Study of the In-vitro Epigenetic Toxicity Effects of Malaoxon, Malathion Dicarboxylic Acid, Cadmium Chloride and Bisphenol-A on PPARγ, PPIA and aP2 gene Expressions

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Abstract

Many environmental pollutants are considered to be obesogens that are encountered to be one of the major non-traditional risk factors for induction of obesity. The aim of the present study is to determine role of malaoxon, malathion dicarboxylic acid (MDCA), cadmium chloride (CdCl₂) and bisphenol-A (BPA); as prevalent pollutants in our locality, Egypt. In vitro study was conducted on pre-adipocytes PCS-210-010 cell line, cells were divided into 5 groups: (I) treated with malaoxon, (II) treated with MDCA, (III) treated with CdCl₂, (IV) treated with BPA, (V) served as control group. LC₅₀ were determined for treated cells, at different concentrations, using MTT assay, expression of PPARγ, PPIA and aP2 genes were estimated using RT-PCR; and adiponectin (ADP) levels were measured spectrophotometrically. Results showed that the studied pollutants significantly upregulated all the studied genes (p<.001) compared to the control group, as well as, ADP levels were significantly increased in treated cells compared to control cells (p<.001). In conclusion, malaoxon, MDCA, CdCl₂ and BPA epigenetically increased the expression of studied genes that play a key role in the process of adipogenesis. These results warranted more depth mechanistic studies for each toxicant to elucidate the main pathway of action.

Keywords: Malaoxon; MDCA; CdCl₂; BPA; PPARγ; PPIA; aP2; Adipogenesis

Introduction

Over the last decades, incidence of obesity has been dramatically increased; nevertheless, most of the inducing factors are referred to high caloric diet and life style, as the main causes for obesity, the contribution of nontraditional factors especially environmental pollutants and stress; have been considered nowadays as major risk factors for its initiation [1].

The human endocrinal system has a major role in regulating metabolism of fats, carbohydrates and proteins to provide the energy needs to our bodies, where it is mainly stored by fat that is held in adipocytes in the adipose tissue. Thus, any alterations of such regulatory mechanisms, mostly results in metabolic imbalance and hence, obesity [2].

Adipogenesis is a process of cellular differentiation that is regulated by a complex gene expression program, peroxisome proliferator-activated receptor γ (PPARγ) gene plays the central role in regulation of adipocyte gene expression control and differentiation in such program [3]. Activation of such gene modulates other numerous genes regulating adipogenesis, such as, adipocyte protein 2 (aP2) that facilitates the transfer of fatty acids between extra- and intracellular membranes [3]. Peptidyl-prolyl cis/trans isomerase A (PPIA), a novel studied gene regarding its adipogenic effects, is implicated in accelerating protein folding. It was recently declared that PPIA plays an important role in the regulation of adipogenesis; and its overexpression might be associated with the occurrence of obesity and type 2 diabetes [4]. Adiponectin (ADP) is an adipocyte-specific hormone that is well known as a critical adipokine which increases insulin sensitivity, thus, any factor that suppress its release is probably induces insulin resistance and metabolic syndrome [5].

In Egypt, the ecosystems are heavily contaminated with a variety of environmental pollutants such as cadmium, malathion as a result of increasing discharge of untreated industrial wastes and agricultural irrigation by wastewater [6], besides, industrial applications of bisphenol-A contained plastics and epoxy resins; are becoming very enormous [7].

These pollutants are considered exogenous substances that human population is ubiquitously exposed on daily basis, either indoors or outdoors, through their different usage and applications; causing adverse health effects and posing risks to human health. They are extensively investigated for their endocrinal disrupting and obesogenic properties [8-10].

Studies that were conducted to elucidate the toxic role of such prevalent toxicants (malathion, cadmium, bisphenol-A) in Egypt on upregulation of adipocyte genes expression, and predisposition to induce pathogenic adipogenesis; are scarce. Thus, the aim of the current study is to evaluate the toxic effects of malathion metabolites (malaoxon and malathion dicarboxylic acid, MDCA), CdCl₂, BPA on expression of PPARγ, aP2 and PPIA genes in pre-adipocytes PCS-210-010 cell line and whether they can affect ADP concentrations in treated cells or not.
Material and Methods

An experimental in vitro study was performed to study the toxic effects of malaoxon, MDCA, CdCl₂ and BPA on gene expression of pre-adipocyte PCS-210-010 cell line. Expression of PPARγ, aP2 and PPIA genes, and estimation of adiponectin concentrations were performed in The Holding Company for Biological Products and Vaccines (VACSERAs), Egypt.

