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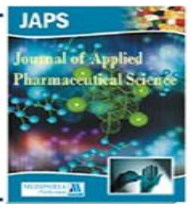

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The enlargements of liver size induced by orotic acid and di (2-ethyl hexyl) phthalate occur in different metabolic pathways

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ABSTRACT

The present study was conducted to elucidate the metabolic pathways by which enlarged liver size of patients undergoing disorders of orotic acid de novo metabolism and those patients of enlarged liver size induced by di(2-ethyl hexyl) phthalate in rats as animal model. The results showed that rats-treated with orotic acid generated liver triglyceride content 400% higher than that of the control accompanied with a significant decrease of phospholipid levels ($P<0.05$). The rates of lipogenic enzymes, both fatty acid synthase (FAS) and phosphatidate phosphohydrolase (PAP), increase accompanying promotions of liver triglyceride content without any changes in fatty acid degradation pathway. However, those rats-treated with di(2-ethyl hexyl) phthalate generated liver phospholipid level significantly higher than of the control accompanied with a markedly decreased the liver triglyceride levels. Both FAS and PAP activities were almost similar with those controls but the rates of fatty acid degradation were increased approximately by 2.5-fold of control. In conclusion: The enlargement of liver size induced by orotic acid is associated with largely retains triglyceride molecules in liver tissues, whereas those induced by di(2-ethyl hexyl) is associated with the induction of phosphorylation generating an increase of liver phospholipid levels.

Keywords: Orotic acid, liver enlargement, hypolipidemia, peroxisome proliferators, apoptosis.

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INTRODUCTION

Liver is one of visceral organs found in human and animal. This organ plays essential role in maintenance of metabolic pathways throughout the bodies (Harrison and Diehl, 2002). Based on cell demands, liver handles biosynthesis and degradation of biomolecules in various metabolic pathways as well as on lipid metabolism. In lipid metabolism, liver plays essential roles in biosynthesis and degradation of the lipid biomolecules. Although deposit lipid biomolecules largely occurs in adipose tissue but their degradation occurs primarily in liver. Various metabolites generated from the biosynthetic and lipolytic pathways of those lipid biomolecules include glucose, amino acids, and lipid derived compounds. Those various derived compounds are secreted into bloodstream and flow into different tissue based on their needs. The rate of those biosyntheses that imbalance with their secretion and degradation enhance this organ size (Fig. 1). Overall, organically growths of liver organs are associated with those biomolecules secretion, lipogenesis, and lipolysis pathways. The abnormally enlargements of liver size are mostly associated with the disorders of lipid metabolism. The disorders of lipid metabolisms in liver tissue generate swelling of the tissue as well as inflammation (Starkel et al. 2003, Tiku et al. 2007, and Suvorava et al. 2009). Although inflammation indicates a response of healthy cells onto cell

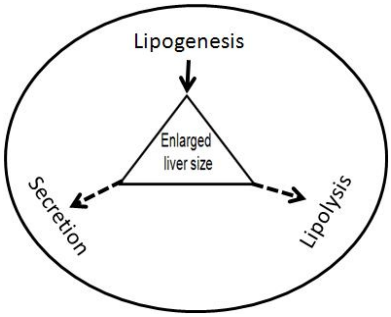

Fig 1. The net liver organ growth determined by the differentiations of secretion, lipogenic, and lipolytic pathways. ⇢ Inhibited pathway. → Induced pathway.

injuries but the cell undergoing inflammation decrease their functions (Ludwig et al. 1990 and Matteoni et al. 1999). The malfunction of cell surface receptor protein, for example, reduces capacities of cell signaling processes; the type 2 diabetes mellitus for example is one of the diseases caused by the malfunction of cell surface insulin-receptor (Raubenheimer et al. 2006 and Alpha-1 Foundation, 2007). Therefore, both cells injured and the inflammation of hepatic tissue cause their cells malfunction and generate metabolic diseases such as steatohepatitis, fibrosis and even cirrhosis as reported (Conway et al. 1989, Cotrim et al. 2000, and Dixon et al. 2001). The abnormally enlargement of liver organ therefore plays important role as one indicator of metabolic disorders.

The orotic acid (intermediate of nucleic acid nucleotides) and di(2-ethyl hexyl) phthalate can induce liver size. The di(2-ethyl hexyl) phthalate known as peroxisome proliferators (PPs) compounds is one of environmental substances normally found in food packed from plastic materials. The enlargement of liver size is known as an early biomarker for hepatocarcinogenesis induced by PPs as reported (Takagi et al. 1992). Although, both those compounds have been known to induce the liver organ size, but so far we know that their metabolic pathways were not clearly described yet. Therefore, the study aimed to describe pathways of liver size enlargement both induced by orotic acid and di(2-ethyl hexyl) phthalate was conducted in present work. The male Sprague-Dawley (SD) rats were used as animal model.

