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Nikki Gillum Posnack¹, Luther M. Swift¹, Matthew W. Kay¹², Norman H. Lee¹, and Narine Sarvazyan¹

¹Department of Pharmacology & Physiology
²Department of Electrical and Computer Engineering
The George Washington University, 2300 I Street NW, Washington, DC 20037

Corresponding Author:
Nikki Gillum Posnack, Ph.D.
2300 I Street NW, Washington, DC 20037
Tel:(202)994-9457
Fax:(202)994-3553
nikkigillum@gmail.com

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Abbreviations:
DEHP – Di(2-ethylhexyl)phthalate
FA – Fatty acid
FAO – Fatty acid oxidation
GO – Gene Ontology
PPARα – Peroxisome proliferator-activated receptor alpha
PVC – Polyvinyl chloride
Abstract

Background: Phthalates are common plasticizers present in medical grade plastics and other everyday products. They can also act as endocrine-disrupting chemicals and have been linked to the rise in metabolic disorders. However, the effect of phthalates on cardiac metabolism remains largely unknown.

Objectives: We sought to examine the effect of Di(2-ethylhexyl)phthalate (DEHP) on the metabolic profile of cardiomyocytes, as alterations in metabolic processes can lead to cell dysfunction.

Methods: Neonatal rat cardiomyocytes were treated with DEHP at a concentration and duration comparable to clinical exposure (50-100 µg/mL, 72hrs). The effect of DEHP on gene expression was assessed by microarray analysis. Physiological responses were examined via fatty acid utilization, oxygen consumption, mitochondrial mass and western blot analysis.

Results: Exposure to DEHP led to upregulation of genes associated with fatty acid transport, esterification, mitochondrial import and β-oxidation. The functional outcome was an increase in myocyte fatty acid substrate utilization, oxygen consumption, mitochondrial mass, PPARα protein expression, and extracellular acidosis. Treatment with a PPARα agonist (Wy-14643) only partially mimicked the effects observed in DEHP samples.

Conclusions: The data suggests that DEHP exposure results in metabolic remodeling of cardiomyocytes, whereby cardiac cells increase their dependence on fatty acids for energy production. This fuel switch may be regulated at both the gene expression and post-transcription levels. Our findings have important clinical implications, as chronic dependence on fatty acids is associated with an accumulation in lipid intermediates, lactate, protons and reactive oxygen species. This dependence can sensitize the heart to ischemic injury and ventricular dysfunction.
Introduction

An estimated 34% of US adults have metabolic syndrome, which can be a precursor to diabetes, obesity, heart disease, stroke and cancer (Ervin 2009). Although the role of lifestyle choices in these disorders cannot be ignored, the dramatic rise in metabolic disease suggests that environmental pollutants may also play a role. Endocrine-disrupting chemicals can interfere with endocrine function, resulting in adverse developmental, reproductive and metabolic effects. Human exposure to endocrine disruptors has been linked to metabolic disturbances (reviewed in Casals-Casas and Desvergne 2011). Endocrine disruptors are known to interact with nuclear receptors, which modulate downstream gene expression by interfering with receptor function and/or transcription factor activity.

Di(2-ethylhexyl)phthalate (DEHP) is a commonly used plasticizer that imparts flexibility to polyvinyl chloride (PVC) products. It has been identified as an endocrine-disrupting chemical (Casals-Casas and Desvergne 2011). Human exposure to DEHP occurs through contact with food packaging, toys, personal care and medical products. The latter is of particular concern, as exposure to DEHP increases dramatically in patients undergoing multiple medical interventions, such as bypass, hemodialysis circuits or long-term use of tubing in intensive care units. This is because DEHP is not covalently bound to the PVC polymer and is highly hydrophobic, allowing it to leach from plastics when in contact with blood and other lipophilic fluids (FDA 2002). A number of animal studies have reported toxic effects of DEHP (reviewed in Carlson 2010), raising concerns about phthalate leaching and human health. Exposure to DEHP results in broad phthalate distribution throughout the body, including the heart (Hillman et al. 1975). Our recent studies revealed that DEHP adversely affects the synchronicity of a cardiac cell network by disrupting connexin-43, the main component of cardiac gap junctions (Gillum et al. 2009). This
effect, which may be attributed to modifications in tubulin and kinesin expression, as well as other gene expression modifications suggested an arrhythmogenic effect of phthalates \textit{in vitro} (Posnack et al. 2011). We sought to expand these studies to examine DEHP’s effect on the metabolic profile of cardiomyocytes, as metabolic alterations can point to additional dysfunctions.