Chemicals

Malaoxon, MDCA, CdCl₂ and BPA analytical standards (≥ 99.9%), and adipocyte cell viability MTT assay kits; were obtained from Sigma-Aldrich, Co. USA. Concentrations (µg/mL to 0.25 µg/mL); were obtained from Sigma-Aldrich, Co. Egypt. Human ADP ELISA kit (SRB/Shanghai Sunred Biotechnology Comp., Ltd.)

Adipocytes' differentiation

Primary Subcutaneous Pre-adipocytes; Normal, Human (ATCC® PCS-210-010’) cell line was obtained from American Type Culture Collection (Ethical approval to conduct the study on human cells was obtained from Institutional Research Board, Mansoura Faculty of Medicine, R.18.03.88), Fibroblast Basal Medium (ATCC® PCS-201-030), Fibroblast Growth Kit-Low Serum Components (ATCC® PCS-201-041), Adipocyte differentiation toolkit (ATCC® PCS-500-050), trypsin-EDTA solution (ATCC® PCS-999-003), D-PBS (ATCC® 30-2200), Tryptic Neutralizing Solution (ATCC® PCS-999-004), Gentamicin-Amphotericin B Solution (10 µg/mL to 0.25 µg/mL); were obtained from Sigma-Aldrich, Co. USA.

Cell viability

All chemicals were dissolved in Roswell Park Memorial Institute medium (RPMI). Adipocyte cells were divided into five groups (I, II, III, IV and V). The first and second groups (I,II) were treated with malaoxon and MDCA respectively; group III was treated with CdCl₂, the fourth group (group IV) was treated with BPA. A matched control group consisted of untreated adipocyte cells was used (group V). All tested chemicals were applied at different concentrations each (0.01, 0.1, 1, 10, 100 µM respectively), and then incubated for 24 hrs to determine LC50 on adipocyte cell line using MTT assay according to the manufacturer instructions. Viability of cells was measured spectrophotometrically using ROBONIK P2000 at a wave length of 450 nm.

RNA isolation and gene analysis studies using Real-time RTPCR

Total RNA was extracted from human adipocytes using the Qiagen RNA extraction kit (Qiagen, Mississauga, Canada). RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA) and RNA quality was determined using a BioAnalyzer (Agilent Technologies, Santa Clara, USA).

First strand cDNA synthesis from the total RNA was performed using QIAGEN Long Range 2 Step RT-PCR Kit (100) (Germany-Cat. No. 205922) according to manufacturer instructions. Real-time PCR was carried out using a SybrGreen reagent [SYBR Green PCR Master Mix (Applied Biosystem, USA, Cat. No.4344463)]. For each reaction, 25 µl containing forward and reverse primers (10 pmol), 12.5 µl Power Sybr® Green PCR master mix reaction buffer, and 2 µl cDNA; were mixed. Melting curve analysis for each set of primers and agarose gel electrophoresis 2% were used to confirm the specificity of the PCR products. A reaction with no template was used as a negative control in each experiment. Standard curves were generated from serial dilutions of a pooled cDNA sample for determination of reaction efficiency and threshold cycle (Ct). Rotor-Gene 1.7.87 real time PCR system is used with its specific software. Reactions were performed in duplicates.

The primer pairs for each gene target4,7,10 were: PPARγ Forward 5’-TGGTGCCCTTCGCTGATGC-3’ Reverse 5’-CTGTGGAAGGCTTTGATGCT-3’. aP2 Forward: GGAAAGCTTGTCTCAGTGAA CCATGGCCTT GACCAAGGAG, PPIA forward primer: 5’-GATCCAGATTTGGCTA TAAAAGGTTCAGAGAACCCCTTATAGCCAAATCTTTTTTGG AAA-3’, PPIA reverse primer: 5’-GGCCCTCTGATGTGTGGAATTTC AAGAGAA ATTCCACACAAACATGATTATGATTTGTACACCCACTTTTATTAGC-3’.

GAPDH forward primer: 5’-GATCCAGATTTGGCTA TAAAAGGTTCAGAGAACCCCTTATAGCCAAATCTTTTTTGG AAA-3’, PPIA reverse primer: 5’-GGCCCTCTGATGTGTGGAATTTC AAGAGAA ATTCCACACAAACATGATTATGATTTGTACACCCACTTTTATTAGC-3’.

