

The metabolic effects of di (2-ethyl hexyl) phthalate medium dose on lipid profiles in serum and liver tissue

Buang Y^{1,2}

¹ Department of Chemistry, Faculty of Science and Engineering, Nusa Cendana University, Kupang, Indonesia

² Laboratory of Applied Biochemistry, Department of Applied Biological Science, Saga University, Saga Shi, Honjo Machi-1, Saga, Japan

Abstrak

Latar belakang: Di (2-ethyl hexyl) phthalate merupakan bahan plasticizer yang banyak digunakan pada kantong darah untuk transfusi. Bahan ini dapat mempengaruhi metabolisme lipid. Penelitian ini bertujuan menyelidiki efek metabolik di (2-ethyl hexyl) phthalate dosis tengah pada profil lipid dalam serum dan jaringan hati.

Metode: Tikus percobaan galur Sprague Dawley diberi diet yang disuplementasi dengan 1,0% di (2-ethyl hexyl) phthalate (kelompok DEHP, n=5) dan diet yang tak disuplementasi (kelompok kontrol, n=5) selama 10 hari. Hewan percobaan dibiarkan mendapatkan makanan secara ad libitum. Kadar lipid dalam serum diukur menggunakan enzyme assay kits. Lipid jaringan hati diekstraksi dan konsentrasinya ditentukan. Sepotong jaringan hati diambil untuk menentukan aktivitas malic enzyme dan carnitine palmitoyl transferase-1 (CPT-1).

Hasil: Kadar lipid serum kelompok DEHP menurun dibandingkan dengan kelompok kontrol ($P < 0,05$), di mana kadar lipid serum (mg/dL) pada kelompok kontrol dan DEHP masing-masing: trigliserida (TG) ($100,5 \pm 16,5$) dan ($31,2 \pm 1,7$); fosfolipid (PL) ($143,3 \pm 7,8$) dan ($88,9 \pm 3,2$); kolesterol total ($88,7 \pm 4,6$) dan ($51,9 \pm 2,3$); dan kolesterol HDL (high-density lipoprotein) ($29,8 \pm 1,0$) dan ($16,1 \pm 0,7$). Kandungan PL hati pada kelompok DEHP meningkat secara bermakna dibandingkan dengan kelompok kontrol ($P < 0,05$); peningkatannya mencapai 15%. Kandungan lipid hati (mg/g jaringan) pada kedua kelompok masing-masing: TG ($40,8 \pm 4,4$) dan ($23,7 \pm 1,3$); kolesterol total ($3,36 \pm 0,29$) dan ($2,33 \pm 0,23$); PL ($36,5 \pm 1,0$) dan ($41,7 \pm 0,6$). Aktivitas malic enzyme dan CPT-1 masing-masing meningkat sebesar 4,35 dan 2,33 kali kelompok kontrol.

Kesimpulan: Di (2-ethyl hexyl) phthalate dosis tengah menurunkan sekresi lipid dari sel-sel hati ke dalam aliran darah. Kandungan TG dan kolesterol total sel-sel hati juga berkurang, sebaliknya kadar fosfolipid hati meningkat. Peningkatan fosfolipid hati disertai peningkatan aktivitas malic enzyme dan CPT-1 merupakan faktor utama penurunan kadar lipid serum, TG dan kolesterol sel-sel hati yang diinduksi oleh di (2-ethyl hexyl) phthalate. (*Med J Indones 2011; 20:20-6*)

Abstract

Background: Di (2-ethyl hexyl) phthalate is the most widely used plasticizer in blood storage bag for transfusion. This substance can modify lipid metabolism. This study was aimed to elucidate the metabolic effects of di (2-ethyl hexyl) phthalate medium dose on lipid profiles in serum and liver tissue.