In order to meet high energy demands, cardiac muscle is able to metabolize multiple substrates. Fatty acids (FA) are the preferred substrate- at least 60% of ATP is generated from fatty acid oxidation (reviewed in Lopaschuk et al. 2010). The remaining energy demand is provided by glucose, lactate and ketone metabolism. A number of studies have addressed the effects of phthalates on metabolism. In contrast, the effect of DEHP on cardiac FA metabolism remains largely unknown (Itsuki-Yoneda et al. 2007; Reubsaet et al. 1990). We show that DEHP exposure results in an upregulation of genes associated with FAO. DEHP-induced genetic modifications resulted in increased FA substrate utilization, increased mitochondrial mass, increased oxygen consumption and extracellular acidosis. The data suggest that these modifications involve upregulation of PPARα (peroxisome proliferator-activated receptor alpha) and its co-activator PGC-1α (peroxisome proliferator-activated receptor gamma, coactivator 1α), as well as alternative pathways.

**Methods**

All animals were treated humanely and with regard for alleviation of suffering.

**Experimental protocol:** Cardiomyocytes were isolated from mixed litters of 1-day old Sprague-Dawley rats (approximately 25 rats/litter; Hilltop Lab Animals, Scottdale PA) by an enzymatic digestion procedure (Arutunyan et al. 2001). Cells were treated with either 1) Dimethyl sulfoxide (DMSO) control, 2) 50 µg/mL DEHP (128 µM), 3) 100 µg/mL DEHP (256 µM), or 4)
50 μM Wy-14643 (PPARα agonist). Unless otherwise noted, cells were treated for 72hr before conducting experiments. Cell toxicity was assessed using a membrane integrity assay (CytoTox-ONE) and the effects on cell proliferation were assessed by measuring lactate dehydrogenase (LDH) release (Promega, Madison WI).

**Microarrays and qRT-PCR validation:** Microarray experiments (Rat 1.0 ST array; Affymetrix, Santa Clara CA) were performed using 6 coverslips of cardiomyocytes for each treatment group (control, 50 μg/mL DEHP) as previously described (Posnack et al. 2011). Hybridization data can be accessed from the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under the series accession #GSE21640. Differentially regulated genes were identified by Student’s t-test with a 1% false discovery rate and 1.5 expression difference cut-off. GeneSpring software was used to identify Gene Ontology (GO) categories (p < 0.01). A polyhierarchical graph specific for the metabolic process category was created and modified using AmiGO visualization (http://amigo.geneontology.org). TIGR Multiexperiment Viewer (MeV; http://www.tm4.org/mev) software was used to visualize genes by GO category, using logarithm (base 2) values with median background correction. Ingenuity Pathway Analysis software (IPA) was used to identify gene networks and canonical pathways. Microarray experiments were validated using qRT-PCR as previously described (Posnack et al. 2011). Quantitation and normalization of relative gene expression was accomplished using the comparative CT method (ΔΔCT). ΔΔCT values were converted to ratios by $2^{-\Delta\Delta CT}$ averaged across replicates. Primer sequences can be found in Supplemental Material (Table S1).

**Fatty acid utilization:** 72hrs after treatment with DMSO control or 50 μg/mL DEHP, cell culture media was changed to a glucose-free media supplemented with 100 μM palmitic acid and 33 μM bovine serum albumin (BSA). An increased rate of FA utilization was detected as a
decrease in media concentration with time. FA concentration was measured over 30hrs using a fluorescence-based assay, in which fatty acids are converted to CoA derivatives for quantification (BioVision, Milpitas CA).

**Immunocytochemistry:** Cardiomyocytes were fixed using a standard 4% paraformaldehyde procedure and stained with PPARα (1:500) and anti-rabbit Cy3 (1:1000). Live cells were loaded with MitoTracker Red (0.1 μM) and imaged at 20X magnification, or loaded and then fixed for high magnification imaging (63X).

