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Effect of di(2-ethylhexyl) phthalate (DEHP) on lipolysis and lipoprotein lipase activities in adipose tissue of rats

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Abstract

The di(2-ethylhexyl) phthalate (DEHP) is an ubiquitous environmental chemical with detrimental health effects. The present work was designed to asses some potential mechanisms by which DEHP causes, among others, a reduced body fat retention. Since this effect could be related to an alteration of adipocyte triacylglycerol (TG) metabolism, we evaluated the effects of dietary DEHP in adipose tissues upon (1) the number and size of fat cells; (2) the basal and stimulated lipolysis and (3) the lipoprotein lipase (LPL) activity. Groups of male Wistar rats were fed for 21 days a control diet alone (control group) or the same control diet supplemented with 2% (w/w) of DEHP (DEHP group). The LPL activity of DEHP-fed rats was increased in lumbar and epididymal adipose tissues. These rats had significantly reduced weight in epididymal and lumbar tissues, together with reduced size of epididymal adipocytes. These alterations do not seem to be associated with higher lipid mobility because neither basal lipolysis nor 'in vitro' stimulated lipolysis by noradrenaline (NA) showed to be modified by DEHP. Based on these results, we concluded that the adipose tissue size reduction induced by DEHP intake is not due to changes in lipolysis nor to a decreased LPL activity. More research is needed to achieve a comprehensive understanding of the potential mechanisms by which DEHP causes, among others, a reduced body fat retention.

Keywords

Di(2-ethylhexyl) phthalate (DEHP), food contaminant, lipoprotein lipase, lipolysis

Introduction

Phthalate esters are industrial chemical additives used primarily to soften and confer flexibility to polyvinil chloride (PVC) plastics. Di(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer in PVC formulations for a wide array of applications including medical devices, food packaging, children's toys and goods.[1-5] DEHP is not chemically bound to the PVC polymer, therefore, it has been shown to leach from PVC formulations into the environment. Thus, DEHF is universally considered an ubiquitous environmental contaminant.[6,7]

Human beings are exposed to these compounds by oral ingestion, inhalation and dermal contact,[8,9] the oral one being one of the most important routes of incorporation.[10]

A great number of toxicological effects and biological alterations due to DEHP exposure have been well documented in animal models.[11-13] Hypolipidaemia, hepatic peroxisome proliferation, hepatomegaly and carcinogenicity have been found.[14-17] Likewise,

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there is comprehensible data showing that DEHP is an endocrine disruptor which, by interfering with steroid hormone action, could affect the reproductive process.[18,19]

We have found that rats consuming a diet supplemented with 2% (w/w) DEHP for 3 weeks showed a reduced gain in body weight, decreased plasma triacylglycerol (TG) levels,[15] impaired glucose metabolism[20] and minor retention of corporal energy. The last alteration has been associated to a lower body protein and fat retention.[21] The decrease in body proteins content was related to a decrease in the nitrogen balance and to a higher urinary excretion of nitrogen. The ability of DEHP to reduce adiposity could be related to pathways that regulate energy expenditure. Moreover, the direct effects on adipocyte TG metabolism should also be considered. In this regard, the mechanisms by which DEHP exerts the fat-lowering effect have not been studied yet. We can consider some potential mechanisms related to lipid metabolism in adipose tissue: decreased lipoprotein lipase (LPL) activity, increased lipolysis or both.

Thus, the present work attempts to assess the possible role of dietary DEHP on metabolic changes observed in rat adipose tissues. So, we investigated the effects of dietary DEHP in adipose tissues on (1) the size of fat cells; (2) the LPL activities and (3) the basal and stimulated lipolysis.

Material and methods

Chemicals

Di(2-ethylhexyl) phthalate (DEHP; 97% pure) was obtained from Reidel-der Haën (Frankfurt, Germany). Macronutrients for diet preparations (corn oil, casein, sucrose, cellulose and corn starch) were commercial grade. Vitamins, minerals and other nutrients were analytical grade. Biochemical reagents, enzymes and standards were from Sigma Chemical Co. (St Louis, MO, USA). All the solvents, reagents and chemicals were of the analytical grade.

