounds, as well as non-POPs are able to interfere with processes involved in diabetes development and/or progression is so far largely unknown. Only for TCDD and a few PCB congeners (POPs), some xenoestrogens (e.g. bisphenol A), organophosphorus compounds (e.g. malathion) and heavy metals (e.g. arsenic) limited mechanistic research has been performed[5].

To enable research on the metabolic disrupting effects of POPs and other environmental pollutants, this research project aims at the selection and evaluation of relevant in vitro systems for investigation of the mechanisms underlying alterations induced by environmental pollutants of processes involved in diabetes. Since pancreatic β-cell dysfunction and reduced insulin sensitivity (insulin resistance) are the core pathophysiological defects in type 2 diabetes, this research focuses both on the effects of environmental pollutants on pancreatic β-cells (function and β-cell mass) and liver cells (insulin responsivity and sensitivity). A first compound that was chosen for investigation of its effect on insulin responsiveness in liver was perfluorooctanoic acid (PFOA). A recent study has reported that augmented blood insulin concentrations, and accordingly increased beta-cell function, as well as elevated insulin resistance could be related to increased serum PFOA[6], whilst others observed no effect[7]. Although there has been some controversy on the epidemiologic association between human PFOA levels and diabetes, their mode of action specifically targeting the energy metabolism, prompts its investigation. We studied if PFOA could alter expression of insulin regulated genes with a key role in insulin responsive pathways, after evaluation of the applicability of the selected liver cell model for studying the insulin metabolism.

2) Materials and methods

2.1 Cell line

H4IIE cells were obtained from American Type Culture Collection (CRL-1458, Manassas, VA, USA) and cultured as recommended.

2.2 Flow cytometric cell proliferation assay

After treatment, cells were harvested and flow cytometric measurements were performed according to Vanparys et al. (2006)[8]. Cell cycle histograms were analyzed using ModFit LT™ 3.0 software packages (Verity Software House Inc., Topsham, ME, USA).

2.3 Real time rt-PCR

Total RNA was extracted using Qiagen RNeasy kit (Qiagen, Antwerp, Belgium) according to the manufacturer's instruction. RNA quantity and quality were evaluated using NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE) and denaturing gel electrophoresis. The RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas GMBH, St. Leon-Rot, Germany) was used to convert 1 µg RNA to first strand cDNA. Quantitative real time rt-PCR analysis was conducted on a Roche Molecular Biochemicals LightCycler 3.5 using cyclophylin as housekeeping gene.

2.4 Microarray analysis

After total RNA extraction with the Qiagen RNeasy kit, RNA was amplified and labeled using The Two-Color Microarrays-Based Gene Expression Analysis (Quick Amp Labeling) protocol from Agilent (Agilent, Diegem, Belgium). Thereafter, 825 ng of Cy3 and Cy5 labeled cRNA was co-hybridized on a 44K Full Genome Rat Microarray (Agilent) for 17h at 60°C in a continuous rotation hybridization oven. Hybridization occurred following a v+2 design, in 2 independent loops. Each condition was applied in triplicates (biological replicates), with intrinsic dye swaps.

Arrays were washed according to the manufacturers protocols and scanned using a Genetix Personal 4100A confocal scanner (Axon Instruments, Union City, CA, USA). The images were analyzed using the Genepix Pro software (Axon Instruments) for spot identification and for quantification of the fluorescent signal intensities. Statistical analysis of microarray data was performed as described in Vergauwen et al. (2010)[9].

Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional annotation was used to reveal biologic processes highly represented in the differential genes list and to calculate the significance/likelihood of their enrichment. Gene ontology terms were considered significant if they had a Bejamini-corrected p-value of less than 0.05.

3) Results and discussion

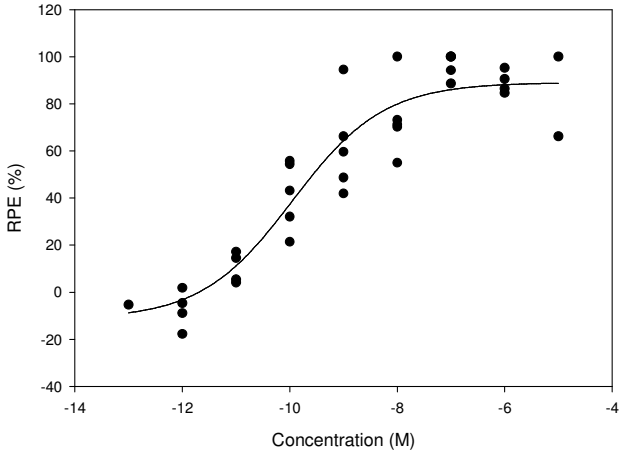

Figure 1. Effect of a concentration range of insulin (M) on growth of H4IIE cells, measured by flow cytometric cell cycle analysis. Growth is expressed as % of the relative proliferative effect (RPE) and was defined as the sum of the % of cells in S and G2M phase. Each dot represents mean of triplicate measurements. The regression line indicates the mean dose response curve of 5 independent experiments.

