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Combined Transcriptomic—^{1}H NMR Metabonomic Study Reveals That Monoethylhexyl Phthalate Stimulates Adipogenesis and Glyceroneogenesis in Human Adipocytes

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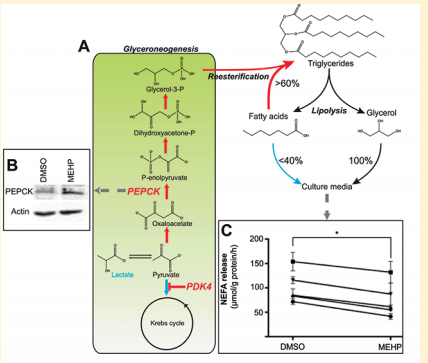
Supporting Information

ABSTRACT: Adipose tissue is a major storage site for lipophilic environmental contaminants. The environmental metabolic disruptor hypothesis postulates that some pollutants can promote obesity or metabolic disorders by activating nuclear receptors involved in the control of energetic homeostasis. In this context, monoethylhexyl phthalate (MEHP) is of particular concern since it was shown to activate the peroxisome proliferator-activated receptor γ (PPARγ) in 3T3-L1 murine preadipocytes. In the present work, we used an untargeted, combined transcriptomic-^{1}H NMR-based metabonomic approach to describe the overall effect of MEHP on primary cultures of human subcutaneous adipocytes differentiated in vitro. MEHP stimulated rapidly and selectively the expression of genes involved in glyceroneogenesis, enhanced the expression of the cytosolic phosphoenolpyruvate carboxykinase, and reduced fatty acid release. These results demonstrate that MEHP increased glyceroneogenesis and fatty acid reesterification in human adipocytes. A longer treatment with MEHP induced the expression of genes involved in triglycerides uptake, synthesis, and storage; decreased intracellular lactate, glutamine, and other amino acids; increased aspartate and NAD, and resulted in a global increase in triglycerides. Altogether, these results indicate that MEHP promoted the differentiation of human preadipocytes to adipocytes. These mechanisms might contribute to the suspected obesogenic effect of MEHP.



KEYWORDS: phthalate, environmental metabolic disruptor, phosphenolpyruvate carboxykinase, glyceroneogenesis, PPARγ

INTRODUCTION

White adipose tissue (WAT) is one of the major contributors to energy homeostasis in humans. Since lipids contribute 70% of WAT, this tissue is also a major reservoir for lipophilic contaminants, which might interfere with the differentiation, metabolism, and secretory functions of adipocytes.[1] Exposure to an increasing number of chemicals is suspected to play a role in the development of the obesity epidemic, and the environmental obesogen concept was introduced recently.[2] In this context, some environmental pollutants are of particular concern because they interfere with nuclear receptors controlling the expression of genes involved in energy homeostasis such as those encoding the peroxisome proliferator-activated receptors (PPARs). PPARγ, the dominant PPAR isoform in human WAT, is an ideal target for these so-called metabolic disruptors, since it regulates lipid storage, induces adipocyte differentiation and survival, and contributes to control insulin sensitivity.[3]

Phthalate esters are industrial chemicals used as plasticizers to improve the pliability of PVC products. Di(2-ethylhexyl) phthalate (DEHP), the most widely used of the phthalate esters, is found in flexible plastics of a wide variety of common products such as medical devices, cosmetics, industrial paints, solvents, or food packaging. DEHP is not covalently bound to its matrix and its propensity to leak can lead to high levels of human exposure. After ingestion, intestinal lipases convert DEHP into its monoester derivative monoethylhexyl-phthalate (MEHP), which is then preferentially absorbed.[4] MEHP can also originate from the

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conversion of DEHP by plasmatic or hepatic lipases. In vitro trans-activation studies have shown that MEHP can activate the three isoforms of PPAR receptors in human and murine cell lines.[5] Furthermore, it has also been demonstrated that, in the murine 3T3-L1 preadipocyte cell line, MEHP activated PPARγ and promoted adipose cell differentiation (adipogenesis).[6] Consequently, MEHP has been considered as a potential obesogen and metabolic disruptor.[7] However, the effects of MEHP on human WAT remain unknown.

Understanding the effects of pollutants in humans raises complex challenges. Extrapolation from animal models is difficult and depends on the conservation of the underlying molecular mechanisms of action between species. Regarding PPAR activators in general, and for DEHP and MEHP in particular, these molecular mechanisms have been shown to differ greatly between rodent models and humans. In rodents, PPAR activators induce sustained hepatic peroxisome proliferation mediated by PPARα, which is likely to influence the metabolic phenotype of these animals.[8] This phenomenon does not occur in humans. Mice treated with DEHP are protected from weight gain induced by a high fat diet, but this phenomenon is reversed in mice expressing the human PPARα.[9] These results clearly demonstrate that the effects of DEHP on WAT can be species-specific and emphasize the importance of using human models to investigate its mechanisms of action.

In vivo, human subcutaneous WAT is constituted of a majority of mature adipocytes representing more than 50% of the total cell count, surrounded by other cell types constituting the stroma-vascular fraction, among which fibroblasts and preadipocytes.[10,11] In the present work, we investigated the effects of MEHP on primary cultures of human adipocytes, which were differentiated in vitro for 11 days. The cultures contained about 30% of preadipocytes and a majority of mature adipocytes. The perturbations induced by MEHP in human adipocytes differentiated in vitro were described using an untargeted systems biology approach combining transcriptomics and ^{1}H NMR-based metabonomics at several time points. We found evidence that MEHP activated two different metabolic pathways in human adipocytes differentiated in vitro, namely, glyceroneogenesis and adipocyte differentiation, thereby increasing triglyceride content.