MATERIALS AND 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 Sprague-Dawley rats aged 5 weeks were housed individually in an air-conditional room (24^{o}C) with a 12-h light/dark cycle. After a 1-wk adaptation period, rats were assigned to three groups (five rats each). Basal 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), 0.20 of choline bitartrate, 0.3 of DL-Methionine, 5 of cellulose, 15 of α-cornstarch, and sucrose to make 100. The orotic acid (as orotic acid group) and di(2-ethyl hexyl) phthalate (as PPs group) diets were prepared by supplementation of 1.0% orotic acid and 1.0 % di(2-ethyl hexyl) phthalate, respectively, to the basal diet at the expense of sucrose. 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 liver lipids and serum

Liver lipids were extracted according to the method of Folch et al.(1957) and concentrations of triglyceride and phospholipids were measured by the methods of Fletcher (1968) and Bartlett (1959), respectively. The total cholesterol content was measured by the methods of Sperry and Webb (1950) with a minor adaptation. Serum triglyceride, phospholipids, and cholesterol were measured using enzyme assay kits from Wako Pure Chemicals according to the manufacturer'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. (2003). Protein concentration was determined by the method of Lowry (Lowry et al. 1951).

Assays of hepatic enzyme activity

The fatty acid synthase (FAS; EC2.3.1.85), phosphatidate phosphohydrolase (PAP, EC3.1.3.4) and carnitine palmitoyl transferase-1 (CPT-1; EC2.3.1.23) activities were determined as previously described (Nagao et al., 2003).

Statistical analyses

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.

RESULTS AND DISCUSION

Liver plays an important role in regulation of metabolisms throughout the bodies. The abnormally enlargements of liver organ are associated with various disorders of metabolic systems and generate metabolic diseases such as steatohepatitis, hypolipidemia (under normal level of lipid content in bloodstream), low levels of albumin in bloodstream, and various other metabolic diseases. The enlarged liver organ follows several metabolic pathways and primary depends on lipid biomolecules. This is because membrane bilayer of biocellular systems are mainly built up by phospholipid compounds. The imbalanced biosynthesis and degradation accompanied with the secretion yields organ size differentiation and develops metabolic diseases. As found in this study that enlargement of liver size (Table 1) induced by orotic acid was contributed by the over accumulation of triglyceride compounds in the tissue accompanied with a decrease of phospholipid contents (Fig. 3). This fact is absolutely different phenomena found in enlargement of liver size induced by di (2-ethyl hexyl) phthalate. The latter treatment indicated that changes of liver size were associated with the excessive contents of phospholipid compounds accompanied with a decrease of liver

triglyceride levels. Therefore, both orotic acid and di(2-ethyl hexyl) phthalate induce enlargements of liver sizes.

Table 1: The growth parameters.

Group	Control	Orotic acid	PPs
Initial body weight (g)	133.0 ± 3.0	133.0 ± 3.0	135.0 ± 3.0
Final body weight (g)	206.0 ± 3.0	202.0 ± 5.0	188.0 ± 6.0
Food intake (g/day)	18.7 ± 0.8a	18.7 ± 0.9[a]	14.1 ± 0.8[b]
Liver weight (g/100 g body weight)	4.0 ± 0.1[a]	5.6 ± 0.2[b]	6.7 ± 0.2[c]

Rats were fed each diet for 10d. Data are mean ± SEM (n = 5 rats/group). Clearly define a & b regarding difference of significance at P <0.05.

As shown in Table 1, both liver relative mass treated with orotic acid and di(2-ethyl hexyl) phthalate are larger significantly than that of the control (P<0.05), in which the liver sizes increased approximately by 40.0% and 67.5%, respectively. Hence, the increased liver sizes are associated with decreases of body weights. Those results also reports that enlargements of liver sizes did not depend on food intake quantities. Although these results did not inform the metabolic pathways yielding those data, but it could be concluded that either orotic acid or di(2-ethyl hexyl) phthalate treatments induce enlargement of liver organ sizes.

Various metabolic pathways induce differentiations of liver organ sizes are including biosynthesis rates accompanied with secretions and degradations of metabolites from/in liver tissues (Fig. 1).

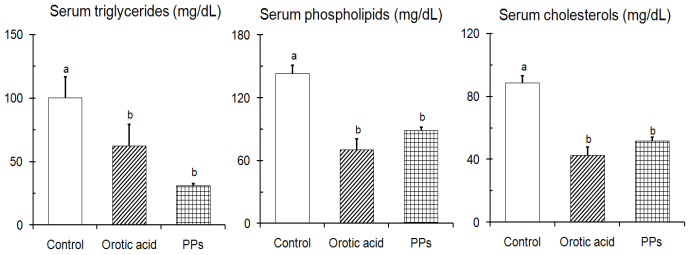

Fig 2. Serum lipid levels.