Methods: Sprague Dawley rats were fed 1.0% di (2-ethyl hexyl) phthalate diet (DEHP group, n=5) or a non-supplemented diet (control group, n=5) for 10 days. The rats were allowed to freely access each food. Serum lipid concentrations were measured using enzyme assay kits. Lipids of liver tissues were extracted and the lipid contents were determined. A piece of liver was prepared to determine the activities of malic enzyme and carnitine palmitoyl transferase-1 (CPT-1).

Results: Serum lipid concentrations (mg/dL) of DEHP group decreased compared to control ($P < 0.05$). The serum triglyceride (TG) concentrations of control and DEHP groups were respectively (100.5 ± 16.5) and (31.2 ± 1.7); phospholipid (PL), (143.3 ± 7.8) and (88.9 ± 3.2); total cholesterol, (88.7 ± 4.6) and (51.9 ± 2.3). The liver TG content of control and DEHP group (mg/g liver) were respectively, (40.8 ± 4.4) and (23.7 ± 1.3); liver cholesterol were (3.36 ± 0.29) and (2.33 ± 0.23); and the liver PL were (36.5 ± 1.0) and (41.7 ± 0.6). Malic enzyme and CPT-1 activities (nmol/min/mg protein) of DEHP group increased compared to control ($P < 0.05$), in which their increases were approximately by 4.35- and 2.33-folds, respectively.

Conclusion: The di (2-ethyl hexyl) phthalate medium dose attenuates lipids secretion from the liver cells into the bloodstream. The increase of liver PL level accompanied with the promotions of malic enzyme and the CPT-1 activities are the key factors of the dietary di (2-ethyl hexyl) phthalate effects in rats to attenuate the lipid secretions from the livers. (*Med J Indones 2011; 20:20-6*)

Key words: Di (2-ethyl hexyl) phthalate, hyperphospholipids, lipolysis, liver lipids, serum lipids

Di (2-ethyl hexyl) phthalate is the most widely used plasticizer in polyvinyl chloride plastic. It was reported that di (2-ethyl hexyl) phthalate used in blood storage bags leaches out in significant amounts into the blood stored and the blood products resulted from exposure of patients to this compound during transfusion.¹⁻³ It was also reported

that after 21 days storage of blood used to transfuse in human recipients, the blood storage bags averagely leaches out 10 mg di (2-ethyl hexyl) phthalate/100 mL blood. Furthermore, a number of reports are available on the toxicity of di (2-ethyl hexyl) phthalate, particularly studied in the liver.^{4,5} Almost all of those studies carried

out the treated doses reached 200mg/100g body weight (BW). Gayathri et al.⁶ administered the rats with a dose of 0.75 mg/100g BW which is equivalence with transfusion of ten unit of blood in a human recipient. These authors did not find the serious toxic effects as evidenced by lack of any histopathological changes in the liver or significant alterations in many biochemical parameters. Overall, the doses of di (2-ethyl hexyl) phthalate that had been treated have ranges 750µg-200mg per 100g BW. Therefore, the dose of 75 mg di (2-ethyl hexyl) phthalate/100g BW that was used in the present study might be considered medium dose.

Currently, di (2-ethyl hexyl) phthalate, a phthalate plasticizer, belongs to a peroxisome proliferators class of rodent nongenotoxic hepatocarcinogens.^{7,8} This phthalate modulates the peroxisome proliferators-activated receptor (PPAR).⁹ PPAR α is known as lipid-activated transcription factors expressed in the liver that belongs to the nuclear hormone superfamily. Numerous authors⁷⁻⁹ reported that di (2-ethyl hexyl) phthalate is the essential transcription factors regulating key cellular functions that include lipid metabolism.

