In vitro and in vivo evidence of bioenergetic metabolism alteration by mono-(2-ethylhexyl) phthalate

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Abstract

Background: To better understand the potential alteration of muscle bioenergetic metabolism by the obesogenic toxicant mono-(2ethylhexyl) phthalate (MEHP) the objectives of this research were to determine the: 1) association between urinary MEHP levels and plasma fatty acid levels in women with obesity who participated in National Health and Nutrition Examination Survey (NHANES) studies, and 2) *in vitro* effects of MEHP on fatty acid, or glucose supported mitochondrial energetics in C2C12 muscle cells.

Results: The association between urinary MEHP from NHANES participants with plasma fatty acid levels was studied via secondary data statistical analyses. ^{14}C-palmitic acid oxidation, Seahorse fatty acid oxidation and glycolysis stress tests and western blot analyses were conducted on C2C12 cells exposed to increasing MEHP concentrations. Increased urinary MEHP in women with obesity was associated with increased plasma gamma-linolenic and arachidonic acid levels. C2C12 myotubes exposed to increasing MEHP concentrations, displayed decreased fatty acid oxidation and mitochondrial bioenergetics. Acyl-CoA synthetase long chain 5 (ACSL5) protein level was also upregulated with increasing MEHP exposure in C2C12 myoblasts. Glycolysis was not significantly modified with increased exposure of C2C12 cells to MEHP.

Conclusions: MEHP exposure may alter fatty acid utilization at the whole-body level in women with obesity and fatty acid utilization in muscle cells. Our findings are consistent with the idea that women with obesity may be particularly susceptible to the effects of MEHP, which alters fatty acid metabolism in muscle cells.

Background

Obesogens are defined as chemical compounds that contribute to impaired lipid metabolism, dysregulation of adipogenesis, and consequently, may contribute to the development of obesity (1). One of these obesogens, di(2-ethylhexyl) phthalate (DEHP), is one of the primary phthalate plasticizers used in North America, and is commonly found in food packaging and medical devices (2,3). DEHP is non-covalently bound into matrices and therefore can leach into the human body and be metabolised via lipases into the monoester derivative mono-(2ethyl hexyl) phthalate (MEHP), one of the major forms to be absorbed (4). While the average tolerable intake for DEHP is 5.8–19 µg/kg/day, individuals exposed to a medical environment can exceed the average daily intake with an increase of ten-fold by the end of cardiopulmonary bypass in adults ([2],5,6) .

Previous human population studies in women and men, have reported a positive association between urinary phthalate metabolites including MEHP and mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) with obesity, while also demonstrating a positive correlation between DEHP metabolite exposure (measured via urinary metabolites) and BMI, waist circumference and adiposity within children (7–9). Studies have also illustrated higher urinary MEHP to MEHHP ratio in women with greater BMI, suggesting a slower rate of oxidative metabolism of the toxicant (10). A decrease in MEHHP, a product of MEHP oxidation, may be a result of a decrease in activity of selected P450 enzymes, which might affect phthalate metabolism (11). This may result in the alteration of fatty acid utilization pointing out the importance of studying the effects of MEHP in subjects with obesity.

Studies have shown that the observation of increased accumulation of lipids in skeletal muscle of subjects with obesity, may be in part due to a decrease in fatty acid oxidation which contributes towards insulin resistance (12). Rodent and human studies have illustrated how chronic high fat feeding can lead to elevated incomplete β oxidation, and impairment in mitochondrial oxidative phosphorylation and biogenesis (13–15). Furthermore, rats consuming increased dietary fat and sucrose, had muscle fiber-specific decrease in ATP production (16,17). Studies have suggested links between type 2 diabetes/insulin resistance and muscle mitochondrial dysfunction, though this remains controversial (18–23).

While little is known about the effects of MEHP on human skeletal muscle, a decrease in muscle strength in the elderly has been shown to be parallel with increasing urinary MEHP, which was hypothesized to be a result of MEHP-induced oxidative stress and inflammation (24). Furthermore, Corbasson et al. (2016) illustrated an association between increased urinary MEHP and decreased lean mass in adults, where lean mass excluded bone mineral content, thus primarily representing muscle mass (25). While adipocytes have been studied extensively and results have demonstrated altered adipocyte lipid metabolism and mitochondrial respiration, little is known about the cellular and molecular effects of MEHP on skeletal muscle fatty acid and glucose metabolism as well as mitochondrial energetics (26).

Thus, we aimed to determine the 1) association between urinary MEHP and plasma fatty acid levels in women with obesity who participated in the National Health and Nutrition Examination Survey (NHANES) 2003–2004 study, and 2) in vitro effects of MEHP on fatty acid or glucose supported mitochondrial energetics in C2C12 muscle cells.