Animals and dietary treatments

Experiments were conducted on male Wistar rats provided by the Comisión Nacional de Energía Atómica (Buenos Aires, Argentina). They were kept acclimatized at 23°C ± 2°C and with a 12-hour light-dark cycle, as well as free access to food and water. Animals were adapted to the animal quarter at least for the term of a week before the beginning of the experiments.

Rats were randomly divided into two groups and fed either a standard diet (control group) or the same standard diet supplemented with 2% (w/w) DEHP (DEHP group). The standard diet was prepared weekly based on the American Institute of Nutrition Ad Hoc Committee recommendation (AIN-93G) formulated for growth, pregnancy and lactation phases of rodents.[22] The diet contains (w/w): 20.0% casein, 7.0% corn oil, 52.9% corn starch, 10.0% sucrose, 5.0% cellulose and 5.1% salt, vitamins and a mix of trace elements. Rats were fed 'ad libitum' for 21 days with either the standard diet or the DEHP-supplemented standard diet. Food intake and body weight were carefully monitored in both groups of animals throughout the experimental period.

All studies on animals were performed in accordance with the principles of the School of Biochemistry regulations, compiled using the Guide to the Care and Use of Experimental Animals in the Laboratory (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996).

Sample collection

According to requirements the animals were anaesthetized with a mixture of acepromazine (1 mg/ kg body weight) and ketamine (100 mg/kg body weight) or they were sacrificed by decapitation between 8:00 am and 10:00 am.

Lumbar and epididymal adipose tissues were removed, weighed and used for adipocytes isolation or stored at –80°C for LPL activity determination.

Adipose tissue LPL activity

LPL activity was measured as described by Martin-Hidalgo et al.[23] Briefly, tissue samples were homogenized in 0.2 M Tris(hidroxymethyl)aminomethane (Tris)-HCl, pH 8.2, at 4°C and delipidated with acetone-diethyl ether. The ketone-powder was dried under nitrogen flow and resuspended in a buffer 0.05 M NH_4OH-NH_4Cl, pH = 8.1, with or without 2 M NaCl. LPL activity was assayed in duplicate by using as substrate: 5.66 μmol triolein, 0.3 mg/mL lecithin, 1% bovine serum albumine, 67 mM Tris, pH 8.0, and 8% heated rat serum (10 min at 56°C). The fatty acids released during incubations were quantified by a titrimetric method.[24] LPL activity was determined by subtracting the non-LPL-dependent

Table 1. Effects of di(2-ethylhexyl) phthalate (DEHP) on food intake, body weight and adipose tissue weights[a]

	Control group	DEHP group
Initial body weight (g)	122 ± 2	121 ± 1
Final body weight (g)	240 ± 5	199 ± 8[b]
Body weight gain (g)	118 ± 9	78 ± 8[b]
Food intake (g/day)	17.2 ± 1.4	16.5 ± 1.0
Body weight gain/food intake (g/g)	0.31 ± 0.03	0.22 ± 0.02[b]
Estimated exposure to DEHP (mg/kg body weight/day)	–	1600[b]
Epididymal Adipose tissue		
Total (g)	3.12 ± 0.20	2.30 ± 0.10[c]
Relative (g/100 g body weight)	1.20 ± 0.07	1.02 ± 0.08
Lumbar adipose tissue		
Total (g)	0.60 ± 0.04	0.40 ± 0.06[c]
Relative (g/100g body weight)	0.25 ± 0.01	0.20 ± 0.01[c]

[a] Values are expressed in mean ± SEM of six animals per group.
[b] Statistically significant differences from respective controls $p < .05$.
[c] Statistically significant differences from respective controls $p < .01$.

activity (high salt) from the total lipolytic activity. Enzyme activity was expressed in micromoles of fatty acids released per gram of tissue per hour.

Fat cells isolation

After the epidydimal fat pads were rinsed in isotonic saline at 37°C, the adipocytes were isolated according to the Rodbell method, with minor modifications.[25,26] Briefly, fat cells were obtained by collagenase digestion (1 mg/mL, 37°C) in Krebs-Ringer bicarbonate buffer (KRB), containing 3.5% of bovine serum albumin (BSA V) and 5 mM of glucose at pH 7.4, under continuous vigorous shaking (90 cycles/min).