**Western blot:** Blots were prepared as previously described (Posnack et al. 2011). Blots were probed with PPARα (1:300) and GAPDH (1:3500, loading normalization). Blots were incubated with anti-rabbit IRdye 800CW (1:5000) and anti-mouse IRdye 680LT (1:10,000).

**Proton production:** Standards were prepared by adjusting the pH of phenol red-containing media samples. An absorbance spectra was acquired for both standards and cell culture samples using a plate reader. The cell culture samples were then normalized against the standards to identify pH modifications.

**Oxygen consumption:** Cardiomyocytes were plated on an XF24 cell culture microplate (100,000 cells/well). Cells were treated for 72hr with DMSO or 50 μg/mL DEHP. An XF24 Analyzer (Seahorse Biosciences, North Billerica MA) was then used to measure oxygen consumption in real time. Prior to XF measurements, cells were incubated for 1hr at 37°C in unbuffered media supplemented with either 1) 100 μM palmitic acid with 33 μM BSA or 2) 25 mM glucose. To validate these results independently, cells were loaded with an oxygen-sensitive probe (MitoXpress) in media supplemented with palmitic acid and BSA. Mineral oil was used to seal the samples and diminish the interference of ambient oxygen. A high rate of
cell respiration depletes the sample oxygen concentration quickly, which is detected as an increase in probe signal.

**Statistical Methods:** A Student’s t-test was used to evaluate the significance of the differences in mean values between two treatment groups. ANOVA and Bonferroni’s Multiple Comparison Test was used to evaluate values between three or more groups. All bar graphs depict the mean value ± standard error of the mean (SEM). Significance is denoted with an asterisk and indicates p < 0.05 (unless a lower value is indicated).

**Results**

**Gene expression profile of DEHP-treated cardiomyocytes:** We studied the effect of DEHP on neonatal rat cardiomyocytes at a dose (50-100 µg/mL) comparable to plasticizer presence in neonates undergoing clinical procedures, such as transfusion or extracorporeal membrane oxygenation (FDA 2002; Gillum et al. 2009). After 72hr exposure, RNA was harvested from cardiomyocytes for DNA microarray experiments. At a 1.5-fold expression difference cut-off, a total of 814 mRNAs were differentially expressed. Of these genes, 334 were upregulated and 480 were downregulated by DEHP treatment.

Gene Ontology (GO) analysis revealed that differentially expressed genes were significantly over-represented in 97 categories (p < 0.01). The lipid metabolic process GO category was the most significantly modified (p < 0.00001). Figure 1A shows a polyhierarchical graph specific for GO categories under the metabolic process ontology. MeV was used to identify genes significantly up or downregulated within four downstream GO categories (Figure 1B), including cholesterol metabolism, FA metabolic process, regulation of FA metabolic process and FA biosynthetic process. The latter two categories are subdivisions of FA metabolic process; genes that overlapped between categories are only displayed once in the heatmap.
images. Genes associated with FA metabolic processes were mostly upregulated in DEHP-treated samples, while genes associated with biosynthesis were mostly downregulated (Figure 1B).

**qRT-PCR validation:** The mRNA expression of 11 genes associated with FA metabolism and regulation were validated using qRT-PCR (Figure 1C). Genes associated with regulation of FA metabolism were upregulated, including: peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor gamma, coactivator (PGC-1α, 1β). Genes encoding FA metabolic enzymes were also upregulated, including: acyl-CoA synthetase (ACSL1), acetyl-CoA acyltransferase (ACAA2), long-chain acyl-CoA dehydrogenase (ACADL), very long chain acyl-CoA dehydrogenase (ACADVL), hydroxyacyl-CoA dehydrogenase (HADHA). Carnitine palmitoyltransferase (CPT1B), which transports fatty acids into the mitochondria, was also upregulated. We also detected an upregulation in enoyl-CoA hydratase (ECH1), an enzyme that functions in the auxiliary step of the β-oxidation pathway and pyruvate dehydrogenase kinase (PDK4), a protein that regulates glucose metabolism.