Some biochemical parameters in lipid metabolism such as serum lipid profiles are constantly altered during normal states or disorders of metabolisms. Very-low density lipoprotein is a lipoprotein handling the lipid transportation from the synthesized lipid in the liver into extra hepatic tissues. Hence, serum lipid profiles generally indicate how lipid metabolism occurred in the liver. Commonly, disorders of lipid metabolism in the liver such as fat infiltration induce hepatic steatosis.¹⁰⁻¹² The impacts of the hepatic steatosis is similar to those seen in patients with alcoholic liver disease and range from mild hepatic steatosis to steatohepatitis, liver fibrosis, and cirrhosis,¹³ and, rarely, to hepatocellular carcinoma.¹⁴

Considering di (2-ethyl hexyl) phthalate is widely used in consumer products in common society such as food packaging materials and children's toys¹⁵ and used for tubing and containers for blood transfusions and blood products, etc., di (2-ethyl hexyl) phthalate constantly and directly or indirectly interacts with human and animal health cells. Therefore, its effect on lipid profiles in serum and liver tissue is of interested to evaluate. The present study was conducted to elucidate the metabolic effects of di (2-ethyl hexyl) phthalate medium dose on lipid profiles in serum and liver tissue using sprague-dawley (SD) rats as animal model.

METHODS

Animals and experimental design

All aspects of the experiment were conducted according to guidelines provided by the ethical committee of experimental animal care at Saga University (Saga, Japan). Male SD-rats aged 5 weeks were housed individually in an air-conditional room (24oC) with a 12-h light/dark cycle. After one week acclimatization, rats were assigned to two groups (five rats each). Control diet (as control group) was prepared according to recommendations of the American Institute of Nutrition (AIN) and contained (in weight %) 20 of casein, 10 of safflower oil, 1 of vitamin mixture (AIN-93), 3.5 of mineral mixture (AIN-93), 10 of sucrose, 0.25 of choline bitartrate, 0.3 of L-Cystein, 0.0014 of t-BHQ, 5 of cellulose, 13.2 of α -cornstarch, and β -cornstarch to make 100. The di (2-ethyl hexyl) phthalate diet (as DEHP group) was prepared by replacement of 1.0% β -cornstarch with di (2-ethyl hexyl) phthalate to the control diet. Considering evidences in food intake and final BW of the dietary di (2-ethyl hexyl) phthalate in male SD-rats, the 1% of food intake is equally to a range of 73-77 mg di (2-ethyl hexyl) phthalate/100g BW dose.¹⁶ The animals received the diets for 10 days. At the end of the feeding period, rats were killed by decapitation after a 9-h starvation. Livers were excised immediately, and serum was separated from blood.

Analyses of serum and liver lipids

Liver lipids were extracted according to the method of Folch et al.,¹⁷ and concentrations of TG, cholesterol, and phospholipids (PL) were measured by the methods used elsewhere.^{10-12,18,19} Serum TG, PL, cholesterol, and glucose were measured using enzyme assay kits from Wako Pure Chemicals according to the manufacture's instructions.

Preparation of liver sub cellular fractions

The mitochondrial and cytosol of liver sub cellular fractions were prepared as previously reported by Nagao et al.¹⁹ Protein content was determined by the method used in our previous studies.^{10-12,18,19}

Assays of hepatic enzyme activity

The malic enzyme (ME, EC1.1.1.40), the carnitine palmitoyl transferase-1 (CPT-1; EC2.3.1.23), glucose 6-phosphate dehydrogenase (G6PDH; EC1.1.1.49), fatty acid synthase (FAS; EC2.3.1.85), phosphatidate phosphohydrolase (PAP, EC3.1.3.4) activities were

determined by the methods used in our previous studies.^{10-12,18,19} The glutathione peroxidase (GSH-Px; EC1.11.1.9) was determined by the methods used elsewhere.^{20,21}

Statistical analyses

All values are expressed as mean \pm standard error of the mean (SEM). Data were analyzed by one-way analysis of variance, and all differences were inspected by Duncan's new multiple-range test using SPSS statistical software (SPSS inc., Chicago, IL, USA).