EXPERIMENTAL PROCEDURES

Cell Culture

Primary cultures of human preadipocytes were provided by Promocell (www.promocell.com) and originated from the subcutaneous adipose tissue of five healthy women, aged 36 ± 5 and with a BMI < 28 kg/m^2. Preadipocyte growth and in vitro differentiation into adipocytes were conducted according to the provider's recommendations. Briefly, preadipocytes were grown in Promocell Preadipocyte Growth Medium containing 5% fetal calf serum, 1 μg/mL hydrocortisone, 10 ng/mL human recombinant growth factor, and 4 μL/mL heparin, until confluence (day 0). Differentiation into adipocytes was then induced using Promocell Preadipocyte Differentiation Medium containing 3 μg/mL ciglitazone, 8 μg/mL biotin, 400 ng/mL dexamethasone, 44 μg/mL 3-isobutyl-1-methylxanthine, 0.5 μg/mL insulin, and 9 ng/mL thyroxine for 3 days (until day 3). Differentiation was pursued for 8 days in Promocell Adipocyte Nutrition Medium containing 3% fetal calf serum, 8 μg/mL biotin, 400 ng/mL dexamethasone, and 0.5 μg/mL insulin (until day 11). On day 11, about 70% of the cells were differentiated into adipocytes and about 30% remained undifferentiated.

Cell Treatment

On day 11, the cells were treated with 100 μM MEHP (Interchim, Montluçon, France) dissolved in DMSO (final concentration 0.1%). This concentration was chosen based on previous findings of the literature because it induced maximal activation of the human PPARγ.[5] To minimize variations due to cell culture conditions, cells from the five primary cultures were grown and treated in parallel for the transcriptomic and metabonomic analyses. Each primary culture was divided between two T75 flasks: one was used for RNA extraction and subsequent transcriptomic profiling and the other for cell pellet recovery and subsequent metabolic profiling. For all other complementary experiments, the same five primary cultures were used.

Transcriptomics and Data Analysis

At 24 h of treatment, total RNA was prepared as previously described.[12] RNA quality was verified on an Agilent Bioanalyzeur 2100. Hybridization, cleaning, and scanning of Human Gene 1.0 ST microarrays were performed according to the manufacturer's instructions (Affymetrix Inc., www.affymetrix.com) on the genomic platform of the Cochin Institute.

Data were normalized using Robust Multi-Array Average expression measure (RMA) Bioconductor package implemented on R (www.bioconductor.org). Log2 normalized data were analyzed using the significant analysis of microarray (SAM) paired test (http://www-stat.stanford.edu/~tibs/SAM/).[13] SAM gives a list of significantly regulated genes and estimates the false positive rate. For the data presented in this study, the false discovery rate was limited to 5%.

Gene ontology analyses were conducted using the bioinformatics tool DAVID (http://david.abcc.ncifcrf.gov:8080/). A combination of an enrichment score of 2 and a corrected p value < 0.05 was considered significant. We also used the link between DAVID and the KEGG metabolic database (http://www.genome.jp/kegg/pathway.html) and selected the pathways that had a p value < 0.05. For more details on DAVID, see ref 14.

^{1}H NMR Metabonomics and Data Analysis

At 24 or 48 h of treatment, cells were trypsinized at room temperature and washed twice in phosphate buffer saline before the cell pellet was frozen at −80 °C. Polar and lipophilic metabolites were extracted from the cell pellets using a chloroform/methanol/water (2:1:3) mixture as described previously.[15] The supernatant containing the aqueous phase was taken, freeze-dried, and dissolved in 50 μL of phosphate buffer 0.2 M (pH 7.4) in D_2O plus 0.001% TSP and transferred to microcapillary tubes. The inferior chloroform phase was taken, dried, and dissolved in 100 μL of $CDCL_3$ plus 0.01% TMS and transferred to microcapillary tubes.

All spectra were acquired on a Bruker Avance 600 MHz spectrometer (Bruker Analytische GmbH, Rheinstetten, Germany), operating at 600.13 MHz, with a spectral width of 12 000 Hz. For aqueous phases, spectra were acquired with a standard one-dimensional sequence [relaxation delay-90°-t1-90°-tm-90°-acquire free induction decay], with a water presaturation applied for 2 s and a mixing time (tm) of 100 ms and a 90° pulse length of 10 μs, using 512 scans and 64K data points. For organic phases, spectra were acquired with a standard 1D sequence with a relaxation delay of 3 s and a 90° pulse length of 3 μs, using 16 scans and 32K data points. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line

broadening. All spectra were manually phased, baseline corrected, and calibrated to TSP (δ0.00). Metabolites were assigned using previously published data[16,17] and additional two-dimensional NMR experiments on selected samples.

To eliminate the variability in water resonance presaturation, the chemical shift region between δ4.66 and 4.88 was removed from all spectra prior to statistical analysis. As previously described,[18] all data were analyzed on full-resolution spectra (33 600 data points), and aqueous phase spectra were normalized to the total peak area. Despite the use of phosphate buffer (pH 7.2), many aqueous phase spectra still displayed subtle shifts. These differences were corrected using an alignment function.[19] For organic phase spectra, the peak area for each signal was integrated (13 peaks). The resulting peak area profile was normalized to a constant sum of the peak area for the two cholesterol signals (δ0.9 and 1.1).

^{1}H NMR profiles from aqueous phases were mean-centered and scaled to unit variance, and ^{1}H NMR profiles from organic phases were mean centered prior to analysis using Matlab software version R2009a (The MathWorks Inc.). ^{1}H NMR profiles were analyzed using orthogonal projection on latent structure-discriminant analysis (O-PLS-DA), where ^{1}H NMR data were used as independent variables (X matrix) and regressed against a dummy matrix (Y matrix) indicating the class of samples (DMSO or MEHP).[20] All O-PLS-derived models were evaluated for goodness of prediction (Q^2Y value) using 5-fold cross-validation. The reliability of each model was established using a permutation test of the Y vector (1000 permutations) in order to determine a p value for each Q^2Y, as previously described.[21] To identify metabolites responsible for discrimination between MEHP- and DMSO-treated cells, the O-PLS-DA correlation coefficients (r^2) were calculated for each variable and back-scaled into a spectral domain, so that the shape of NMR spectra and the sign of the coefficients were preserved.[18] The weights of the variables were color-coded, according to the square of the O-PLS-DA correlation coefficients. Correlation coefficients extracted from significant models were filtered so that only significant correlations above the threshold defined by Pearson's critical correlation coefficient ($P < 0.05$; $r^2 = 0.38$ when $n = 10$) were considered significant.