Adipocytes were filtered through nylon mesh and washed three times with collagenase-free KRB buffer in order to eliminate the stroma and blood vessels. The isolated fat cells were then resuspended in collagenase-free buffer at 37°C.

Fat cells size

The microscopic method of Di Girolamo[27] was used to measure cell diameters. The sizing of at least 200 adipocytes was carried out by the same operator throughout the study to improve precision. The number of fat cells in similar intervals of 2.5 μm in diameter was treated as a single variable, and the average value obtained for each interval was used to plot an histogram representing the group.[26] The mean diameter and mean volume for the entire fat cell population were calculated from the histogram.[27,28]

Fat cells lipolysis

Measurements of lipolytic activity were performed by incubating isolated adipocytes (1-2.5 × 10^5 cells) in KRB buffer, with continuous gentle shaking (30 cycles/ min) at 37°C under 95% O_2 – 5% CO_2 in both, basal state and in presence of different concentrations of noradrenaline (NA): 0.1, 1, 10 and 100 μM (stimulated lipolysis). The reaction was stopped by immersing incubations tubes into an ice-water bath.[29] Two aliquots were then taken for enzymatic determinations of glycerol released in the incubation buffer as a valid index of lipolysis. The glycerol is phosphorylated to glycerol-3-phosphate by ATP in presence of glycerokinase. Glycerol-3-phosphate is then converted to dihydroxyacetone phosphate by glycerophosphate dehydrogenase, leading to the formation of NADH. The enhancement in NADH concentration measured by an increase in the fluorescence is proportional to the amount of glycerol.[30]

The lipolysis was expressed as ηmol of glycerol released per μmol of TG from isolated adipocytes. For the expression, other aliquots of adipocyte suspensions were taken for determination of TG by the method of Folch.[31]

Results

Food intake, body weight and adipose tissue weights

As shown in Table 1, the animals of DEHP group had an average intake comparable to that observed in rats fed standard diet. However, body weight gain for 3

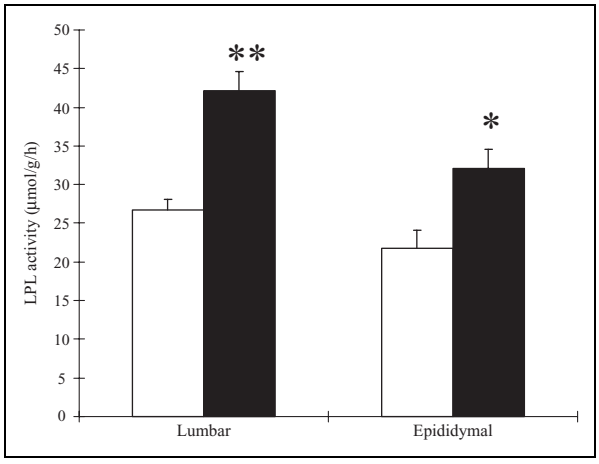

Figure 1. Effects of di(2-ethylhexyl) phthalate (DEHP) on lipoprotein lipase (LPL) activity in lumbar and epididymal adipose tissue. Control group (open bars) and DEHP group (solid bars). Each value represents mean ± SEM of six animals. Statistically significant differences from respective controls *$p < .05$ and **$p < .01$.

weeks was significantly reduced in animals fed with a diet supplemented with 2% DEHP, resulting in a mean final weight gain 34.4% lower than in the control group.

Table 1 shows that at the end of the experimental period, the epididymal and lumbar adipose tissues weights of DEHP-fed rats were significantly reduced (26% and 33%, respectively) compared to age-matched control animals.

LPL activity

The LPL activities in lumbar and epididymal adipose tissues were significantly increased when the animals were fed with 2% DEHP in the diet (Figure 1). In addition, the percentage of increase was higher in lumbar than in epididymal adipose tissue (60% and 41%, respectively).

Adipocyte size and TG content

The morphometric analysis revealed reduced diameter and volume of epididymal adipocytes in DEHP group compared to those of the control group (Table 2). Moreover, in adipose tissues of the DEHP group, the adipocyte TG content (μmol/cell) decreased significantly.