RESULTS

Dietary di (2-ethyl hexyl) phthalate promoted liver weight

The daily food intake is shown in Table 1. The food intake of DEHP group decreased in comparison to control. The low level of food intake in the group was equivalent with the reduction of body weight. However, the weights of liver were significantly higher than that of the control group ($P < 0.05$).

Effects of di (2-ethyl hexyl) phthalate on glucose blood level, serum and liver lipid levels

As shown in Figure 1, the lipid levels in serum of DEHP group decreased significantly ($P < 0.05$), in which serum TG, PL, total cholesterol, and HDL-cholesterol levels decreased approximately by 70%, 38%, 41%, and 46%, respectively. Although failed to reach significant level, the glucose blood level decreased by approximately 9%.

Table 1. The metabolic effects of di(2-ethyl hexyl) phthalate on growth parameters

Group	Control	DEHP*
Initial body weight (g)	132.8 \pm 3.4	134.6 \pm 2.5
Final body weight (g)	206.1 \pm 3.5 ^a	188.4 \pm 6.3 ^b
Food intake (g/day)	18.7 \pm 0.8 ^a	14.1 \pm 0.8 ^b
Liver weight (g/100 g body weight)	4.0 \pm 0.1 ^a	6.7 \pm 0.2 ^b

Values are expressed as mean \pm SEM of five rats. Clearly define a & b indicates significant difference at $P < 0.05$. *DEHP, di(2-ethyl hexyl) phthalate

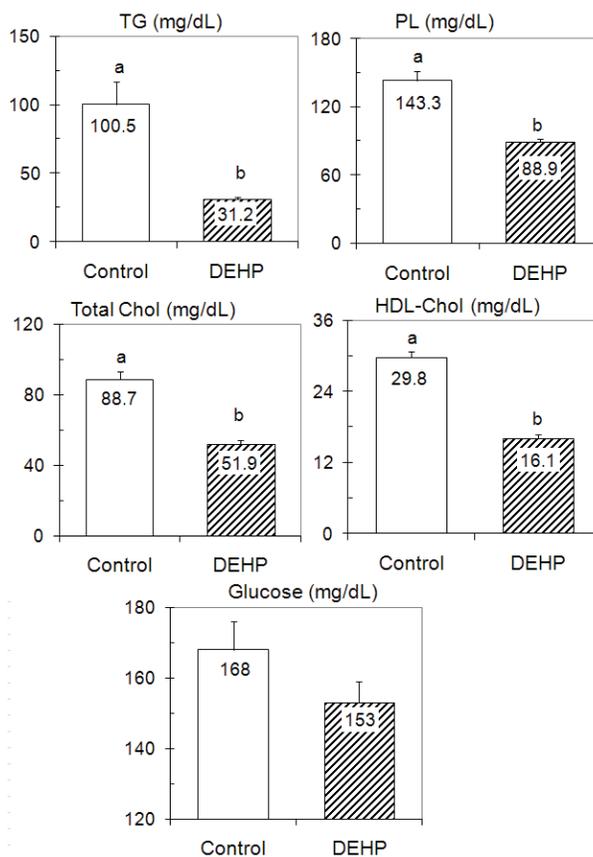


Figure 1. Serum lipids and glucose levels

Values are expressed as mean \pm SEM of five rats. Clearly define a & b regarding difference of significance at $P < 0.05$.

Figure 2 shows the differences in liver lipid contents. Dietary di (2-ethyl hexyl) phthalate promoted significantly the liver PL content than that of the control group ($P < 0.05$). The level was promoted approximately by 15%. Both liver

TG and liver cholesterol contents, however, decreased significantly in the group ($P < 0.05$). The decreases were approximately by 73% and 44%, respectively.