Rats were fed 1.0% orotic acid (orotic acid group) and 1.0% di(2-ethylhexyl) phthalate (PPs group) supplemented diets or a non supplemented diet (Control group) for 10 days. Rats were killed by decapitation after a 9-h starvation. Values are expressed as mean± SEM of five rats. Clearly define a & b regarding difference of significance at P <0.05.

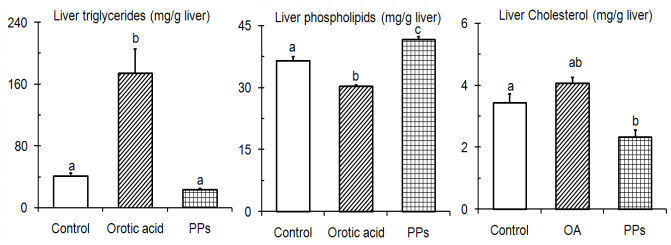

Fig 3. The liver lipid contents Values are expressed as mean± SEM of five rats. Clearly define a & b regarding difference of significance at P <0.05.

As shown in Fig. 2, the serum lipid contents found in rats treated with orotic acid and di(2-ethyl hexyl) phthalate decreased significantly compared to control group (P<0.05). The serum triglyceride contents of PPs group lower than that of the orotic acid group but slightly higher found in serum phospholipids and cholesterol contents. The differentiations of serum triglyceride contents found in those two treated groups conclusively indicate that both treatments induce metabolic system in different pathways. This is in consistent with lipid contents found in liver tissues (Fig. 3).

The data reported in Fig. 3 shows liver triglyceride contents of rats treated with orotic acid were markedly higher than that of two other groups. The liver triglyceride content reached 5-fold of the control group. Therefore, the decrease levels of serum triglyceride indicated that most of triglyceride molecules are retained in liver tissue (Fig. 3) instead of secretion (Fig. 2). The enhancements of liver triglyceride contents are followed by the reduction of liver phospholipid (P<0.05). Both these data are in agreement with the reports of Aflaki et al. (2011) that triglyceride accumulation activates the mitochondrial apoptosis pathway. Hence, the increased apoptosis are reasonable with a decrease of liver phospholipid contents, in which phospholipid is a cell membrane bilayer constituents. The differentiations of those two lipid substances, triglyceride and phospholipid, were followed by slightly increase liver cholesterol level. Overall, these results suggested that enlargement of liver organ induced by orotic acid is associated with the disorders of triglyceride metabolism in that organ. Similar with those rats treated with orotic acid, the rats treated with di(2-ethyl hexyl) phthalate generated enlargements of liver organs (Table 1). Hence, among the lipid components determined in liver organ, the liver phospholipid levels were significantly higher than that of the two other groups (Fig.3, P<0.05). This result is in agreement with the report of Yanagita et al. (1987) that di (2-ethyl hexyl) phthalate enhances hepatic phospholipid synthesis. Both triglyceride and cholesterol contents were lower than that of the control group. This is in agreement with the data reported by Hasmal et al. (2000) that di(2-ethyl hexyl) phthalate suppress apoptosis but induces DNA and peroxisome proliferator activated-receptors α (PPAR-α)-mediated gene expression. The increased phospholipid content retained in liver might be reasonable with a decrease of secretion (Fig. 2). Considering di(2-ethyl hexyl) phthalate is a peroxisome proliferator compound inhibiting apoptosis (Hasmal et al. 2000) and the data reported in Fig. 3, the enlargements of liver organs therefore are associated with the enhancements of liver phospholipid contents accompanied with suppression of apoptosis and induction of DNA biosynthesis and PPAR-α-mediated gene expression. Overall, taking all data reported by Takagi et al. (1992), Hasmal et al. (2000), Aflaki et al. (2011) and the results of present study could be concluded that enlargements of liver size induced by orotic acid is associated with a promotion of apoptosis according to reports of Aflaki et al. (2011) because the liver triglyceride content markedly increased accompanied with a significant decrease of liver phospholipid levels (Fig. 3); however the enlargement of that organ induced by di(2-ethyl hexyl) phthalate might be associated with induction of hepatocarcinogenesis according to reports of Takagi et al. (1992)

accompanied with an inhibition of apoptosis according to data reported by Hasmal et al. (2000) because data found in present study show that liver phospholipid level significantly increased accompanied with a decrease of the triglyceride level (Fig. 3).