Results

MEHP and plasma omega-6 polyunsaturated fatty acid levels in women with obesity participating in the NHANES 2003-2004 Study

Participants' characteristics are presented in Table 1. While the majority of the plasma omega-6 fatty acid levels did not display a significant association with urinary MEHP, a positive association was observed between urinary MEHP levels and gamma-linolenic acid (0.436 ± SE: 0.182; p=0.019) as well as arachidonic acid (7.62 ± SE: 1.73; p=0.000) in women with obesity (Table 2). Furthermore, r^2 adjusted model value explained 35% of the individual variation observed in arachidonic acid levels. Interestingly, it is MEHP which has the largest effect size (f^2=0.212) in the linear model explaining variance in plasma arachidonic acid levels. However, while r^2 adjusted model value explained 24% of the individual variation observed in gamma-linolenic acid levels with a major influence of ethnicity, MEHP still influenced the model with an effect size of f^2=0.056.

Gamma-linolenic acid is a precursor of arachidonic acid. Moreover, increasing adult human dietary gamma-linolenic acid or arachidonic acid induces an increase in tissue and plasma arachidonic acid content (27,28). Plasma arachidonic acid is a major source of skeletal tissue arachidonic acid (29). Unmetabolized free arachidonic acid is converted into arachidonoyl-CoA by acyl-coenzyme A synthetases long-chain (ACSL), which play an important role in fatty acid metabolism and are believed to be involved in pathophysiological events(30–34). Based on previous work on rat fibroblasts where these enzymes were shown to play a role in arachidonic acid metabolism, we studied the effects of MEHP on exogenous long-chain fatty acid oxidation, mitochondrial respiration, and glycolysis in C2C12 myotubes (35).

Evaluation of MEHP cytotoxicity on C2C12 cells

Prior to studying the effects of increasing concentrations of MEHP on C2C12 myotubes, its cytotoxicity was studied. There was no significant difference in the number of apoptotic and necrotic cells with increasing concentrations of MEHP exposure on C2C12 myotubes, measured by condensed nuclei and PI positive cells (Figure 1). Furthermore, cell death always remained below 5% of the cell population.

The effects of MEHP on exogenous fatty acid oxidation and mitochondrial respiration in C2C12 myotubes

To characterize the metabolic effects of MEHP, ^{14}C-palmitic acid oxidation as well as palmitate-induced respiration were studied in C2C12 myotubes. There was an overall decrease in total fatty acid oxidation following the exposure of myotubes to increasing concentrations of MEHP (Figure 2 a; p=0.035). Specifically, a decrease was observed between the control 0.1% DMSO (1.183 nmol/hr/mg ± 0.035) and MEHP exposed cells of 10 µM (1.033 nmol/hr/mg ± 0.048; p=0.011), 100 µM MEHP (1.076 nmol/hr/mg ± 0.012; p=0.048) and the highest concentration 300 µM MEHP (1.034 nmol/hr/mg ± 0.022; p=0.012). This reduction in fatty acid oxidation, was accompanied by a reduction in spare respiratory capacity (p=0.012) when studying the muscle cell effects of MEHP on oxygen consumption rate (OCR) (Figure 2 b and c). A decrease in spare respiratory capacity was observed between 0.1% DMSO (1.87 pmol/min/µg ± 0.121), 10 µM (1.85 pmol/min/µg ± 0.177) and the higher concentrations of MEHP, 100 µM (1.10 pmol/min/µg ± 0.279; vs 0.1% DMSO p=0.042; vs 10 µM DMSO p=0.047) and 300 µM (0.65 pmol/min/µg ± 0.281 vs 0.1% DMSO p=0.005; vs 10 µM DMSO p=0.005). While a trend towards a decrease in maximal respiration was observed between 10 µM (1.75 pmol/min/µg ± 0.382) and higher concentrations of 100 µM (0.62 pmol/min/µg ± 0.263) and 300 µM MEHP (0.60 pmol/min/µg ± 0.466), results were not significant. No difference in basal respiration rate was observed between C2C12 myotube exposure to different MEHP concentrations. Furthermore, metabolic effects were only observed during fatty acid-driven oxidation, as mitochondrial stress test demonstrated no difference with exposure to increasing concentrations of MEHP (Supplementary Figure 1).