Determination of Intracellular Triglycerides

Human preadipocytes from the same five women were differentiated in vitro for 11 days before treatment with 100 μM MEHP or 0.1% DMSO for 24 or 48 h. At day 0, day 3, day 6, day 9, and day 11 of differentiation, as well as after 24 and 48 h of treatment with MEHP or DMSO, cells were trypsinized, washed twice with PBS, suspended in 100 μL of PBS, and frozen at −80 °C. Intracellular triglycerides were measured using the "TG enzymatic PAP 150" kit from Biomérieux (Marcy l'Etoile, France) and normalized to protein content determined using the bicinchonic acid method (Pierce biotechnology, Rockford, IL). Each measurement was performed in triplicate.

Real Time Quantitative PCR (RT-QPCR)

Human preadipocytes from the same five women were differentiated in vitro for 11 days before treatment with MEHP 100 μM or DMSO 0.1% for 4, 8, or 24 h. Total RNA extraction was performed as for the transcriptomic study. Reverse transcription, RT-QPCR and data analysis were performed as previously described[12] using primers described in Supplemental Table 1, Supporting Information and RPL13A as housekeeping gene.

Western Blot

Human preadipocytes from the same five women were differentiated in vitro for 11 days before treatment with 100 μM MEHP or 0.1% DMSO for 18 h. Proteins were extracted using RIPA buffer according to the manufacturer's instructions (Santa-Cruz Biotechnology, Heidelberg, Germany). Cell lysates were centrifuged for 15 min at 14000g and 4 °C to retrieve the cytosol. Western blotting with anti-PEPCK-C was conducted as described previously,[22] using actin as an internal standard to ensure the quantity of loaded protein.

Determination of Nonesterified Fatty Acids (NEFA) Released in Culture Medium

Human preadipocytes from the same five women were differentiated in vitro for 11 days before treatment with 100 μM MEHP or 0.1% DMSO for 18 h. They were fasted for 3 h in DMEM without glucose, containing 3% (w/v) fatty acid-free bovine serum albumin, and then transferred in a Krebs Ringer Bicarbonate buffer containing 5 mM pyruvate for 2 h. This medium was collected and NEFA was measured using a colorimetric method with the Free Fatty Acids Half-Micro Test kit from Roche Diagnostics GmbH (Mannheim, Germany). Each measurement was performed in triplicate.

RESULTS

Transcriptomic Effects of MEHP on Human Adipocytes

To assess the effects of MEHP on human WAT, primary cultures of subcutaneous preadipocytes ($n = 5$) were differentiated in vitro into adipocytes for 11 days and treated with MEHP (100 μM) or with DMSO (vehicle, 0.1% final). An untargeted global profiling approach combining transcriptomics and ^{1}H NMR-based metabonomics was used, starting with transcriptomic profiling at 24 h of treatment. Principal component analysis (PCA) conducted on the entire transcriptomic profile clearly separated MEHP and DMSO-treated human adipocytes, indicating that MEHP strongly modified gene expression (Figure 1A). Using a paired SAM test, we selected 227 genes significantly differentially regulated between MEHP- and DMSO-treated adipocytes: 142 upregulated and 85 downregulated (Supplemental Table 2, Supporting Information). The biological pathways modulated by MEHP were then investigated using gene ontology analyses of the 142 upregulated transcripts or of the 85 downregulated transcripts in the DAVID (Figure 1B) and KEGG databases (Figure 1C). The same gene ontology analysis using all 227 genes together identified the same biological pathways and is therefore not reported here.

The analysis of upregulated transcripts first showed that the most significantly activated biological pathway was the "PPAR signaling pathway" which contained 12 genes ($p = 3 \times 10^{-7}$, Supplemental Table 3, Supporting Information). This result indicated that MEHP probably activated PPARγ, the most highly expressed PPAR isoform in WAT. MEHP treatment also enhanced metabolic processes involved in lipid metabolism, as illustrated by the following significant terms in the gene ontology analyses: "Carboxylic acid metabolic process" ($p = 2.7 \times 10^{-4}$), "Fatty acid biosynthesis" ($p = 2 \times 10^{-3}$), "Fatty acid binding" ($p = 1.0 \times 10^{-2}$), "Polyunsaturated fatty acid biosynthesis" ($p = 1.7 \times 10^{-2}$) and "Fatty acid metabolism" ($p = 1.9 \times 10^{-2}$).

Finally, the analysis of down-regulated transcripts showed that MEHP disrupted genes involved in cell shape and in cell−environment interactions, as indicated by the terms

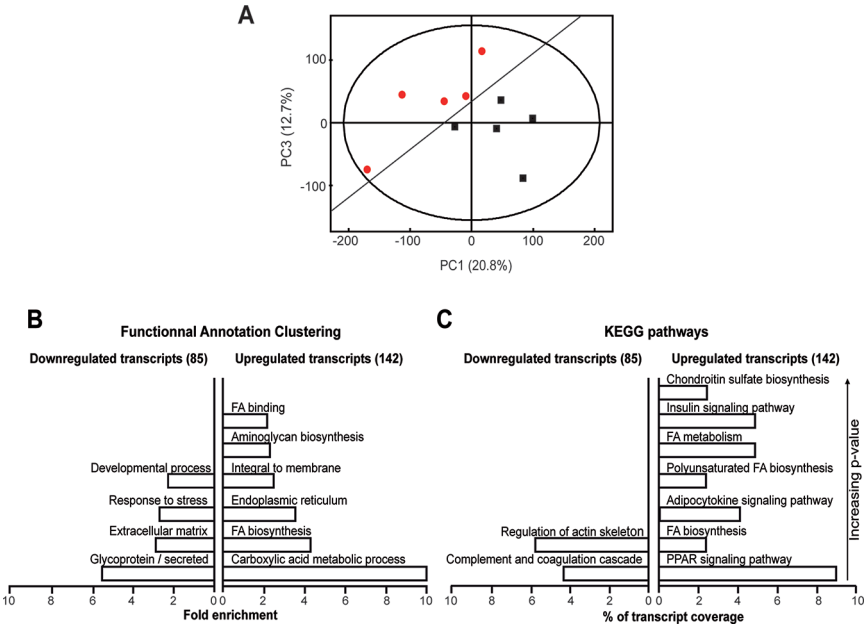

Figure 1. Transcriptomic effects of MEHP on human adipocytes. Human preadipocytes ($n = 5$) were differentiated in vitro into adipocytes for 11 days and then treated with MEHP (100 μM) or DMSO (0.1%, vehicle) for 24 h. (A) PCA analysis was conducted using expression data from the entire microarray (32 322 probesets). Black: DMSO, red: MEHP. (B, C) Gene ontology analyses were conducted on the 142 significantly upregulated and on the 85 downregulated transcripts, using DAVID (B) and KEGG (C) databases. $P < 0.05$ was used as cutoff for selecting significantly enriched biological pathways.