3.1 Rationale of selection of cell line

As the H4IIE cell line has been shown to perform gluconeogenesis in a physiological relevant way (i.e. stimulated by glucagon and glucocorticoids and inhibited by insulin), this cell line was eligible because it possesses at least one of the features, necessary for a good model system for insulin signaling research. Another advantage of H4IIE cells is that this cell line has been extensively used as cell bioassay for screening of environmental extracts for Ah-active compounds. As such, it has proven its value as a stable screening system for environmental samples[10].

3.2 Applicability analysis of H4IIE cell line

3.2.1 Flow cytometry

Cell cycle analysis using flow cytometry on propidium iodide stained cells was applied to study effects of a range of insulin concentrations on cell proliferation. The results of the proliferative effect of insulin after 24h of exposure are shown in figure 1. A dose-response curve is presented and cell growth is indicated by relative proliferative effect (RPE), which means that data are expressed in terms of percentage of growth stimulation, relative to the concentration at which growth stimulation was maximal (100%).

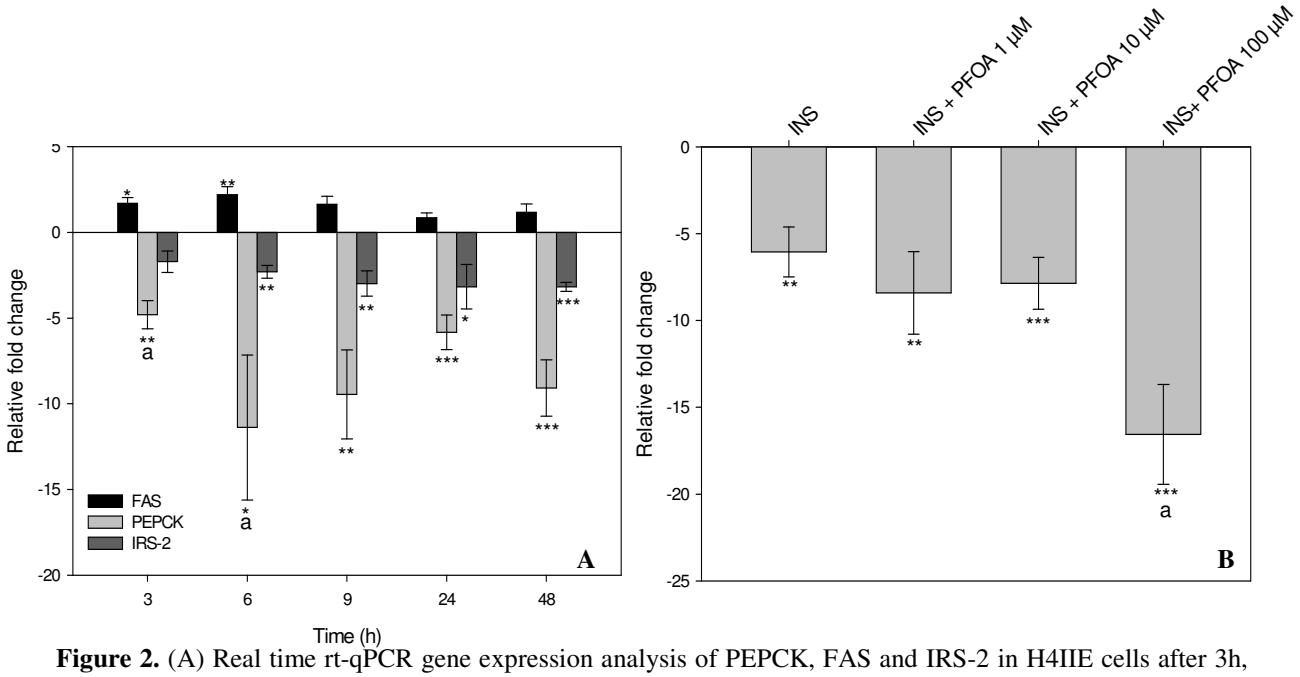

Figure 2. (A) Real time rt-qPCR gene expression analysis of PEPCK, FAS and IRS-2 in H4IIE cells after 3h, 6h, 9h, 24h and 48h of exposure to 100 nM insulin. (B) Real time rt-qPCR gene expression analysis of PEPCK after 24h exposure to 100 nM insulin and/or 1, 10 or 100 µM PFOA. Data are represented as the mean fold change relative to the solvent control (mean ± stdev; n = 3). *indicates significant differential expression compared to solvent control (Students T-test, *p<0.05, **p<0.01, ***p<0.001), letters indicate significant differences between exposures (ANOVA, Tukey post-hoc test, p<0.05).