The effects of MEHP on cellular glycolysis levels

Based on altered fatty acid metabolism in C2C12 myotubes exposed to MEHP, the effects of the toxicant on glucose utilization were studied, since we hypothesized that the latter may act as a compensatory pathway for ATP production. Specifically the extracellular acidification rate (ECAR) was studied in the Seahorse XF analyzer. ECAR measured during mitochondrial stress test, illustrated a trend towards an increase in basal glycolysis levels following increased exposure to MEHP (Supplementary Figure 1). Furthermore, ECAR measured by the glycolysis stress test in C2C12 myotubes, also illustrated a trending increase in basal glycolysis levels, specifically between 0.1% DMSO control (1.10 ± 0.097) and 300 µM MEHP (1.40 ± 0.135) (Figure 3).

The effects of MEHP on metabolism related proteins

After examining the effects of MEHP exposure on muscle fatty acid and glucose utilization/metabolism, we studied the toxicant's effect on levels of acyl-CoA synthetase long chain protein 5 (ACSL5), a key protein in long-chain fatty acyl-CoA production from free fatty acids. Increasing the exposure of C2C12 myotubes to MEHP appeared to have no effect on ACSL5 (data not shown). However, there was a decrease in ACSL5 levels (p=0.032) with increasing MEHP in myoblasts (Supplementary Figure 2), specifically between 10 µM (Volume Intensity: 1.00 ± 0.075) and higher concentrations including 100 µM (Volume Intensity: 0.464 ± 0.137; p=0.007) and 300 µM MEHP (Volume Intensity: 0.467 ± 0.116; p=0.008) when adjusted for 0.1% DMSO control. These results reveal alteration in ACSL5 levels in myoblasts exposed to MEHP toxicant.

Discussion

MEHP, is the monoester hydrolyzed form of the plasticizer DEHP. While much is known about the metabolic effects of MEHP on the general population, how this toxicant influences the metabolism of an individual with obesity remains to be elucidated. Studies have shown that individuals with obesity have elevated intra-myocellular accumulation of lipids in skeletal muscle, in part due to a reduction in fatty acid oxidation thus contributing towards insulin resistance (12). As previous research by Corbasson et al. (2016), has illustrated an inverse association between urinary MEHP and lean mass (which excluded bone mineral content, thus primarily muscle mass), our research was focused on studying the effects of MEHP on muscle cell metabolism. Our results implicate an alteration in fatty acid utilization by MEHP in muscle cells as well as suggest changes in specific fatty acid utilization at the whole-body level in individuals with obesity.

Our secondary data statistical analyses focused on assessing the association between urinary MEHP and plasma fatty acid levels, given that previous studies illustrated that increased DEHP exposure is reflected in urinary levels of the monoester derivative (36). As we were interested in analysing the effects of MEHP on fatty acid transport and mitochondrial oxidation alteration, we used plasma fatty acid levels as a proxy for mitochondrial bioenergetic function (37). Specifically, previous prospective studies have shown increased omega-6 fatty acids from erythrocytes to be positively associated with weight gain (38). Our results showed a positive association between plasma levels of gamma-linolenic acid and arachidonic acid with urinary MEHP levels. Furthermore, we observed that MEHP has a large effect on plasma arachidonic acid levels. Plasma arachidonic acid is a major source of skeletal tissue arachidonic acid and increases in plasma arachidonic acid and gamma linolenic acid levels are linked with an increase in arachidonic acid in skeletal muscle (28,29). As our secondary statistical analyses included esterified fatty acids that are found in the phospholipids, triglycerides and cholesterol esters, increased levels of

gamma-linolenic acid and arachidonic acid that we observed with increased urinary MEHP, may also relate to increase in fatty acids channeled towards lipid biosynthesis. Our findings suggest an alteration of both fatty acid metabolism and utilization of fatty acids at the whole-body level with increased urinary MEHP levels. Due to a potential disruption in their enzymatic activity of MEHP oxidizing cytochrome P450 enzymes, individuals with obesity may be more susceptible to fatty acid metabolic alteration caused by MEHP, as compared to healthy individuals (10). Consequently, this could make them more prone to fatty acids metabolism dysfunction. However, this will need to be confirmed by future studies.