**Gene network analysis:** IPA and PathVisio (www.pathvisio.org) were used to identify biological pathways pertinent to lipid metabolism. A mitochondrial FAO pathway overlaid with mRNA expression fold change values is shown in Figure 2A. Upregulation of genes involved in multiple parts of the FAO pathway was observed in DEHP-treated samples. These included triglyceride hydrolysis (PNPLA2), FA esterification (ACSL1), mitochondrial import (CPT1A, CPT1B, SLC25A20) and β-oxidation (ACADL, ACADVL, HADHA, HADHB). Conversely, ACSL3, which encodes an acyl-CoA synthetase enzyme involved in FA biosynthesis, was downregulated.
In addition to FA metabolism and FA elongation, alternative lipid canonical pathways were also altered, including those related to steroid, ketone, arachidonic acid, linoleic acid and phospholipid metabolism and degradation (Figure 2B). IPA toxicity analysis also revealed functionally grouped gene sets that are altered in response to a xenobiotic exposure (DEHP). As expected, PPARα activation and FA metabolism genes were upregulated (Figure 2C). In comparison, cholesterol biosynthesis was downregulated. Gene sets associated with detoxification pathways, including glutathione depletion and cytochrome P450, were also upregulated in DEHP samples. Toxicity gene sets associated with mitochondrial membrane depolarization and NRF2 (NF-E2-related factor 2) mediated oxidative stress response were also upregulated. These are of interest, as increased FAO is likely to enhance reactive oxygen species production, and NRF2 upregulates antioxidant genes (Gao et al. 2007).

**Increased FAO and mitochondrial oxygen consumption:** To determine if gene expression changes resulted in a physiological response, the rate of FA utilization was measured over 30hrs by modifying the cell culture media to contain only FA (glucose free). After 6 hours, DEHP-treated samples metabolized significantly more FA, as indicated by a drop in FA concentration in cell media samples (Figure 3A). This effect became more pronounced at later time points.

FAO requires a greater amount of oxygen per molecule of ATP produced compared with glucose oxidation. Therefore, increased reliance on FA substrates for energy production should also increase oxygen consumption. To further investigate the effect of DEHP on FAO, we measured oxygen consumption rates in cardiomyocytes cultured with either 100 μM palmitic acid or 25 mM glucose using an extracellular flux analyzer. Oxygen consumption rates were increased in DEHP-samples supplemented with palmitic acid, and decreased in DEHP samples supplemented with glucose (Figure 3B). This latter may signify a lack of substrate flexibility in
DEHP-treated samples, whereby glucose utilization is diminished. These data were independently validated by measuring total oxygen consumption using an oxygen sensitive probe, in the presence of 100 µM palmitic acid (Figure 3C).

**Increased PPARα expression:** The PPARα nuclear receptor acts as a lipid sensor that modifies transcriptional responses to metabolic status. Activation (or inhibition) of PPARα via exogenous compounds can interfere with metabolic homeostasis. Phthalates are known PPARα agonists (Bility et al. 2004). PPARα protein expression was increased in DEHP-treated samples compared with control, as shown by immunostaining (Figure 4A) and western blot analysis (Figure 4B).

**Increased mitochondrial mass:** Activation of PPARα and its transcriptional co-activator protein, PGC-1α, can increase mitochondria biogenesis (Wu et al. 1999). Since both PPARα and PGC-1α are upregulated in DEHP samples we examined mitochondrial mass using MitoTracker. As a positive control, PPARα was activated directly via Wy-14643, a specific PPARα agonist (Ren et al. 2010). Assessment of mitochondrial structure showed an increase in mitochondrial mass, particularly in cells treated with a high concentration of DEHP (100 µg/mL) or Wy-14643 (Figure 5A, B).

**Increased proton production:** FAO is inversely proportional to glucose oxidation. Byproducts of FAO inactivate the pyruvate dehydrogenase complex (PDC), thereby diverting pyruvate to lactate via lactate dehydrogenase (LDH). Therefore, increased FAO can uncouple glycolysis from glucose oxidation, leading to proton and lactate accumulation (Figure 6D). To confirm this pathway we performed pH measurements on treated cells. After 3-days treatment, cells exposed to a high concentration of DEHP showed significant extracellular acidosis (Figure 6A). Acidosis increased with time, after 6-days treatment 100 µg/mL DEHP samples reached a pH 5.
µg/mL DEHP samples were also more acidic after 6-days treatment, although to a lesser extent. However, Wy-14643 treated samples were more basic (pH 7.5) compared with controls. The observed pH changes were not attributed to chemical additives (Figure 6A, media columns). Since pH changes can also reflect cell culture overgrowth or cell death, we examined cell viability to rule out these effects. Wy-14643-treated samples had a decrease in cell viability (2.5% of control) and total cell number (20% of control), which may explain the shift to a more basic pH. However, neither cell viability nor total cell number was altered in DEHP samples (Figure 6B, C). DEHP-induced acidosis was independently verified in extracellular flux experiments; DEHP samples were more acidic than controls after 1hr incubation in unbuffered media (data not shown).