Results

In vitro study

Cell viability: MTT assay shows that estimated LC50 for the studied toxicants after incubation 24 h; were 0.064 uM for malaoxon, 0.086 uM for MDCA; 0.053 uM for CdCl₂ and 0.059 uM for BPA (Table 1).
µM: Micromole; MDCA: Malathion dicarboxylic acid; CdCl₂: cadmium chloride; BPA: bisphenol-A.

Table 1: MTT assay at different concentrations of malaoxon, malathion dicarboxylic acid (MDCA), cadmium chloride (CdCl₂) and bisphenol-A (BPA) in pre-adipocytes PCS-210-010 cell line.

**PPARγ, aP2 and PPIA gene expressions using RT-PCR**

Statistically significant increases in the expression of the studied genes compared to the control group (P<0.001). The mean values of their expression are shown in Table 2 and Figure 1.

| Sample data | Gene expression (IU/ml ) |
|-------------|-------------------------|
| Ser. | Sample code | Used conc. uM | PPARγ | aP2 | PPIA |
| 1 | Maloxone. | 0.064 | 13.3805 | 26.9638 | 27.635 |
| 2 | MDCA | 0.086 | 13.3805 | 6.93188 | 32.0276 |
| 3 | CdCl₂ | 0.053 | 4.42575 | 4.67748 | 19.8296 |
| 4 | BPA | 0.059 | 6.28263 | 33.0271 | 16.09 |
| 5 | Control | | 1 | 1 | 1 |

MDCA: Malathion Dicarboxylic Acid; CdCl₂: Cadmium Chloride; BPA: Bisphenol-A; FLD: Fold Change.

Table 2: PPARγ, PPIA and aP2 gene expressions in treated pre-adipocytes PCS-210-010 cell line after exposure to malaoxon, malathion dicarboxylic acid (MDCA), cadmium chloride (CdCl₂) and bisphenol-A (BPA).

**Adiponectin concentrations**

The mean values of adiponectin concentrations in treated pre-adipocytes were 1.613, 1.089, 1.427 and 0.7915 mg/dl for malaoxon, MDCA, CdCl₂, BPA, respectively, compared to 0.4832 mg/dl of control samples, with statistically significant difference between each toxicant compared with the control group (P<0.001).

**Statistical analysis**

Data were analyzed using SPSS version 19 using student's t-test for unpaired samples. Significance was set at P-value less than 0.05.

**Discussion**

Worldwide, a dramatic increase in the prevalence rates of obesity was observed as a significant public health problem that threatens human population [7]. Adipogenesis is a process for the differentiation of pre-adipocytes into mature adipocytes; a step that is essential for lipid storage and metabolism inside the human body and regulated mainly by a set of essential gene programs [11]. PPARγ is considered a major regulator for gene networks occurring in adipogenesis, lipid metabolism and metabolic homestasis [12,13]. aP2 is fatty acid binding protein 4, expressed in adipocytes and is important for adipogenesis and has profound effects on glucose and lipid metabolism, especially for vascular health. It is known to be regulated by the transcription factors of the PPARγ and C/EBP family members [7].

Many environmental pollutants are well known with their endocrinal disrupting properties. Bisphenol-A, a chemical that human is heavily exposed to; and widely used in plastics industry, has been linked extensively to obesity and promoting adipogenesis in either animal or human studies especially in the stage of pre-adipocytes differentiation [7,14]. aP2 is fatty acid binding protein 4, expressed in adipocytes and is important for adipogenesis and has profound effects on glucose and lipid metabolism, especially for vascular health. It is known to be regulated by the transcription factors of the PPARγ and C/EBP family members [7].

Malathion is a widely used pesticide; known to
have endocrine disrupting effects [9]. It is proposed that malathion toxicity depends mainly on its bioactivation to the toxic metabolites mainly Malaoxon [16].

These environmental pollutants are known to be obesogens that promote adipose tissue differentiation pathways at several key points at the molecular level, where they can interfere with nuclear transcriptional regulators that control lipid homeostasis [2]. The precise mechanism by which endocrinal disruptors promote adipocyte differentiation is mainly through PPARγ regulation that is used as proxy bio-marker for screening of environmental obesogens [11].