Unmetabolized free arachidonic acid levels are controlled by enzymes such as acyl-coenzyme A synthetases long-chain, which activate fatty acids and play a role in directing fatty acids into different metabolic pathways, and thus are believed to be involved in pathophysiological events (30–34). Since our secondary analyses illustrated urinary MEHP's positive association with plasma levels of gamma-linolenic acid and arachidonic acid, we studied the effects of MEHP on muscle cells. Previous work illustrated that MEHP directly activated PPARgamma in C2C12 cells and promoted adipogenesis (39). Interestingly, from our results, exposure of C2C12 myotubes to increased concentrations of MEHP resulted in a disruption in mitochondrial respiration, particularly a decrease in spare respiratory capacity. Spare respiratory capacity, indicates the ability of cells to respond to increases in energy demands. While ACSL5 protein levels remained constant in C2C12 myotubes exposed to MEHP, a significant decrease in ACSL5 protein expression levels was observed within myoblasts. ACSL5, which is located on the exterior of the mitochondrial outer membrane, contributes towards the conversion of long-chain free fatty acids to fatty-acyl CoA. The decreased in expression of this key protein within myoblasts may contribute to altered fatty acid metabolism in proliferative cells. However, this needs to be confirmed by further studies. Thus, exposure of C2C12 myotubes to increasing concentrations of MEHP alters fatty acid oxidation and oxidative phosphorylation pathway. Previous research has demonstrated an upregulation of 3T3-L1 adipocyte respiration with increased exposure to MEHP, while exposure of isolated rat liver mitochondria to MEHP showed an inhibition in palmitic acid oxidation as well as a reduction in overall mitochondrial respiration (26,40). This illustrates a tissue-specific effect of MEHP on mitochondrial respiration and fatty acid metabolism.

We observed a trend towards an increase in basal glycolysis between C2C12 control myotubes and myotubes exposed to 300 μM MEHP. This increase in basal glycolysis is consistent with a compensatory mechanism in the face of impaired mitochondrial energetics.

The present study focuses on the effects of MEHP at both the cellular and whole-body levels in women with obesity. To our knowledge, this is the first study measuring the effects of MEHP on plasma omega-6 polyunsaturated fatty acid (gamma-linolenic acid and arachidonic acid) levels in women with obesity. Future work should examine the metabolic effects of MEHP in men with obesity.

Conclusion

In conclusion, our current results expand knowledge on the effects of MEHP on cellular and whole-body metabolism. Increased MEHP exposure contributes towards metabolic dysfunction at both the muscle cell and whole-body levels, supporting previous studies on the influence of muscle metabolism on systemic metabolic abnormalities in individuals with insulin resistance and dysregulated glucose metabolism. Altogether our findings are consistent with the conclusion that women with obesity may be more susceptible to the metabolic effects of MEHP.

Methods

NHANES 2003–2004 Study Data Analysis

The NHANES study has been previously described (41). Briefly, NHANES is an American based survey designed to study the health and nutritional status of both adults and children in United States. NHANES provides extensive data on demographic, dietary and biological laboratory/body measurements. Because the continuous NHANES survey for 2003–2004 was the most recent survey that provided data on plasma fatty acid levels, we selected this population for our secondary statistical analyses. Inclusion criteria for the following human population statistical analyses were women with obesity (BMI ≥ 30 kg/m^2) who had data collected for omega-6 fatty acid levels, urinary MEHP levels, age and ethnicity.

Studied variables have been previously described (42). BMI was measured using standard calculations (weight in kg/ height in m^2). Urinary MEHP was measured by high performance liquid chromatograph-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) using isotopically-labeled phthalate metabolites as internal standards. Plasma fatty acid levels were measured from 100 μl of plasma obtained from individuals who had fasted for ≥ 8 hours.

NHANES 2003–2004 Study Statistical Analyses

Backward stepwise regression analyses were conducted to study the association between urinary MEHP and plasma fatty acid levels. Covariates that were included within the regression models, were continuous variables (age and BMI), and categorical variable (ethnicity: Non-Hispanic White, non-Hispanic Black, Mexican American and other Hispanic). Ethnicity was coded by dummy variables while using non-Hispanic White as the reference level. Urinary MEHP was also included to determine whether it was a significant factor for plasma fatty acid levels. Prior to conducting the statistical analyses, one extreme outlier, based on standard deviation (SD) = 3, was found for urinary MEHP levels and removed from data analysis. Independent variables considered in the regression analyses were also weighted by using sample weight values provided by NHANES. Cohen's f^2 value was calculated, to measure the effect size for each of the independent variables. This value calculates to what extent a specific independent variable contributes to the overall regression model. Descriptive statistics was also conducted to determine the mean, SD and total participants (n), for all dependent variables studied. All statistical analyses were conducted using Minitab software 17, while a p value < 0.05 was considered statistically significant.

Cell Culture

The C2C12 mouse myoblast cell line (ATCC® CRL-1772™) was grown in standard Dulbecco's Modified Eagle's medium (DMEM; Gibco) containing 10% Bovine Serum (BS; Gibco), 1% L-glutamine (Life Technologies) and 1% penicillin/streptomycin (Life Technologies). C2C12 cells were differentiated into myotubes for 7 days in DMEM supplemented with 2% BS.