Discussion

DEHP is a widely used phthalate found in FDA-approved medical devices, tubing and intravenous bags. DEHP toxicity has been reported for multiple organs in animals (Carlson 2010), and human studies have raised concerns pertaining to the male reproductive tract and development (Swan 2008). Additionally, public health studies have correlated phthalate exposure with metabolic disorders in humans (Stahlhut et al. 2007; Svensson et al. 2011). In contrast, the cardiac effects of DEHP remain largely ignored, and little has been published on the effects of DEHP on cardiac metabolism (Itsuki-Yoneda et al. 2007; Reubsaet et al. 1990). To the best of our knowledge, our study is the first to thoroughly assess the effects of DEHP on substrate metabolism in cardiac cells, at clinically relevant doses.

DEHP absorbed by the body is metabolized to Mono(2-ethylhexyl)phthalate (MEHP) and 2-ethylhexanol. Oral exposure results in rapid enzymatic hydrolysis to MEHP. However, the rate of DEHP conversion to MEHP is substantially slower intravenously. In clinical settings
DEHP exposure primarily occurs via the intravenous route which avoids rapid first pass metabolism (i.e., intravenous fluids, extracorporeal membrane oxygenation or cardiopulmonary bypass circuits) (Carlson 2010; FDA 2002). Our previous studies (Gillum et al. 2009) revealed that DEHP, and not MEHP, adversely affects the *electrical properties* of cardiac cells (i.e., slowed conduction velocity, asynchronous beating, diminished connexin-43 expression). However, DEHP’s metabolites may also influence *cardiac metabolism*; indeed, MEHP exposure also affected metabolic gene expression (Supplemental Material, Figure S1).

We showed that DEHP treatment of cardiomyocytes results in increased expression of genes associated with FA metabolism. Figure 6D illustrates genes of interest that were modified in our experiments (Lopaschuk et al. 2010). Myocyte metabolism of FA proceeds as follows: FA enter the myocyte (step #1 in Figure 6D) via fatty acid translocase (CD36), and are transported by fatty acid binding protein (FABP). Once inside the cell, FA undergo esterification (#2). ACSL1, the predominant isoform found in the heart (Durgan et al. 2006), catalyzes the synthesis of acyl-CoA from FA. Cardiac overexpression of ACSL can result in severe cardiomyopathy due to increased FA uptake and an ensuing accumulation of lipids (Chiu et al. 2001). Acyl-CoA is then converted to acyl-carnitine by CPT1 (#3), which facilitates entry into the mitochondria. CPT1 activity is tightly controlled by its inhibitor, malonyl-CoA (#4), which is regulated by AMP-activated kinase (AMPK). AMPK promotes conversion of malonyl-CoA to acetyl-CoA (#5), thereby alleviating the inhibitory signal on CPT1. Acyl-carnitine is translocated across the mitochondrial inner membrane (#6) by acylcarnitine translocase (SLC25A20). Once inside the mitochondria, FA enter the β-oxidation spiral (#7), whereby acyl-CoA molecules are converted to acetyl-CoA. This four step conversion is catalyzed by numerous enzymes, including Acadl, Acadvl, Hadha, Hadhb, Decr1 and Acaa2. Each cycle
through the β-oxidation spiral produces one molecule of each: FADH₂, NADH and acetyl-CoA (#8). Acetyl-CoA is transferred to the citric acid cycle for ATP production (#8), and FADH₂ and NADH byproducts are transferred to the electron transport chain (#9). Overall, our data shows that DEHP treatment influences nearly every step in the FA metabolic pathway.