The histograms of adipose cell size distribution (at a 2.5-μm interval) at the end of the experimental period shows no differences between groups (Figure 2).

Table 2. Effects of di(2-ethylhexyl) phthalate (DEHP) on diameter and triacylglycerol (TG) content of epididymal adipose tissue

	Control group	DEHP group
Cell diameter (μm)	56 ± 1.2	52.3 ± 1.4[b]
Cell volume (ρl)	121 ± 5.0	94 ± 6.0[b]
TG (μmol/cell)	126 ± 4.0	112 ± 6.0[c]

[a] Values are expressed in mean ± SEM of six animals per group.
[b] Statistically significant differences from respective controls $p < .01$.
[c] Statistically significant differences from respective controls $p < .05$.

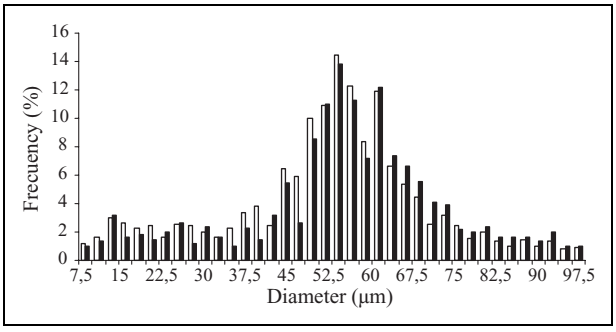

Figure 2. Representative histograms distribution of adipocytes cells mean diameters isolated from epididymal depots of rats fed a control group (open bars) and DEHP group (solid bars). The histograms were plotted by sizing at intervals of 2.5 μm, 200 adipocytes from each individual rat. Six animals were included in each group.

Basal and stimulated lipolysis of fat cells

There were no differences in adipocyte basal lipolysis between both groups (ηmol glycerol/ μmol TG/ 60 min; control group: 0.94 ± 0.15 versus 1.08 ± 0.23 in DEHP group). Figure 3 shows the dose-response curve for NA-stimulated lipolysis in epididymal adipocytes of both groups. Addition of NA markedly increased lipolysis as compared to unstimulated cells, reaching the maximal response at 1 μM of NA. However, lipolytic response to NA in the epididymal adipocytes was not affected at any of the concentrations examined when DEHP was compared to control group.

Discussion

The present work provides new insight into the effect of DEHP intake on lipolysis and LPL activity, with regard to the potential contribution to the known fat-lowering effect of this plasticizer. At least to our knowledge, no studies were conducted to investigate this issue.

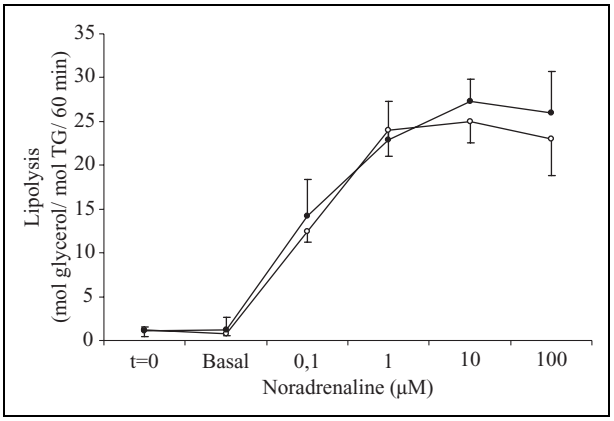

Figure 3. Effects of di(2-ethylhexyl) phthalate (DEHP) on basal and estimulated lipolysis in epididymal adipose tissue. Control group (ϒ); DEHP group (λ).Values are expressed in mean ± SEM of six animals per group.