MEHP Exposure

MEHP (Accu Standard- please see company's precautionary and hazard statements for proper use of chemical) acute exposure design consisted of either C2C12 myoblasts or myotubes exposed to 10, 50, 100 and 300 μM of MEHP (in 0.1% DMSO) for 24 hours. Concentrations of MEHP used were physiologically relevant and previously published by Chiang et al. 2016 (26). MEHP solutions were added into culture medium. Cells exposed to 0.1% DMSO and untreated cells served as controls.

MEHP Cytotoxicity Test

C2C12 myotubes were exposed to MEHP (10, 100 and 300 μM of MEHP) for 24 hours with n = 3 independent experiments. 1:500 dilution of Propidium Iodide (Sigma; 1 mg/ml) and 1:1000 dilution of Hoechst® 33342 (ThermoFisher; 5 mg/ml) were added and mixed into culture medium. Following 10 min incubation at 37 °C, cells were imaged using the Zeiss Axio Observer D1 Inverted DIC Fluorescence Microscope (20x, 0.80 NA, Air, Plan-Apo (DIC II) Objective; Blue (Ex:390/22 Em:460/50) and Red (Ex:560/40 Em:630/75)).

^{14}C-Palmitic acid Oxidation Experiment

Following 24 hour exposure of C2C12 myotubes in a 12-well plate to 10, 100 and 300 μM of MEHP, total palmitic oxidation was assessed using ^{14}C-palmitic acid, as described previously (43). Briefly, ASP (acid-soluble products) from both culture medium and cells were extracted (44–46). The remainder of the medium in the glass vials containing benzethonium hydroxide was used for determining CO_2 production. Prior to scintillation counting in a PERKIN ELMER Tri-Carb 2910 TR liquid scintillation counter, all experimental vials were incubated overnight in the dark at room temperature. Three independent experiments were conducted. Total fatty acid oxidation = CO_2 produced + ASP measured from cells and culture medium. All values were adjusted to protein concentration which was determined using the Biorad Protein Assay Dye Reagent.

Mitochondrial Stress Test, and Analyses of Glycolysis and Fatty Acid Oxidation

Following exposure of C2C12 myotubes to 10, 50, 100 and 300 μM MEHP for 24 hours in 96-well Seahorse plates, mitochondrial stress tests, glycolysis stress tests, and fatty acid oxidation tests were conducted following Agilent's Seahorse protocols. The 50 μM concentration was only used for mitochondrial stress test and glycolysis stress test. As we were only interested in measuring total fatty acid oxidation, etomoxir addition (which measures endogenous fatty acid oxidation) was omitted from the protocol. Prior to initiating the Seahorse XF assay, 30 μl of palmitate-BSA (stock concentration: 1 mM) or BSA (stock concentration: 0.17 mM) as control were added into the appropriate wells. Final concentrations for drugs utilized in the fatty acid oxidation and mitochondrial stress test were: oligomycin (3 μM); Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 2 μM), antimycin (4 μM), and rotenone (5.6 μM). Final concentrations for drugs utilized in the glycolysis stress test were oligomycin (3 μM) and FCCP (2 μM), glucose (100 mM) and 2-deoxy-D-glucose (2-DG; 1M). All experimental results were normalized to results from control untreated cells. Three independent experiments were conducted for the mitochondrial stress test, 5 for the glycolysis stress test and 3 for the fatty acid oxidation test.

Western Blot Analysis

Active Motif nuclear kit- "Preparation of Whole Cell Extract from Cells" protocol was utilised for the extraction of proteins from C2C12 cells exposed to MEHP (10, 50, 100, 150, 300 μM) for 24 hours, with n = 3 independent experiments. Protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific).

A standard western blot protocol was implemented using 12% SDS-PAGE gels. All primary and secondary antibodies were prepared in 5% BSA-PBST. Membranes were incubated with the following primary antibodies: ACSL5 goat polyclonal IgG 1:200 (SantaCruz; sc-47999), GAPDH rabbit polyclonal IgG 1:15000 (Abcam; ab22555), β-actin mouse monoclonal IgG 1:1000 (SantaCruz; sc-47778), Tom20 rabbit polyclonal IgG 1:1000 (SantaCruz; sc-11415). Secondary antibodies used were rabbit anti-goat IgG-HRP 1:5000 (SantaCruz; sc-2768), goat anti-rabbit IgG-HRP 1:5000 (SantaCruz; sc-2004), and goat anti-mouse IgG-HRP 1:5000 (SantaCruz; sc-2031). Amersham ECL prime western blotting detection reagent and VersaDoc MP 4000 system (Biorad) with the Quantity One 4.6.9 Software (Chemi Hi Sensitivity 0.5x Gain Application) were used to detect and analyze protein bands. As there was an increased variation in protein band intensity between the different western blot membranes for the C2C12 cells exposed to MEHP, the following western blot quantitative analyses were normalized to results from untreated control cells.