"Glycoprotein/secreted" ($p = 5.7 \times 10^{-6}$), "Extracellular matrix" ($p = 8.9 \times 10^{-3}$), and "Regulation of the actin cytoskeleton" ($p = 5 \times 10^{-2}$).

^{1}H NMR Based Metabolic Profiling of MEHP-Treated Human Adipocytes

The metabolic disruptions induced by MEHP in human adipocytes were evaluated in the same cell cultures ($n = 5$) at 24 and 48 h of treatment using ^{1}H NMR profiling on both the organic and the aqueous fractions of cell extracts. Metabolic alterations induced by MEHP treatment were first investigated in the polar extracts of human adipocytes (Figure 2A). To assess the differences between the metabolic profiles of MEHP- versus DMSO-treated human adipocytes, we constructed O-PLS-DA models at 24 and 48 h as described in Experimental Procedures. The metabolic profiles of MEHP-treated adipocytes differed markedly from those of DMSO-treated cells, at both 24 h (parameters of the O-PLS-DA performed with one predictive component and one orthogonal component: $R^2X = 0.63$, $R^2Y = 0.87$, $Q^2Y = 0.73$, $p = 0.001$, Supplemental Figure 1, Supporting Information) and 48 h of treatment (parameters of the O-PLS-DA performed with one predictive component and one orthogonal component: $R^2X = 0.65$, $R^2Y = 0.94$, $Q^2Y = 0.73$, $p = 0.009$; Figure 2B). The metabolites responsible for this discrimination were determined by computing their correlation coefficient (r^2) derived from the O-PLS-DA models (Supplementary Table 4, Supporting Information). At both time points, MEHP-treated adipocytes displayed lower intracellular levels of lactate and of several amino acids, including valine, glutamine, phenylalanine, and tyrosine, as well as higher levels of aspartate. MEHP treatment also influenced the level of metabolites involved in phospholipid metabolism, as indicated by higher levels of phosphoethanolamine and choline, and lower levels of glycerophosphocholine in MEHP-treated adipocytes. Finally, NAD^+ and creatine levels were increased in MEHP-treated adipocytes.

In the apolar organic cell extracts, signals from various lipophilic molecules such as cholesterol, glycerol linked to phospholipids and/or to triglycerides and fatty acids were detected (Figure 2C). As most of these signals derived from fatty acids (either free or esterified in triglycerides), the conventional method of normalization, dividing each spectral data point by the total spectral area, was not adequate. For example, a global increase of triglyceride content in one group would be missed using this method. Therefore, ^{1}H NMR peaks were integrated and normalized to cholesterol signals (δ0.68 and δ1.01). This normalization resulted in a simplified data matrix containing 13 variables. At 24 h of treatment, no difference was observed between MEHP- and DMSO-treated human adipocytes ($Q^2Y < 0$, data not shown). At 48 h of treatment, significant differences were observed between the organic metabolic profiles of MEHP-treated compared to DMSO-treated human adipocytes (parameters of the O-PLS-DA performed with one predictive component and two orthogonal components: $R^2X = 0.93$, $R^2Y = 0.98$, $Q^2Y = 0.68$, $p = 0.03$). The discrimination between MEHP- and DMSO-treated human adipocytes was mainly due to a higher level of the protons involved in fatty acid double bonds (δ5.35, signal J, $r^2 = 0.41$, $p = 0.046$) in MEHP-treated adipocytes (Figure 2D). Two of the three glycerol signals showed increases of borderline significance in MEHP-treated adipocytes (δ5.28, signal I, $r^2 = 0.26$, $p = 0.13$ and δ4.33, signal H, $r^2 = 0.29$, $p = 0.1$).

Assessment of Intracellular Triglyceride Content in Human Adipocytes

The previous untargeted transcriptomic and metabonomic observations demonstrated that MEHP significantly disrupted