In the present study, BPA induces gene expression of both PPARγ, aP2 around (6- and 33- folds higher than control group. These results indicate its role in adipogenesis pathway, a fact that was confirmed by several other previous studies; especially at high does more than 80 µM [5,10,11]. Boucher et al. [14] studied a global transcriptional profiling in primary human adipocytes exposed to BPA. They declared that BPA unregulated a number of genes involved in TGs and lipid metabolism and accumulation. A study was conducted by Valentino et al. [17], they declared that 1 nM BPA did not roughly affect adipocyte differentiation in 3T3-L1 pre-adipocytes and PPARγ mRNA levels were unchanged. In studies that were conducted by Atlas et al. [7] and Boucher et al. [18], they studied the effects of BPA on differentiation of 3T3-L1 cells. Unlike our results, they found that PBA unregulated aP2 (2-5 fold) at a dose of 1nM and 10-fold at higher concentrations; at both protein and mRNA levels as a direct transcriptional effect and independent from the PPARγ levels which remained unchanged. Discrepancy of such results with that of the present study may be due to different used concentrations or explained by other mechanisms such as non-classic ER-pathways. In accordance to the results of the present study, a study that conducted by Ariemma et al. [10], to estimate the chronic effect of BPA on adipogenesis and mastering gene expression in 3T3-L1 pre-adipocytes. They found that PPARγ was significantly increased after 8-days of exposure to BPA from the start of the differentiation process.

Notwithstanding, BPA was extensively studied as regards its role in induction of adipogenesis and its effect on PPARγ and aP2 genes. Very limited studies were found to investigate such role regarding CdCl₂, meanwhile, to the best of our knowledge, this study was the first to study the relation between malathion and the studied genes.

In the present work both cadmium and malathion metabolites (malaoxon and MDCA) induces gene expression of PPARγ [4-, 13- and 13-fold respectively] and aP2 increased (5-, 27- and 7-fold respectively) compared to control group. Interestingly the results of CdCl₂ in the present study are not in accordance with Kawakami et al. [19] and Lee et al. [20]. In the first study, authors assessed chronic exposure to Cd and the possible alteration of epidymidal white adipose tissue in mice and the adipocyte differentiation. They found that mRNA expression levels of peroxisome PPARγ2 decreased in the Cd-treated groups, as well as, adiponectin mRNA expression levels. Similarly, the study conducted by Lee et al. [20], they investigated the effects of cadmium at a dose of 3 µM on cellular differentiation of 3T3-L1 preadipocyte cells. They found that cadmium suppressed the expression of PPARγ proteins, as key transcriptional activators for adipogenesis, in a dose-dependent manner. These discrepancies of results may be due to different used concentrations. On the other hand, the adipogenic effects of malathion and its active metabolites were not investigated. However, their potential adipogenic effects were explained by either due to reducing the estradiol levels [11].

One of the interesting genes that was recently involved with adipogenesis in this research is PPIA. No much studies were done to investigate the exact role of this gene regarding adipogenesis induction, it was reported that, PPIA was found to be increased in patients with type 2 diabetes as well, it increased during 3T3L1 adipocyte differentiation [4]. In their study, they confirmed that intracellular PPIA can be considered as an important regulator of adipogenesis, affecting mainly C/EBPB and not PPARγ. To best of our knowledge, this study was the first to relate the expression of PPIA to induced adipogenesis in pre-adipocytes treated cells with such environmental pollutants. The studied pollutants (malaoxon, MDCA, CdCl₂ and BPA), significantly increased the expression of PPIA by 28-, 32-, 20-, 16-fold respectively compared to control group, an interesting finding that warranted further investigation.

Adiponectin is the key regulator of insulin sensitivity and tissue inflammation in the human cells; consequently, factors suppressing ADP release are known to promote insulin resistance and developing metabolic syndrome [21].

In the present work, ADP levels were significantly increased in adipocyte treated cells with malaoxon, MDCA, CdCl₂ and BPA (p<0.001). This finding contradicts with most of the previous literature that confirmed that most of these toxicants caused reduction in the levels of ADP in their studies [5,21,22]. Hugo et al. [5] found that BPA at low nanomolar concentrations suppressed ADP release from human adipose tissue and mature adipocytes. On the other hand, Ariemma et al. [10] found that chronic exposure to BPA on T3T-L1 has no significant difference on the level of ADP expression.

In the present study, malathione metabolites, cadmium and bisphenol-A upregulate several important genes that are implicated in mastering adipogenesis programming pathways, and enhancing adipocytes differentiation and growth and lipid homeostasis. However, in-depth studies are warranted to understand of the mechanism of action for each toxicant to enhance our knowledge toward their roles as this would help to specify the exact mechanistic regulation of such toxicants in affecting the regulation of adipogenesis.

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