C2C12 in vitro Studies Statistical Analyses

One-way ANOVA statistical analyses with Fisher individual test for difference of means was conducted using Minitab 16 software.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the [National Health and Nutritional Examination Survey-NHANES 2003-2004] repository, [https://wwwn.cdc.gov/nchs/nhanes/ContinuousNhanes/Default.aspx?BeginYear=2003]

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

AR participated in the study design and majority of the experiments including the statistical

analyses conducted within this paper. AR also wrote the draft of the manuscript. SS conducted the work studying cytotoxicity effects of MEHP on C2C12 myoblasts. KM conducted the MEHP

exposure studies on C2C12 myoblasts for protein expression analyses using western blots. DP

participated in interpreting the data and revised the article critically. MH participated in

interpreting the data and revised the article critically. FT participated in the study design,

interpreted the data and revised the article critically. All authors read and approved the final

manuscript.

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Abbreviations

MEHP- Mono-(2ethylhexyl) phthalate

MEHHP- Mono(2-ethyl-5-hydroxyhexyl) phthalate

DEHP- Di(2-ethylhexyl) phthalate

NHANES- National Health and Nutrition Examination Survey

ACSL5- Acyl-CoA Synthetase Long Chain 5

BMI- Body Mass Index

HPLC-ESI-MS/MS- High Performance Liquid Chromatograph-Electrospray Ionization-tandem Mass Spectrometry

ASP- Acid Soluble Products

FCCP- Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

PI- Propidium iodide

OCR- Oxygen consumption rate

ECAR- Extracellular acidification rate

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Tables

Table 1: Characteristics of women with obesity enrolled in NHANES 2003-2004

Variable	Number (n)	Mean	Standard Deviation (SD)
Age (yr)	89	46.66	18.64
BMI (kg/m^2)	87	35.94	5.17
MEHP (ng/ml)	88	7.85	13.72
Gamma-Linolenic Acid (µmol/l)	88	50.91	21.77
Arachidonic Acid (µmol/l)	88	880.4	221.0

Table 2: Backward regression analysis studying the modulators of plasma omega-6 fatty acid (Arachidonic Acid (AA) and Gamma-Linolenic Acid (GLA)) levels within adult women with obesity who participated in NHANES 2003-2004 study

Dependent Variable	Independent Variable /Term	Unstandardized Coefficient	Standard Error	Cohen's f^2	Variable p-value	r^2 adjusted model	Model p-value
AA						0.3465	0.000
	Constant	649.7	64.5	-	0.000		
	Age	3.57	1.22	0.064	0.004		
	MEHP	7.62	1.73	0.212	0.000		
	Ethnicity			0.109	0.003		
	Non-Hispanic Black	81.4	46.1		0.082		
	Mexican American	-102.3	66.4		0.127		
	Other Hispanic	-364	132		0.007		
GLA						0.235	0.000
	Constant	35.84	6.81	-	0.000		
	Age	0.386	0.129	0.079	0.004		
	MEHP	0.436	0.182	0.056	0.019		
	Ethnicity			0.528	0.049		
	Non-Hispanic Black	-8.42	4.87		0.088		
	Mexican American	-14.60	7.01		0.041		
	Other Hispanic	-25.1	13.9		0.076		