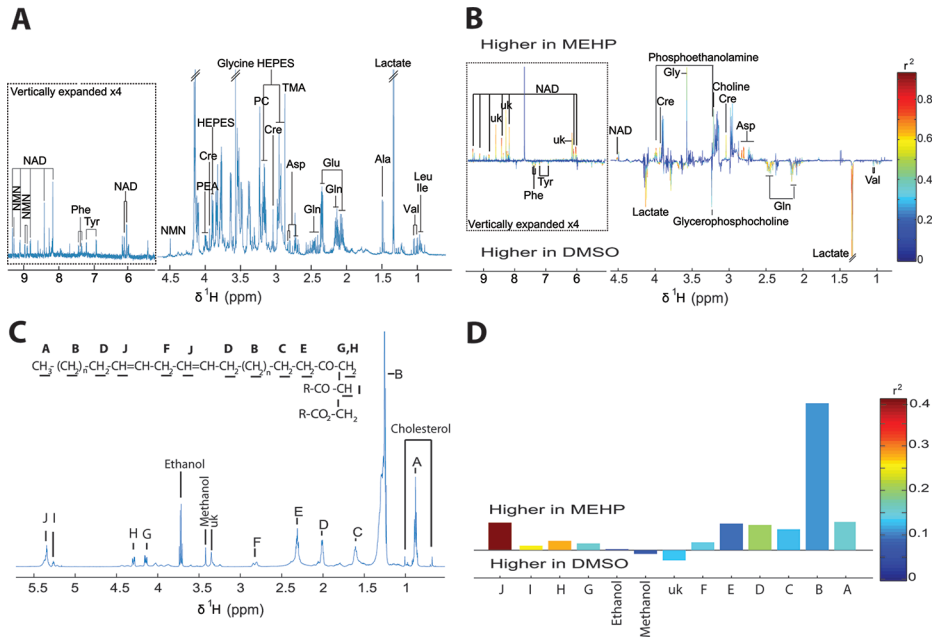

Figure 2. ^{1}H NMR-based metabonomic investigation MEHP effects on human adipocytes. (A) Representative ^{1}H NMR spectra (600 MHz) derived from an aqueous extract of human adipocytes. (B) Plot of O-PLS-DA coefficients related to the discrimination between ^{1}H NMR spectra from aqueous extracts of human adipocytes treated with MEHP ($n = 5$, top) vs. DMSO ($n = 5$, bottom) for 48 h. O-PLS-DA parameters: $R^2X = 0.65$, $R^2Y = 0.94$, $Q^2Y = 0.73$, $p = 9 \times 10^{-3}$, one predictive and one orthogonal component. (C) Representative ^{1}H NMR spectra (600 MHz) derived from an organic extract of human adipocytes. uk: unknown compound. (D) Plot of O-PLS-DA coefficients related to the discrimination between ^{1}H NMR spectra from organic extracts of human adipocytes treated with MEHP ($n = 5$, top) vs. DMSO ($n = 5$, bottom) for 48 h. O-PLS-DA parameters: $R^2X = 0.93$, $R^2Y = 0.98$, $Q^2Y = 0.68$, $p = 0.03$, one predictive and two orthogonal components. Leu: leucine; Ile: isoleucine; Val: valine; Glu: glutamate; Gln: glutamine; Asp: aspartate; TMA: trimethylamine; Cre: creatine; PC: phosphocholine; uk: unknown compound; PEA: phosphoethanolamine; NMN: *N*-methylnicotinamide; NAD: nicotinamide adenine dinucleotide; Tyr: tyrosine; Phe: phenylalanine.

lipid metabolism in human adipocytes. However, using standard 1D NMR spectroscopy, the increase in glycerol signals was of borderline significance, and we were unable to differentiate between the glycerol linked to phospholipids and the glycerol linked to triglycerides. Therefore, in order to confirm and refine the previous results, we measured the triglyceride concentration using an enzymatic reaction at several stages of human adipocyte differentiation and after MEHP treatment (Figure 3).

Intracellular triglyceride accumulation increased linearly during the in vitro differentiation protocol. Eleven days after the hormonal induction of differentiation, triglyceride accumulation reached its maximal level and the cells were then treated with MEHP. At 24 h, the intracellular triglyceride content was not affected by MEHP treatment. In contrast, at 48 h, the triglyceride level was significantly higher in MEHP-treated compared to DMSO-treated adipocytes (213.6 ± 2.9 μg/mg protein vs. 168.8 ± 9.9 μg/mg protein, respectively, $p = 0.02$, Figure 3). These results therefore confirmed the increased glycerol signals highlighted by ^{1}H NMR after 48 h of MEHP treatment.

Early Effects of MEHP on Human Adipocytes

Transcriptomic and metabonomic results were integrated using untargeted statistical modeling (Supplemental Figure 2, Supporting Information). Integration of transcriptomic and metabonomic data at the same time point (24 h of treatment) produced poor statistical models, as illustrated by the low number of p values < 0.1 (p values in blue, Supplemental Figure 2, Supporting Information). In contrast, integration of metabonomic profiles obtained at 48 h of treatment and transcriptomic profiles at 24 h produced an overall shift of the p values toward

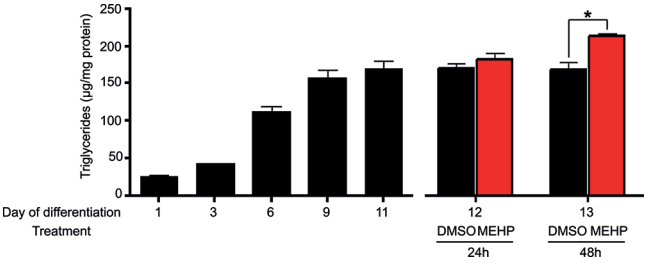

Figure 3. Intracellular triglycerides in human adipocytes. Human preadipocytes ($n = 5$) were differentiated in vitro into adipocytes for 11 days and then treated with MEHP (100 μM) or DMSO (0.1%, vehicle) for 24 or 48 h. Black: DMSO, red: MEHP. *: $P < 0.05$ compared to DMSO-treated cells, Wilcoxon signed-rank test.

lower values, indicating stronger associations between these two data sets (p values in red). This result demonstrated that the metabolic alterations after 48 h of MEHP treatment correlated well with the transcriptomic alterations after 24 h, whereas the metabolic alterations after 24 h of treatment failed to correlate with the transcriptomic data, suggesting that MEHP may induce earlier transcriptomic alterations.

To further explore this hypothesis, we performed complementary transcriptomic experiments at earlier time points and we assessed the expression of selected genes involved in key adipocyte metabolic processes (e.g., differentiation, glyceroneogenesis, fatty acid synthesis...) after 4, 8, and 24 h of treatment with MEHP (Figure 4). At 24 h of treatment with MEHP, most of the tested genes were significantly upregulated, which

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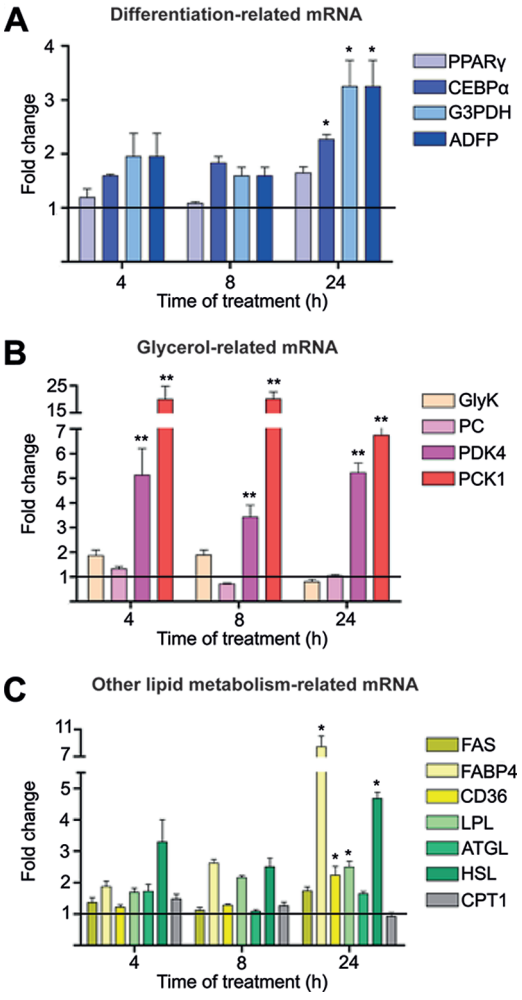