Previously, a deleterious effect with DEHP on whole body energy metabolism was observed in rats fed a diet supplemented.[21] Nitrogen balance studies showed that it decreased significantly in animals fed with DEHP-diet and these findings were associated with a lower final carcass retention of nitrogen.[21] Furthermore, in both groups the total energy consumption and digestibility of diets was equal, suggesting that these changes were related neither to a reduced food intake nor to a nutrient malabsorption.[21] In addition, it was demonstrated that these lower protein retention is paralleled to the lower body fat accumulation in rats of DEHP group. We suggested that this effect could be mediated, at least in part, by a specific effect of DEHP on metabolic changes of adipose tissues. Since the doses used in this experimental animal model are near to the lowest observed effect level (LOEL)[32] for the plasticizer DEHP, this study did not preclude that some general toxicological actions can also be involved in the reduction of adipose tissue (size and weight).

Initially, we evaluated LPL activity in adipose tissues in order to establish relationship between influx and utilization of circulating TG by adipose tissues. The LPL enzyme takes part in the clearance of circulating lipoprotein-TG and as a result of this process, the fatty acids transported into the cell may either be re-esterified into TG or immediately oxidized. The LPL activity in epididymal and lumbar adipose tissues increased when the animals were fed with 2% DEHP. In this work, we demonstrated that TG content of adipocyte isolated from epididymal adipose tissue was reduced in DEHP group, correlated with lower adipocyte sizes. Thus, the higher uptake of fatty acids, due to an increased LPL activity would not be correlated to a higher re-esterification of fatty acids to TG in epididymal adipose tissue.

The effect of DEHP intake on lipolysis of adipose tissue and its potential contribution to the fat-lowering action was studied. Lipolysis was evaluated in epididymal fat pads because it is well known that this fat store is very sensitive to a variety of lipolytic stimuli.[33] Specifically, the lipolysis occurs within all TG-storing tissues so that lipids can be mobilized for oxidative or non-oxidative process. It has been shown that lipolysis in fat cells reflects the catalytic activity of the hormone-sensible lipase, which is the key enzyme in the limiting step of rate moving of TG on adipose tissue.

For this purpose, epididymal adipocytes from control and DEHP-fed rats were incubated with NA, a beta-adrenergic agonist that stimulates the hormone-sensible lipase activity. Based on the results of basal, as well as, stimulated lipolysis in isolated adipocytes showing no differences between DEHP and control groups at each level of the effector NA from 0 to 100 μM, support the concept that the reduction in adipose tissue size produced by DEHP in rats is not due to changes in lipolytic activity. Nevertheless, these results do not exclude the possibility that other or different levels of hormones could be involved in an increased lipolysis in 'vivo.' Hormone-sensible lipase activity is up-regulated by a number of hormones such as NA, glucagon, TSH, thyroid hormones (T_3, T_4) and others. Previous results of our laboratory showed that the levels of TSH, T_3 and T_4 were not modified by the effect of DEHP.[21] At least to our knowledge, there are no results showing alterations in levels of other hormones that could be related to lipolysis regulation.

Other mechanisms might intend to clarify the changes observed in fat body of DEHP-fed rats. Phthalate esters integrate a group of compounds known as peroxisome proliferators, which, in rodents, resulted in a pleotropic response mediated by the peroxisome proliferator-activated receptors (PPARs).[34,35] PPARs belong to a superfamily of nuclear hormone receptors that control genes involved, between others, in lipid metabolism. It is known that MEHP (major metabolite of DEHP) is a $PPAR_\alpha$ and $PPAR_\delta$ agonist.[35-37] The activation of $PPAR_\delta$ in vivo in adipocytes stimulates fatty acid oxidation and TG utilization.[38] This mechanism could explain the greater activity of LPL observed and the decreased adipocytes TG content. On the other hand,

the activation of $PPAR_{\delta}$ induces expression of genes encoding for uncoupling protein involvement in the regulation of energy metabolism. Thus, a higher and continuous oxidation of fatty acids, without synthesis of ATP, might lead to a waste of energy and a reduction of the stored fat in adipose tissue. This could be another mechanism responsible for the decrease of corporal fat and weight in the animals fed with DEHP.

In summary, the present study demonstrates that the reduction in adipose tissue size and body fat content induced by DEHP intake in rats might not be due to an increased lipolytic activity nor to a reduced adipose tissue LPL activity. More research is needed to achieve a comprehensive understanding of the potential mechanisms by which DEHP causes, among others, a reduced body fat retention.

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