In DEHP-treated samples we observed a decrease in oxygen consumption in the presence of glucose, and an increase in proton generation. These results confirm that FAO inhibited glucose oxidation, a phenomenon known as the “Randle cycle” (Randle et al. 1963). Increased FAO leads to inhibition of the pyruvate dehydrogenase complex (PDC). PDC is a multi-enzyme complex that converts pyruvate to acetyl-CoA, thereby linking glycolysis with the citric acid cycle. Byproducts of FAO increase pyruvate dehydrogenase kinases (PDK) activity, which functions to inactivate the PDC enzyme, pyruvate dehydrogenase (PDH, #10). Inactivation of PDC prevents the conversion of pyruvate to acetyl-CoA, and instead diverts pyruvate to lactate production via LDH (#11). Consequently, an increase in FAO and decrease in PDC activity uncouples glycolysis and glucose oxidation. This scenario, whereby glycolysis is unaltered but pyruvate oxidation is inhibited, can result in an accumulation of protons and lactate. Notably, increased lactate levels have been observed in the muscle of DEHP-treated animals (Martinelli et al. 2006). We found that DEHP-treatment resulted in an upregulation in both LDH (microarray analysis, data not shown) and PDK4. This change in gene expression likely explains the extracellular acidosis observed in DEHP samples. A similar phenomenon was observed when adipocytes were exposed to MEHP, which led to increased PDK4 expression and diverted pyruvate away from the citric acid cycle (Ellero-Simatos et al. 2011).

We hypothesize that the described changes in metabolism are partially, but not exclusively, related to upregulation of PPARα and its co-activator PGC-1α. Both PPARα and
PGC-1α act as lipid sensors that modify transcriptional responses to metabolic status (Lopaschuk et al. 2010). PPARα is abundant in metabolically active tissues, such as the heart, and we observed DEHP-induced modifications to a number of PPARα target genes (Figure 6D). The influence of PPARα on metabolism has been elucidated in both gain- and loss-of-function experiments. Transgenic mice with cardiac-restrictive PPARα overexpression develop a metabolic phenotype that mimics the diabetic heart, including: high FAO rates, low glucose oxidation rates, and gene expression changes including PDK4 upregulation (Finck et al. 2002). Moreover, PPARα overexpressing hearts showed signs of cardiomyopathy. Treatment with the PPARα agonist Wy-14643 similarly increased PDK4 expression in both cardiac (Zungu et al. 2009) and skeletal muscle (reviewed in Burri et al. 2010). In comparison, PPARα-null mice have decreased rates of FAO and increased rates of glucose oxidation (Campbell et al. 2002).

Phthalates are known PPARα agonists (Bility et al. 2004), and many of the effects observed in our studies can be attributed to PPARα upregulation. However, DEHP-induced toxicity has also been reported in multiple organs of PPARα-null mice (Ito et al. 2007; Peters et al. 1997; Ward et al. 1998). The genes modified by DEHP in PPARα-null mice included those involved in xenobiotic metabolism, including targets of constitutive androstane receptor (CAR) or pregnane X receptor (PXR), and cholesterol biosynthesis which is regulated by other transcription factors, including retinoid X receptor (RXR) (Ren et al. 2010). This is of interest to our studies, as our data illustrated differences between DEHP and PPARα agonist treatment (Wy-14643), suggesting involvement of an alternative receptor.

We propose that increased AMPK expression is an additional mechanism by which DEHP can exert its effects on metabolism. AMPK is an energy sensor-it turns on ATP-producing catabolic processes and turns off ATP-consuming anabolic processes. AMPK can be
activated by increased FA supply, increased AMP:ATP ratio, and ischemia. In turn, AMPK activation leads to an upregulation of PPARα and CPT1β, as a way to dispose of FA via β-oxidation (Lopaschuk et al. 2010). The downstream effects of AMPK activation, including mitochondrial biogenesis, have been attributed PGC-1α (Jager et al. 2007). Increased AMPK activity was also the main mechanism attributed to an insulin-resistant phenotype observed in PDK4 overexpressing hearts, which included increased FAO, decreased glucose oxidation and increased lactate levels in myocardial tissue (Chambers et al. 2011). Lastly, AMPK activity is associated with an inhibition of cholesterol synthesis (Henin et al. 1995), a result we observed in DEHP-treated samples. Despite crosstalk between PPAR and AMPK- PPARα agonists modulate AMPK activity independently of the PPAR nuclear receptor (Chanda et al. 2009).

All-in-all, our data suggests that DEHP exposure results in metabolic remodeling of cardiomyocytes, whereby cardiac cells increase their dependence on FA for energy production. This dependence on FA may be regulated at both the gene expression and post-transcription levels. This phenomenon should be examined further in both in vitro adult cardiomyocytes and in vivo.