Figure 4. MEHP induced early transcriptomic alterations in human adipocytes. mRNA expression of genes involved in adipocyte differentiation (A), glycerol metabolism (B), and other lipid-related metabolic pathways (C) in MEHP-treated (100 μM) or DMSO-treated (0.1%) human adipocytes. Data represent fold changes compared to DMSO-treated human adipocytes (mean ± SEM, $n = 5$). *: $P < 0.05$, **: $p < 0.01$ compared to DMSO-treated cells, Wilcoxon signed-rank test.

confirmed the microarray results. On the contrary, at earlier time points, only two genes were upregulated: the phosphoenolpyruvate carboxykinase 1 (PCK1) and the pyruvate dehydrogenase kinase 4 (PDK4), which encode the two main enzymes involved in glyceroneogenesis.[23,24]

Glyceroneogenesis is a metabolic pathway that converts glucogenic precursors such as pyruvate or lactate into glycerol-3-phosphate, which is then used for triglyceride synthesis (Figure 5A). It has been demonstrated that the cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C), the protein encoded by the PCK1 gene, is the key enzyme in glyceroneogenesis.[25] Therefore, we assessed PEPCK-C protein expression after MEHP treatment. After 18 h of MEHP treatment, the PEPCK-C protein content was increased (Figure 5B). Furthermore, glyceroneogenesis contributes to regulate the release of non-esterified fatty acids (NEFA) from WAT in the circulation.[25] NEFA release from adipocytes indeed represents a balance between fatty acid breakdown by lipolysis, and fatty acid reesterification into triglycerides, which are then released into

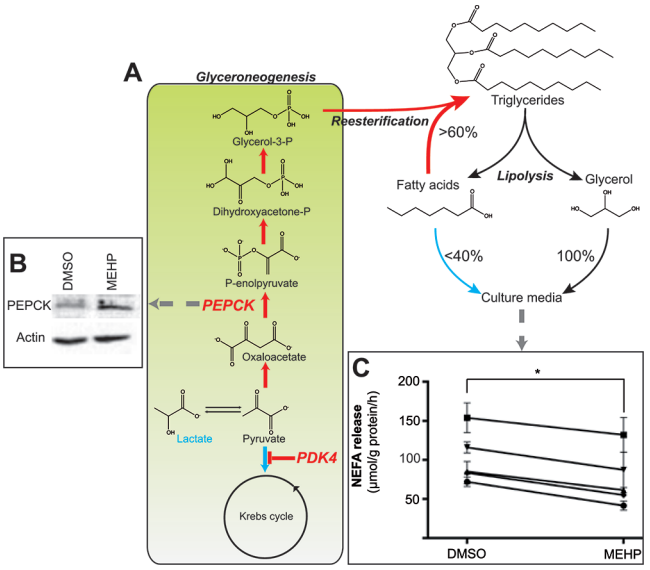

Figure 5. MEHP induced early metabolic alterations in human adipocytes. (A) General description of the glyceroneogenesis and NEFA reesterification pathways in adipocytes (adapted from ref 25). (B) PEPCK-C protein expression in human adipocytes treated with MEHP (100 μM) or DMSO (0.1%) for 18 h. Representative Western blot. (C) NEFA release in the culture medium of human adipocytes treated with MEHP (100 μM) or DMSO (0.1%) for 18 h and fasted for 3 h. Results represent a mean ± SEM for three replicates per individual ($n = 5$). *: $P < 0.05$ compared to DMSO-treated cells, Wilcoxon signed-rank test.

the bloodstream (Figure 5A). The effects of MEHP on NEFA reesterification were investigated in human adipocytes by evaluating NEFA release into the culture medium during fasting. At 18 h of treatment with MEHP or DMSO, human adipocytes were transferred for 3 h into a glucose-deprived culture medium, in which the NEFA content was determined. NEFA release was significantly lower in MEHP-treated than in DMSO-treated adipocytes (75 ± 13 μmol/g protein/h vs 100 ± 12 μmol/g protein/h respectively, $p = 0.03$, Figure 5C).

DISCUSSION

MEHP Enhances the Expression of Genes Associated with PPARγ Activation in Human Adipocytes Differentiated in Vitro

This study is the first attempt to decipher the effects of the pollutant MEHP in a human model of WAT. We used an untargeted combined transcriptomic−^{1}H NMR-based metabonomic approach to derive hypotheses about the mechanisms of action of MEHP, which were then confirmed using targeted molecular methods. Transcriptomic profiling of human adipocytes differentiated in vitro, established that 24 h of MEHP treatment significantly induced the expression of 12 genes involved in "PPAR signaling pathway" ($p = 3 \times 10^{-7}$, Figure 1, Supplementary Table 3, Supporting Information). Seven of these 12 genes have a known consensus sequence for PPAR binding (PPAR response element) in their promoter: PCK1,[26] PDK4,[27] adipocyte differentiation related protein (ADRP),[28] lipoprotein lipase (LPL), fatty-acid translocase (CD36), acyl CoA synthetase 1 (ACS1), and adiponectin (AdipoQ).[3]