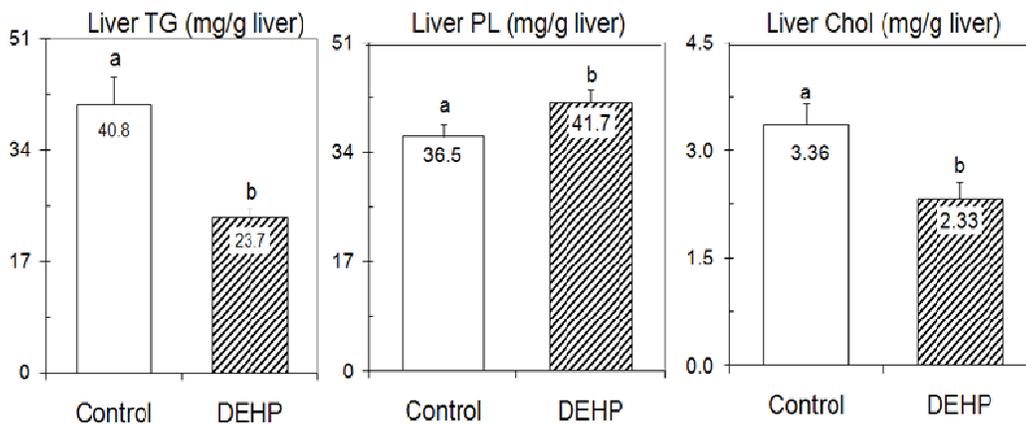


Figure 2. The liver lipids content

Values are expressed as mean \pm SEM of five rats. Clearly define a & b indicates significant difference at $P < 0.05$

Effects of di (2-ethyl hexyl) phthalate on the activities of malic enzyme and carnitine palmitoyl transferase-1

Figure 3 shows the activities of enzymes determined in present study. Compared to control, both malic enzyme and CPT-1 activities of DEHP group increased significantly ($P < 0.05$), in which the increases were

approximately by 4.35- and 2.33-folds, respectively. The activities of G6PDH and PAP slightly increased that were approximately by 12% and 5%, respectively. However, FAS activity decreased slightly. Furthermore, the activity of antioxidant enzyme represented by GSH-Px decreased significantly ($P < 0.05$).

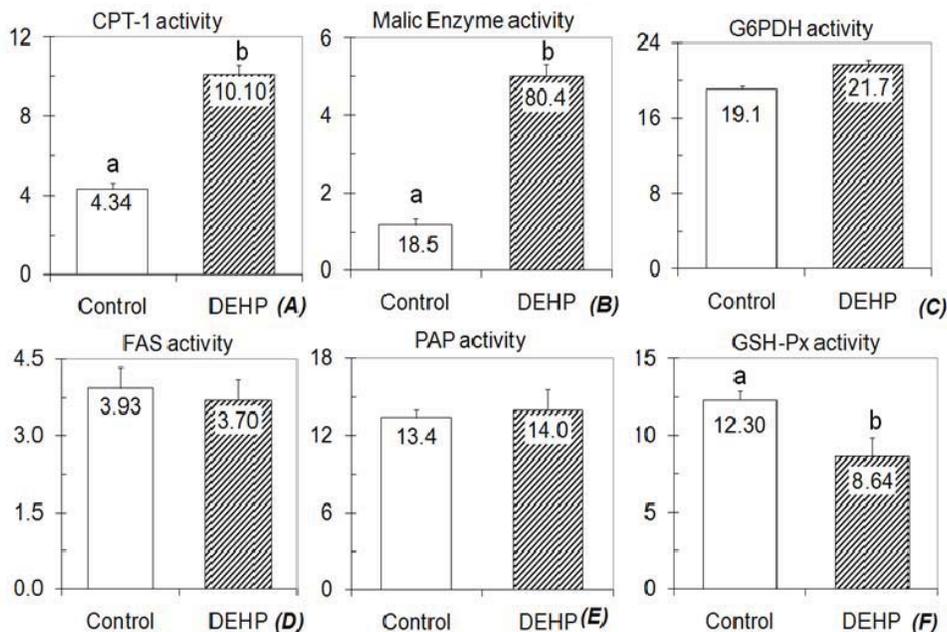


Figure 3. Metabolic responses regulated by di (2-ethyl hexyl) phthalate on liver enzyme activities (in nmol/min/mg protein)