Conclusions
The heart exhibits energy substrate flexibility; however, a number of conditions are associated with alterations in glucose and/or FA metabolism. Diabetic cardiac dysfunction is partly driven by metabolic abnormalities, chiefly a loss of energy substrate flexibility. The diabetic phenotype consists of a reduced responsiveness to insulin, decreased glucose utilization and increased dependence on FA. Chronic dependence on FA as a fuel source is associated with an accumulation in lipid intermediates, lactate, protons and reactive oxygen species. Therefore, dependence on FA can sensitize the heart to ischemic injury and ventricular dysfunction.
Diabetic mouse models also display an increase in myocardial oxygen consumption. The metabolic profile seen in DEHP-treated samples has a striking similarity to that seen in diabetic cardiomyopathy (Lopaschuk et al. 2010). One can suggest that the adverse metabolic cascade typically initiated by high levels of circulating triglycerides in diabetic patients could also be initiated by chronic phthalate exposure. This parallel to diabetes has important public health implications that should be further addressed.
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**Figure Legends**

**Figure 1.** DEHP-treatment alters the expression of genes associated with metabolic and biosynthetic processes

A) Polyhierarchical graph with Metabolic Process at the top of the ontology tree. Significantly different GO categories are highlighted in gray (p < 0.01). Arrows show directionality of hierarchy.  
B) Heatmap images show differential gene expression in GO categories (p < 0.01). Each row represents an individual gene and each column represents an independent experiment.  

*Upregulated = red, downregulated = green*  
C) qRT-PCR of genes associated with FA metabolism and regulation; changes were significantly different for all genes (n=4). *Mean value +/- SEM*

**Figure 2.** DEHP-treatment upregulates genes associated with FAO, and modifies lipid canonical and detoxification pathways

A) Mitochondrial FAO pathway overlaid with mRNA expression fold change values (numbers to right of each gene). *Upregulation = positive value, Downregulation = negative value*  
B) Modified canonical pathways associated with lipid metabolism. Columns show the percentage of genes up/downregulated.  
C) Modified detoxification pathways. *Upregulation = red, Downregulation = green*

**Figure 3.** DEHP-treatment enhances FA utilization and oxygen consumption

A) DEHP-treatment increases the rate of FA utilization, detected by a decrease in FA concentration with time (n=3).  
B) Oxygen consumption rates were maximal in DEHP samples supplemented with palmitic acid, and minimal when supplemented with glucose.  
C. Total oxygen consumption was increased in DEHP samples supplemented with FA. Increased phosphorescence signal signifies oxygen depletion (n=4). *Mean value +/- SEM*
Figure 4. DEHP-treatment increases PPARα protein expression

A) PPARα (red) staining intensity and total area were increased in DEHP-treated samples. Staining intensity values are shown using rainbow color in a 2.5 dimensional plot. High intensity = red, low = blue, size bar = 50µM; mean fluorescence values +/- SEM  

B) PPARα protein expression was increased in DEHP-treated samples normalized to GAPDH (n=3).

Figure 5. DEHP and Wy-14643-treatment increase mitochondria mass

A) Top panel: live imaging of cells loaded with MitoTracker red showed increased staining in DEHP (100 µg/mL) or Wy-14643 samples (20X, size bar = 100 µM). Middle panel: top panel images are represented in 2.5 dimensional plots, staining intensity is shown in rainbow color (high intensity = red, low = blue). Lower panel: cells were fixed to eliminate contraction movement (63X, size bar = 20 µM).  

B) Total mitochondrial staining area was higher in DEHP (100 µg/mL) or Wy-14643 (50 µM) samples (n=3). Mean fluorescence values +/- SEM

Figure 6. DEHP-treatment results in proton accumulation (A, B, C) which is likely due to modifications in fatty acid and glucose oxidation pathways (D)

A) pH measurements from media samples with chemical additives, either in the presence or absence of cells (media only; n=3).  