Figures

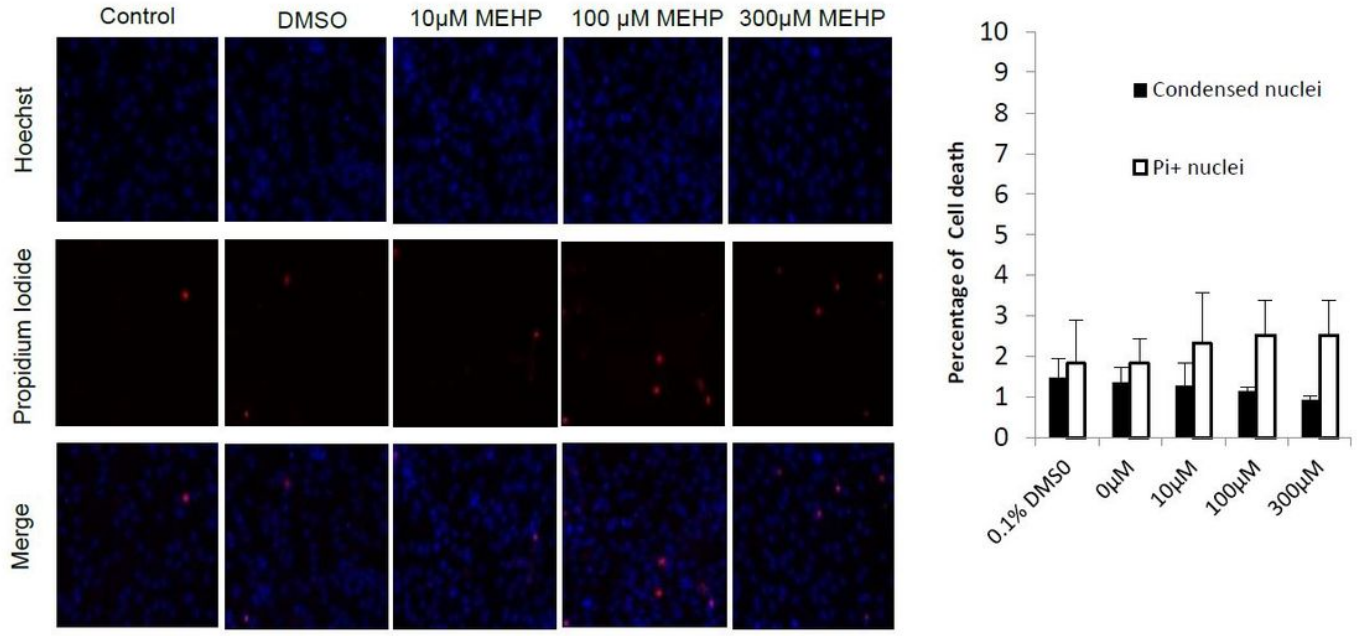

Figure 1

MEHP cytotoxicity in C2C12 myotubes - Differentiated myotubes were exposed to varying concentrations of MEHP (10, 100 and 300 μM) for 24 hours followed by staining with Hoechst 33342 and Propidium Iodide (PI), both DNA staining dyes. Cells that have undergone apoptosis display nuclei condensation, which is detected via Hoechst 33342, while apoptotic and necrotic cells would be detected by PI, which is unable to cross the membrane of live cells. No significant difference in cytotoxicity was detected between varying concentrations of MEHP exposure to C2C12 myotubes as seen on bar graph (cell death being less than 5% for all conditions). Images were taken at 20x with Zeiss AxioObserver.D1 Microscope (n=3; 6 images/condition taken with 20x objective)