In keeping with our findings, studies in 3T3-L1 preadipocytes showed that MEHP was a selective PPARγ activator.[6] In this

murine cell line, MEHP induced the selective recruitment of transcription cofactors by PPARγ, leading to induction of the expression of a restricted subgroup of genes compared to treatment with rosiglitazone, a full agonist of PPARγ. For example, in 3T3-L1 preadipocytes, 10 days of MEHP treatment induced the expression of adipoQ, CD36, ACS1, LPL, fatty acid binding protein 4 (FABP4), cAMP element binding protein alpha (CEBPα), and liver X receptor alpha (LRXα), whereas rosiglitazone but not MEHP induced the expression of glycerol kinase (GlyK) and oxidized low density lipoprotein receptor 1 (Olr1). This selective gene activation indicated that MEHP-induced PPARγ target gene expression depended on the promoter context. This work in 3T3-L1 cells also highlighted the fact that PPARγ transactivation by MEHP differed according to the analyzed cell line, which emphasizes the importance of confirming these results in human cells, where the cellular coregulator equipment might differ. Using human adipocytes differentiated in vitro, we observed a similar gene induction pattern after MEHP treatment as in 3T3-L1 preadipocytes, i.e., induction of the expression of FABP4, adipoQ, CD36, ACS1, LPL, CEBPα, and LRXα genes but not of GlyK or Olr1 genes (Supplementary Table 2, Supporting Information, Figure 4). However, in previous studies GlyK and Olr1 were activated by full agonists of PPARγ in human adipocytes.[29,30] Thus, our results showed a restricted gene induction profile in MEHP-treated human adipocytes compared to full PPARγ agonists, indicating that MEHP might be a selective PPARγ activator in human adipocytes.

MEHP Has a Pro-Differentiating Effect on Human Adipocytes Differentiated in Vitro

PPARγ is a key regulator of adipogenesis, the process through which fibroblast-like preadipocytes are converted into mature adipocytes. In 3T3-L1 murine preadipocytes, MEHP-dependent PPARγ activation promotes adipogenesis.[6] In the present work, human preadipocytes were first differentiated in vitro for 11 days. Intracellular triglycerides accumulation, a phenotypic marker of differentiation, increased linearly during in vitro differentiation (Figure 3). Triglyceride accumulation peaked 11 days after the hormonal induction of differentiation, and the cells were then treated with MEHP for one or two additional days. Although a large proportion of cells (60−70%) were already differentiated at this stage, one possibility was the resumption of differentiation in the remaining preadipocytes.

Adipocyte differentiation is a very complex phenomenon that converts fibroblastic preadipocytes into mature adipocytes. This process involves dramatic increases in triglyceride content, drastic morphologic changes, and enhanced transcription of adipocyte-specific genes.[31] In the present work, MEHP treatment was associated with numerous transcriptomic alterations confirming that MEHP induced further differentiation of humans adipocytes differentiated in vitro. First, a well-described hallmark of the adipogenesis process is the dramatic alteration in cell shape as the cells convert from a fibroblastic to a spherical cell shape. These morphological modifications occur simultaneously with changes in the levels and types of extracellular matrix components.[31] Here, we showed that MEHP treatment for 24 h disturbed genes linked to "actin cytoskeleton regulation" and "extracellular matrix" (Figure 1), indicating an effect on cell shape and on interactions of cells with the extracellular matrix. For instance, MEHP regulated the expression of two collagen genes (COL4A6 *1.35, COL8A6 *-1.56, Supplementary Table 2, Supporting Information). This switch in the expression of collagen genes is a well-described event in adipogenesis.[31] Second, MEHP enhanced the expression of genes involved in all metabolic pathways leading to triglyceride synthesis and storage in human adipocytes: channeling and transport of fatty acids (LPL, CD36, FABPs), production of glycerol-3-phosphate (glycerol-3P dehydrogenase (G3PDH), PCK1) and of fatty acid-CoA esters (acylCoA synthase 2 (ACSF2), acylCoA synthase long chain 3 (ACSL3) and ACSL5), and de novo lipogenesis (acetylCoA carboxylase (ACCb), fatty acid synthase (FAS)) (Supplementary Table 2, Supporting Information).

In addition to these transcriptomic alterations, metabolic profiling revealed drastic changes in MEHP-treated cells. First, MEHP increased the signals of protons involved in lipid double bonds, as well as the aspartate and NAD contents (Figure 2). All these metabolic changes have been reported previously during the differentiation of 3T3-L1 preadipocytes to adipocytes.[32−34] Indeed, fatty acid desaturation and aspartate transfer for putrescine synthesis were previously shown to be required for 3T3-L1 conversion into adipocytes.[35,36] Second, MEHP decreased the intracellular concentrations of lactate and glutamine, possibly via increased use of these substrates for de novo lipogenesis.[37,38] Finally, ^{1}H NMR-based metabolic profiling, as well as subsequent enzymatic assays, demonstrated that after 48 h of treatment, MEHP further increased the intracellular triglyceride level (Figure 3). Altogether, these results demonstrate that MEHP had a pro-differentiating effect on human adipocytes differentiated in vitro.

MEHP Activated Glyceroneogenesis and Enhanced NEFA Reesterification Early in Mature Human Adipocytes

Interestingly, early modulations in the metabolic profiles of MEHP-treated human adipocytes occurred prior to the increase in intracellular triglycerides. In addition, at 24 h of treatment with MEHP, untargeted statistical integration of both transcriptomic and metabonomic profiles revealed poor correlations. This result suggested that MEHP induced early modulation of gene expression in human adipocytes, independently from the differentiation process. This hypothesis was confirmed by the strong and selective induction of two genes, PCK1 and PDK4, at only 4 h of treatment with MEHP (Figure 4). PDK4 inhibits the activity of the pyruvate dehydrogenase complex, which is involved in the synthesis of acetyl-coA from pyruvate. PDK4 induction therefore contributes to alter the pyruvate toward oxaloacetate production. PEPCK-C, the product of PCK1, synthesizes phosphoenolpyruvate from oxaloacetate. Therefore, in WAT, combined activation of PCK1 and PDK4 genes results in the redistribution of the pyruvate flux toward glyceroneogenesis.[24] As previously discussed, glyceroneogenesis is a metabolic pathway enhanced during fasting that converts glucogenic precursors such as pyruvate, lactate, or amino acids into glycerol-3-phosphate, which is subsequently used for triglyceride synthesis (Figure 5A). Here, glyceroneogenesis activation by MEHP in human adipocytes was further confirmed by the observation that MEHP increased PEPCK-C protein levels. Since PEPCK-C activity correlates directly with its expression level,[39,40] this observation reflected an increased glyceroneogenic flux in MEHP-treated human adipocytes.