B, C) pH changes in Wy-14643 samples, but not DEHP samples, may be explained by decreased cell viability (2.5% of control) or by a reduction in cell number (20% of control; n=3). Mean values +/- SEM  

D) DEHP-treatment increases the expression of multiple genes in the FAO pathway. Inhibition of PDH via PDK4 expression causes lactate accumulation, as does an upregulation of lactate dehydrogenase (LDHA). Common associations between metabolic regulators and FAO genes are shown; genes significantly altered in DEHP samples compared with controls are highlighted in dark gray. Phthalates can activate nuclear receptors (PPARα, estrogen related receptor gamma- ESRRG)
and their co-activators (PGC-1α). PGC-1α and PPARα are associated with increased FAO, mitochondria biogenesis, and lactate transport. AMPK activity can also increase FAO gene and PDK4 expression, via PGC-1α and PPARα.
Figure 2

A

Lipases
LipC
LipE
LipF

Panpl2

Triacylglycerol

Glycerol

Fatty Acid

Acetyl-CoA

Hexadecanoyl-CoA

Tetradecanoyl-CoA

Acyl-CoA

(Cr)

Glyeryl-3-Phosphate

Dihydroxyacetone Phosphate

Glyceraldehyde-3-Phosphate

See Glycolysis MAPP

See TCA Cycle MAPP

See Beta Oxidation 2 MAPP

B

Percentage

Upregulated

Downregulated

C

Percentage

Cholesterol Biosynthesis

LPS/IL-1β Mediated Inhibition of RXR Function

GSH Depletion Phase II Reactions

Decreases Depolarization of Mitochondria and Mitochondrial Membrane

LXR/RXR Activation

PPARα/RXRα Activation

NRF2‐mediated Oxidative Stress Response

Cytosine P450 Panel Substrate is a Sterol

LXR/RXR Activation

NRF2‐mediated Oxidative Stress Response

Cytosine P450 Panel Substrate is a Sterol
Figure 3

A

Fatty Acid Concentration in media (μM)

Control

DEHP (50 μg/mL)

1 Hour 3 Hour 6 Hour 24 Hour 30 Hour

B

Oxygen Consumption Rate (pmol/min)

Control (glucose) DEHP (glucose) Control (fatty acids) DEHP (fatty acids)

C

Oxygen Consumption (A.U.F.)

Control (fatty acids) DEHP (fatty acids)
**A**

Control

![Image](image1)

DEHP

![Image](image2)

**B**

![Image](image3)
Figure 5

A

Control

DEHP (50 μg/mL)

DEHP (100 μg/mL)

Wy-14643 (50 μM)

B

MitoTracker Red Area

Control

DEHP (50 μg/mL)

DEHP (100 μg/mL)

Wy-14643 (50 μM)
Figure 6

A 3 day treatment  6 day treatment

|          | pH     |          |          |
|----------|--------|----------|----------|
| Control  | 8.00   | 7.50     |          |
| DEHP (100 μg/mL) media | 7.00 | *        |          |
| Wy14643 media |     |          |          |

|          |         |          |          |
|----------|--------|----------|----------|
| Control  | 8.00   |          |          |
| DEHP (50 μg/mL) | 7.50 | *        |          |
| Wy14643 (50 μM) |     |          |          |

B 100%

|          |         |          |          |
|----------|--------|----------|----------|
| Control  | 100    |          |          |
| DEHP (50 μg/mL) | 90 | *        |          |
| DEHP (100 μg/mL) | 90 |          |          |
| Wy14643 (50 μM) | 90 |          |          |

C

|          |         |          |          |
|----------|--------|----------|----------|
| Control  | 0.75   |          |          |
| DEHP (50 μg/mL) | 0.50 |          |          |
| DEHP (100 μg/mL) | 0.25 |          |          |
| Wy14643 (50 μM) | 0.00 |          |          |

D

|          | PPARα  | PGC-1α  | ERRγ    | AMPK    |
|----------|--------|---------|---------|---------|
| CD36     | ✔      | ✔       | ✔       | ✔       |
| FABP     | ✔      | ✔       | ✔       | ✔       |
| CPT1     | ✔      | ✔       | ✔       | ✔       |
| PDK      | ✔      | ✔       | ✔       | ✔       |
| LDH      | ✔      | ✔       | ✔       | ✔       |
| PGC1α    | ✔      | ✔       | ✔       | ✔       |
| FAO      | ✔      | ✔       | ✔       | ✔       |
| PPARα    | ✔      | ✔       | ✔       | ✔       |