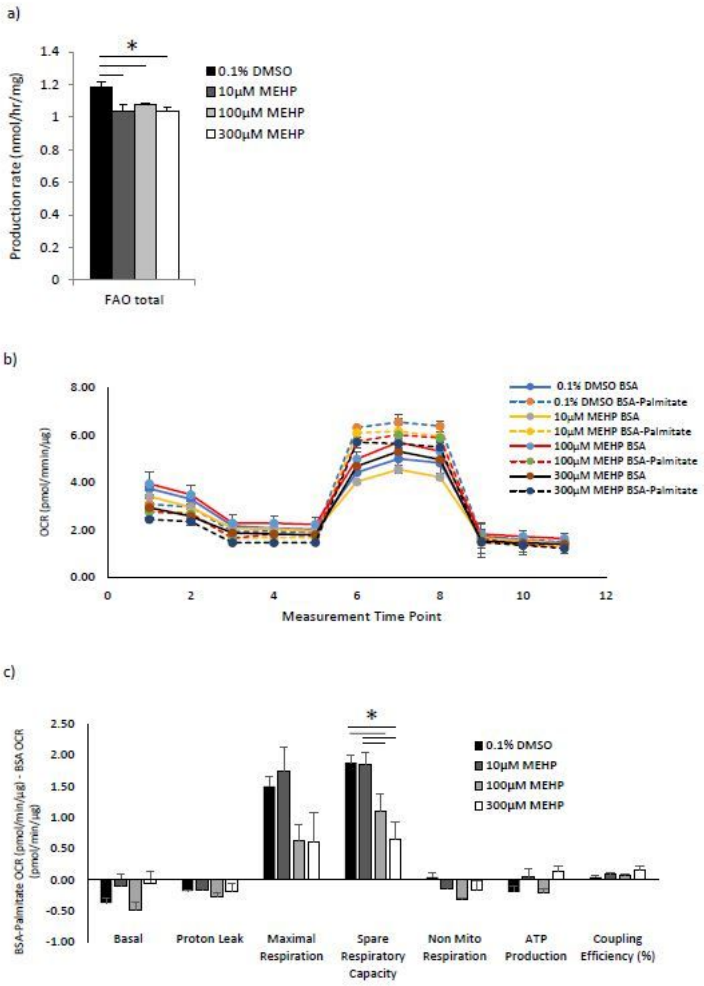

Figure 2

Effects of MEHP on C2C12 myotube fatty acid oxidation and mitochondrial respiration - Differentiated myotubes were exposed to varying concentrations of MEHP (10, 100 and 300 μM) for 24 hours. a) Assessment of total fatty acid oxidation by measuring palmitate- driven exogenous fatty acid oxidation after incubation with radiolabelled 14C-palmitate. n=3. b and c) Fatty acid oxidation assays by measuring mitochondrial oxygen consumption rate (OCR) in presence of palmitate (n=3). Represented in both a time course plot (b) and quantitative graph (c) * represents $p < 0.05$

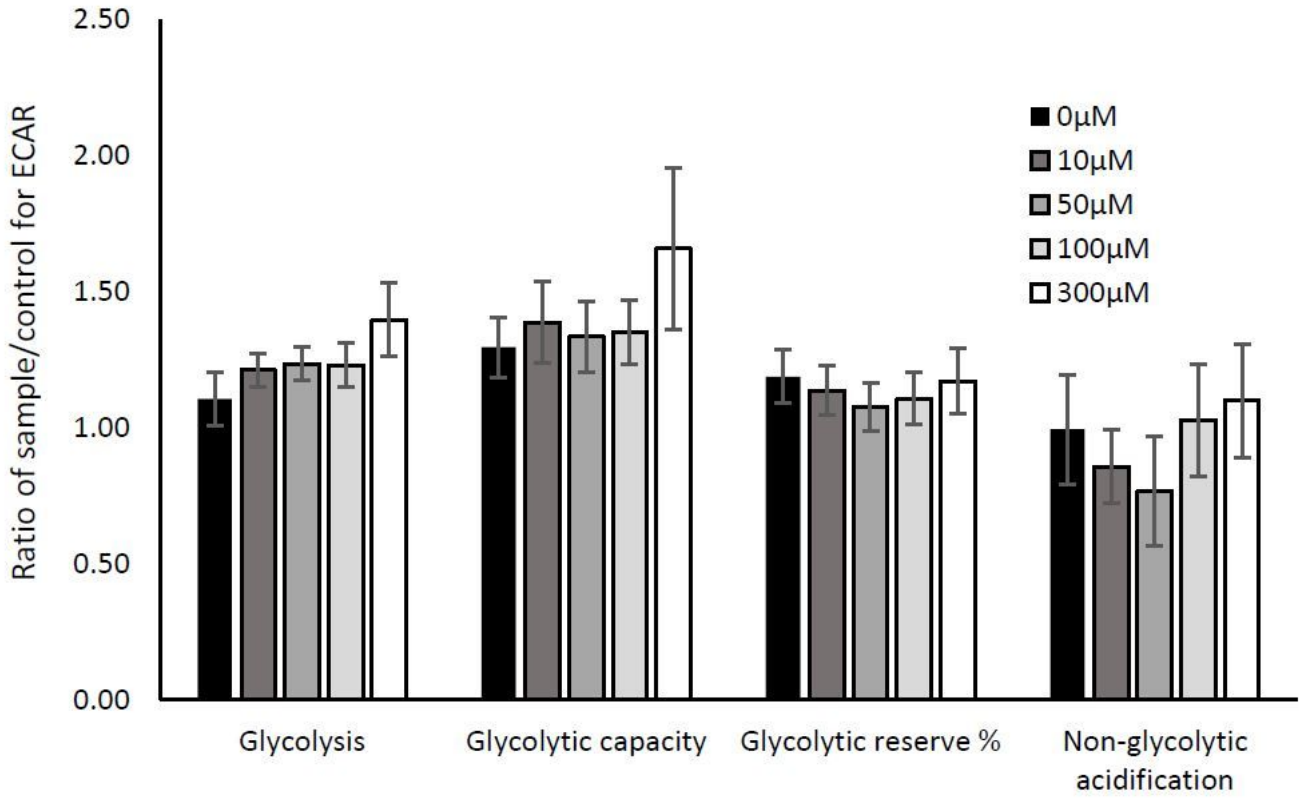

Figure 3

Assessment of the effects of MEHP on cellular glycolysis levels in the presence of exogenous glucose - Differentiated myotubes seeded onto XF96-well plates were exposed to varying concentrations of MEHP (10, 50, 100 and 300 µM) for 24 hours. Extracellular acidification rate (ECAR) was assessed using the Seahorse XF Cell Glycolysis Stress Test (n=5)

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