Values are expressed as mean \pm SEM of five rats. Clearly define a & b indicates significant difference at $P < 0.05$

DISCUSSION

Di (2-ethyl hexyl) phthalate modulates the PPAR⁹ and is an essential transcription factors regulating lipid metabolism.^{7-9,16} The present study found that orally intake of 1.0% *di (2-ethyl hexyl) phthalate* diet reduced markedly serum lipid level. The intake also attenuated liver TG and cholesterol contents, however promoted liver phospholipids level. The malic enzyme and CPT-1 activities increased markedly. The promotions of these enzyme activities and the liver phospholipids level are the key factors of the metabolic effects of di (2-ethyl hexyl) phthalate in attenuations of serum lipids and the liver TG and cholesterol contents.

The di (2-ethyl hexyl) phthalate dose applied in present treatment was 1.0% of food intake; and by considering those food intakes and the body weights of the animal treated (Table 1), the dose of the present treatment was of 75 mg di (2-ethyl hexyl) phthalate per 100 g BW. This dose was in the range of currently treated which is 750 µg-200 mg per 100 g BW.⁶ As shown in Table 1, the liver weight of DEHP group increased significantly compared to control ($P < 0.05$). The decrease in final body weight however might be suitable with low food intakes of the rats in the group. Overall, the applied 75 mg di (2-ethyl hexyl) phthalate/100 g BW dose to the rats developed hepatomegaly.

Hepatomegaly is known as an abnormal enlargement of the liver. The enlargements of the liver size by this treatment (Table 1) indicate a disorder of metabolism including lipid metabolism in the liver tissue. Therefore, serum and liver lipids levels were important to determine. The decreases of serum lipid levels (Fig. 1) suggested that dietary di (2-ethyl hexyl) phthalate attenuated lipids secretion from the liver tissue into the bloodstream. The decrease of lipid levels secreted from the liver therefore might cause their content in the liver decreased. The decrease of liver TG and cholesterol content accompanied with a promotion of liver PL level (as shown in Fig. 2) is consistent with the reports of Yanagita et al.¹⁶ that intake of 1% di (2-ethyl hexyl) phthalate in rats induces hyperphospholipids of the liver tissue but attenuates TG and cholesterol levels. Therefore, di (2-ethyl hexyl) phthalate intake in the present study attenuated the serum lipid level and also the liver TG and cholesterol levels, but promoted liver PL content.

The increased PL content of liver tissue might indicate a promotion of PPAR α , the PPAR expressed in the liver. Burns and Heuvel²² reported that phosphorylation modulates PPAR α and affects these receptors activities

to control a variety of target genes involved in lipid homeostasis. Latruffe et al.²³ and Howarth et al.²⁴ found that di (2-ethyl hexyl) phthalate administered to rats lead to increase the volume and density of peroxisomes in the liver cells. Both those later authors also reported that during peroxisome proliferations, di (2-ethyl hexyl) phthalate stimulate the transcription of genes encoding enzymes of lipid metabolism, particularly fatty acid beta-oxidation in a PPAR-dependent manner. Our previous study found that the increased fatty acid degradation is associated with the promotion of liver PL level.¹¹ The promotions of liver PL level might indicate that those receptors are activated and therefore fatty acid degradation increased. The increased fatty acid degradation (Figure 3A) accompanied with unchanged activities of FAS and PAP between the groups might indicate a decrease of fatty acyl-CoA level, a substrate provided for TG biosynthesis; therefore liver TG content decreased (Figure 2). Furthermore, the increased fatty acid degradation in the liver tissue might indicate an enhancement of Krebs cycle to deliver adenosine triphosphate. The enhancement of Krebs cycle rate can be elucidated from some parameters of anaplerotic reactions.