The reproducibility of the growth stimulating effect of insulin was evaluated by calculating the mean of the half maximal effective concentration or EC_{50}-value, based on five independent experiments. The dose-response curve in figure 1 was generated by expressing mean RPE at each insulin concentration and was used to calculate the mean EC_{50} value, which was 117.0 pM (95% CI: 585.6 fM – 23.4 nM) with a coefficient of variation of 23.2 %. This high stability and reproducibility indicated that the H4IIE cell line is sensitive for at least one of the known (non-metabolic) actions of insulin. The results of flow cytometric cell cycle analysis were used to select concentrations at which the metabolic response of H4IIE cells on insulin was studied. The concentrations 10 pM (~ EC_{25}), 1 nM (~ EC_{75}) and 100 nM (~ EC_{100}) insulin were selected.

3.2.2 Real time-PCR analysis

Because insulin acts partly at the transcriptional level real-time rt-qPCR analysis was used for further selection of the conditions, to be analyzed with microarray technology. Expression of 3 key genes, known to be transcriptionally regulated by insulin, was measured after 3h, 6h, 9h, 24h and 48h of exposure to 100 nM insulin. Target genes were: phosphoenolpyruvate carboxykinase (PEPCK), which regulates gluconeogenesis together with glucose-6-phosphatase; insulin receptor substrate 2 (IRS-2), which is the main mediator of hepatic insulin signaling; and fatty acid synthase (FAS), a key enzyme of hepatic lipogenesis, responsible for the synthesis of long chain saturated fatty acids (e.g. palmitate and stearate).

Looking at the expression profiles of these three genes in function of time (fig. 2A), it is clear that FAS is regulated only at very early time points (after 3h and 6h exposure), whilst for IRS-2 a more delayed response on transcription activity is observed (i.e. repression was significant after 6h of exposure). PEPCK downregulation is present at any investigated time point. Furthermore it can be concluded that the direction of insulin mediated transcriptional regulation i.e. upregulation of FAS[11,12] and downregulation of IRS-2[11] and PEPCK[11,12], came up to expectations in the H4IIE cell line. This was a first indication of the presence of a physiological relevant insulin response. Because a distinction could be made between genes with rapid insulin-dependent regulation and some with a more delayed response, two time points, i.e. 6h and 24h of insulin exposure, were selected to ascertain coverage of both early and late responses in further microarray analysis.

3.2.3 Microarray analysis

To further evaluate if H4IIE cells are a representative model system to study insulin signaling, a microarray experiment was exerted in which the conditions 6h and 24h exposure to 100 nM were analyzed. The results (table 1) showed that the H4IIE cell line has an extensive and physiological highly relevant insulin response system. As such, this hepatocyte model is a valid *in vitro* system to investigate possible effects of pollutants on insulin sensitivity and action, insulin signaling, etc.

3.3 Case-study: PFOA

It is know that PFOA, and other perfluorinated compounds, have severe effects on lipid metabolism. As insulin is an important mediator of this energetic pathway and as information on possible associations between PFOA and insulin resistance is accumulating, we sought to investigate if PFOA might be able to alter insulin action. Using the H4IIE cell line, we focused on the expression of the 3 previously selected key genes in cells that were exposed to insulin, PFOA and a combination of both. As can be seen for PEPCK (fig. 2B), it seems that PFOA fortifies the inhibitory effect of insulin, which might be an indication that PFOA affects, at least in part, insulin regulation of glucose metabolism. Although these first results on the combined effects of insulin and PFOA are promising, much more research is needed to be able to estimate the importance of the observed effects. Also with regard to extrapolation to the in vivo situation, caution is advised. In further experiments, the combined insulin/PFOA effect will be investigated in more detailed, using physiologic endpoints and transcriptome and proteome analysis.

Table 1. Insulin responsive pathways present in H4IIE cells. GO enrichment analysis was performed with DAVID and GO terms were considered significant if $p<0.05$ (Bejamini-corrected p-value). Functional annotation clustering was used and clusters with an enrichment score above 1.3 were considered to be significantly enriched. A key word was given to each of the significantly enriched clusters. Key words that were found in the functional annotation clustering data set are represented in bold. These are the insulin-regulated processes, present in H4IIE cells, the others are known insulin-regulated hepatic processes which were not represented in the obtained gene expression data set.

Most important insulin-regulated processes			
Carbohydrate metabolism	Lipid metabolism	Protein metabolism	Cell cycle
✓ ↑ glucose and amino acid uptake ✓ ↑ **glycolysis** ✓ ↓ **gluconeogenesis** ✓ ↑ glycogen synthesis	✓ ↑ **fatty acid synthesis** ✓ ↓ **fatty acid breakdown** ✓ ↑ **cholesterol synthesis**	✓↓ **proteolysis** ✓↑ protein synthesis	✓↑ **mitosis**

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