Furthermore, glyceroneogenesis contributes to regulate NEFA release from WAT to the circulation. NEFA release from adipocytes results from a balance between fatty acid breakdown by lipolysis and fatty acid reesterification into triglycerides, which are then released into the bloodstream. Fatty acid reesterification accounts for a large proportion of lipolytically derived NEFA (30−70%) and therefore requires glycerol-3- phosphate synthesis,

which under fasting conditions originates from glyceroneogenesis (Figure 5A). In the present work, we demonstrated that NEFA release was reduced in MEHP-treated adipocytes after fasting. This change might also result from a decrease in lipolysis or β-oxidation. However, at early time points, we found no decreases in the expression of genes involved in lipolysis, such as the adipocyte triglyceride lipase (ATGL) or the hormone-sensitive lipase (HSL), or in β-oxidation, such as the carnitine palmitoyl transferase 1 (CPT1). Similarly, a recent study showed no effect of DEHP on lipolysis in rat adipose tissue.[41] Altogether, our results indicate that more NEFA were reesterified into triglycerides under MEHP treatment, for which glycerol backbone was provided by the concomitant enhancement of glyceroneogenesis.

Finally, it has been demonstrated that PEPCK-C was expressed only in mature adipocytes and that PPARγ was unable to activate PCK1 in preadipocytes.[42] Therefore, we conclude that PCK1 induction and the subsequent increases in glyceroneogenesis and NEFA reesterification observed in human adipocytes differentiated in vitro after MEHP treatment occurred in the mature adipocytes present in the cell culture.

MEHP Exposure and Expected Consequences for Humans

Although the human exposure to DEHP is ubiquitous, plasma levels of DEHP and MEHP are thought to remain generally low because of rapid urinary excretion.[43] Plasmatic concentrations of 38 mg/L (~100 μM) DEHP and of about 1.37 μg/mL (~3 μM) MEHP have been measured in healthy individuals.[44,45] However, several parameters such as daily use of cosmetics, mouthing behavior in children, neonatal intensive care, frequent blood transfusion or dialysis can increase phthalate exposure 4−14-fold compared to control populations.[46−48] Furthermore, DEHP is a lipophilic compound that can accumulate in WAT.[44] We found no data on MEHP accumulation in WAT. However, exposure data suggest that, under certain circumstances, MEHP levels in plasma and WAT may reach 100 μM, the concentration for which we observed metabolic effects in human preadipocytes and adipocytes.

In this work, we have shown that MEHP increased the triglyceride content in human adipocytes differentiated in vitro, which may be explained by, at least, two concomitant mechanisms: an early increase in glyceroneogenesis and NEFA reesterification occurring in mature adipocytes, followed by differentiation of the remaining preadipocytes. In previous studies, both mechanisms were activated in animal and human WAT by other PPARγ agonists, such as the antidiabetic drugs thiazolidinediones.[22,49] Both mechanisms contribute to the global improvement in carbohydrate and lipid homeostasis observed during thiazolidinedione treatment. However, they also both result in increased triglyceride accumulation in WAT and are thought to participate in the weight-gain promoting effect of these drugs. These mechanisms may thus contribute to the suspected obesogen effect of MEHP and of other phthalates capable of activating PPARγ. Epidemiological studies have indeed shown a positive correlation between urinary MEHP concentrations and waist circumference in healthy men[50] and between other phthalate metabolites and body mass index.[51]

In addition, glyceroneogenesis activation by MEHP may also interfere more generally with energy homeostasis in exposed populations. The effect of PEPCK-C in WAT on both glyceroneogenesis and reesterification of NEFA to triglycerides is crucial to prevent diabetes. PEPCK-C dysregulation interferes with the triglyceride/fatty acid cycle, which regulates lipid synthesis and transport between WAT and the liver, leading to the development of insulin resistance in mice.[52] In another study, Franckhauser et al. demonstrated that transgenic mice overexpressing PEPCK-C in WAT remained insulin sensitive under a high-fat diet but developed severe obesity and were more hyperinsulinemic, glucose intolerant, and insulin resistant than wild-type controls.[53] Thus, PEPCK-C overexpression in adipose tissue increased the susceptibility to diet-induced obesity and insulin resistance. These results, together with our present work, suggest that phthalate exposure might be of particular concern in populations already susceptible to insulin resistance because of an unbalanced diet.

CONCLUSION

This study in a human WAT model provides the first evidence that the environmental pollutant MEHP promotes triglyceride storage by altering the metabolism of both preadipocytes and mature adipocytes. The earliest effects of MEHP were increases in glyceroneogenesis and NEFA reesterification in mature adipocytes, which interfered with the regulation of fatty acid release by human WAT. After a longer treatment time, MEHP had a pro-differentiating effect on the preadipocytes remaining in the cell cultures. These results provide convincing new evidence that MEHP interferes with endogenous lipid metabolism in human WAT and should therefore be considered as a potential obesogen, as well as a more general metabolic disruptor.

ASSOCIATED CONTENT

Supporting Information

Supplemental Table 1: List of primers used for RT-QPCR. Supplemental Table 2: Genes significantly differentially regulated in human adipocytes after 24 h of MEHP treatment. Supplemental Table 3: Genes involved in the "PPAR signaling pathway". Supplemental Table 4: Variations of metabolite signals in the O-PLS-DA models related to the discrimination between ^{1}H NMR spectra from aqueous extracts of human adipocytes treated with MEHP or DMSO. Supplemental Figure 1: O-PLS-DA coefficients related to the discrimination between ^{1}H NMR spectra from aqueous extracts of human adipocytes treated with MEHP (n = 5, top) or DMSO (n = 5, bottom) for 24 h. Supplemental Figure 2: Statistical integration of transcriptomic and metabonomic alterations induced by MEHP in human adipocytes. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

WAT: white adipose tissue; PPAR: peroxisome proliferator-activated receptor; DEHP: di(2-ethylhexyl) phthalate; MEHP: monoethyl-hexyl phthalate; PCA: principal component analysis;

O-PLS-DA: orthogonal partial least-squares-discriminant analysis; NEFA: nonesterified fatty acids; PCK1: phosphoenolpyruvate carboxykinase (gene); PEPCK-C: phosphoenolpyruvate carboxykinase-cytosolic (protein); PDK4: pyruvate dehydrogenase kinase 4

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