Anaplerotic reaction is a filling up mechanism of Krebs cycle catalyzed by several enzymes. One biocatalyst involved in this mechanism is malic enzyme. Malic enzyme converses pyruvate to malate, a member of Krebs cycles. The increased malate concentration promotes the rate of Krebs cycle. The markedly increased malic enzyme activities found in DEHP group (as shown in Figure 3B) suggested that di (2-ethyl hexyl) phthalate intake in present dose could promote pyruvate conversion into malate. The increased conversion of pyruvate into malate accompanied with the increased G6PDH activity (Figure 3C) might induce rates of glycolysis to metabolize glucose and therefore deliver pyruvate. The pyruvate further plays as a starting molecule in biosynthesis of malate and ribose, a key molecule in nucleic acid nucleotide formations. This mechanism was reasonable because serum glucose level decreased (Figure 1) although it failed to reach significant level ($P < 0.05$). Hence, the decreased serum glucose level might indicate a promotion of glucose uptake by the liver cell as results of gradient concentration occurred between bloodstream and liver cytosolic cellular fractions. Furthermore, the decreased activities of antioxidant enzyme represented by GSH-Px might indicate that toxicity of medium dose of di (2-ethyl hexyl) phthalate used in present treatment might still appear in liver. Overall, considering all alterations of those enzyme activities, the flow chart

describing anaplerotic reaction induced by di (2-ethyl hexyl) phthalate was depicted in Figure 4.

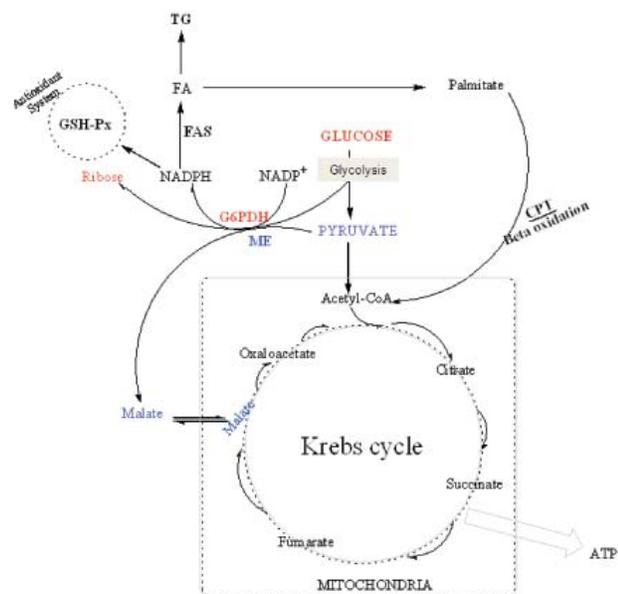


Figure 4. The pathways of anaplerotic reactions and the fatty acid degradation

Red, pentose phosphate pathway; blue, Pyruvate converted into malate catalyzed by malic enzyme

GSH-Px, glutathione peroxidase; NADP, nicotinamide adenine dinucleotide phosphate; G6PDH, glucose 6-phosphate dehydrogenase; ME, malic enzyme; FA, fatty acid; FAS, fatty acid synthase; TG, triglyceride; CPT, carnitine palmitoyl transferase; ATP, adenosine triphosphate

In conclusion, the ingestion of di (2-ethyl hexyl) phthalate in rats decreased levels of serum and liver lipids, except liver phospholipids. The promotion of liver phospholipids content induced activities of malic enzyme and CPT-1. Both these enzymes play essential roles in metabolic responses regulated by the di (2-ethyl hexyl) phthalate in lipid metabolism. The application of di (2-ethyl hexyl) phthalate in accurately dose might be one of the beneficial strategies in development of hypolipidemic drugs to secure the vessel blood from the attached lipids.

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