Both triglyceride and phospholipid molecules play essential roles in enlargements of liver organ based on the inducing factors that are orotic acid and di(2-ethyl hexyl) phthalate compounds, in case of present study. Both triglyceride and phospholipid compounds contain three and two fatty acids, respectively. Therefore, the increased triglyceride and phospholipid contents in liver tissue induced by those different factors conclusively indicate an increase of fatty acid contents in these tissue cells. Hence, the fatty acids play as the substrates for various lipogenic and lipolytic enzymes include FAS, PAP, and CPT-1. The increasing lipogenic enzyme activities promote the fatty acid content generated and vice versa. However, the retained fatty acid is counterbalanced by their degradation as well as the secretion. The FAS enzyme catalyzes fatty acid biosynthesis whereas PAP prepares diglyceride molecule from phosphatidate. The fatty acid generated by FAS enzyme esterifies diglyceride to provide triglyceride molecules. As shown in Fig. 4, the FAS and PAP enzyme activities in orotic acid-treated rats were higher than that of the control group. These data conclusively indicate that both fatty acid and triglyceride molecule contents increase induced by the orotic acid compound. The increased triglyceride yielded by the synthesis accompanied with a decrease of degradation through fatty acid β-oxidation catalyzed by CPT-1 (Fig. 4) and the secretion (Fig. 2) promote accumulation of this compound in liver (Fig. 3). Both FAS and PAP levels in di(2-ethyl hexyl) phthalate-treated rats, however, were almost similar with control. These results are reasonable because the retained triglyceride contents in liver were similar with their control.

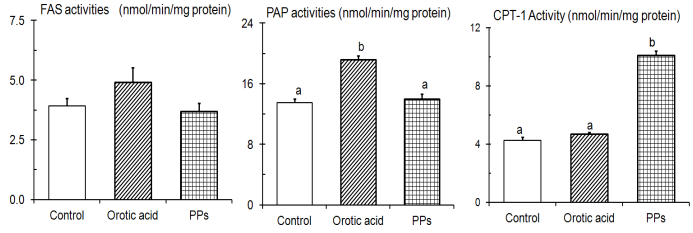

Fig 4. The activities of lipogenic and lipolytic enzymes Values are expressed as mean± SEM of five rats. Clearly define a & b regarding difference of significance at P <0.05.

The significantly decreased triglyceride content in serum compared to orotic acid group (Fig. 2) might indicate a promotion of liver triglyceride lipase (liver TGL) activities. This enzyme hydrolyzes triglyceride molecules to generate fatty acids. Hence, the increased liver TGL activities are reasonable because the PAP activity was almost similar with its control but CPT-1 level, which indicates a promotion of fatty acid β–oxidation, markedly increased (Fig. 4). This result is in agreement with the report of Burn and Heuvel (2007) that phosphorylation modulates PPAR-α. The modulated PPAR-α promotes CPT activities as well as fatty acid β-oxidation. This is consistent with the reports of Latruffe et al. (2000) and Howarth et al. (2001) that di(2-ethyl hexyl) phthalate stimulate the transcription of genes encoding fatty acid ß-oxidation and increase the volume and proliferations of peroxisomes in the liver cells respectively and therefore inhibits apoptosis as reported (Roberts et al. 2000). This is also consistent with our previous study (Buang et al. 2005) that the increased fatty acid ß-oxidation is associated with the promotion of liver phospholipid level in polyunsaturated fatty acid dependent manner. Taking all those findings and considering the peroxisome as a single membrane organelle, the increasing volume and proliferation of peroxisome organelles might be reasonable to increase phospholipid content. Overall, the results of this study conclusively indicate that the enlargement of liver sizes in di(2-ethyl hexyl) phthalate-treated rats is associated with modulations of phosphorylation.

CONCLUSION

Both orotic acid and di(2-ethyl hexyl) phthalate are inducible factors of liver organ enlargements. The enlargement of liver size induced by orotic acid is associated with induction of lipogenic pathways accompanied with a retardation of fatty acid β-oxidation and inhibition of secretion. However, the enlargement of liver size induced by di(2-ethyl hexyl) phthalate is not associated with those lipogenic levels but with modulation of phosphorylations. Therefore, the enlargements of the liver size induced by orotic acid and di(2-ethyl hexyl) phthalate occur in different metabolic pathways.

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REFERENCES

Aflaki E, Radovic B, Chandak PG, Kolb D, Eisenberg T, Ring J, Fertschai I, Uellen A, Wolinski H, Kohlwein SD, Zechner R, Levak-Frank S, Sattler W, Graier WF, Malli R, Madeo F, Kratky D. Triglyceride Accumulation Activates the Mitochondrial Apoptosis Pathway in Macrophages. J Biol. Chem. 2011, 286 (9): 7418.

Alpha-1 Foundation Version 1.6. What you need to know about Alpha-1 Antitrypsin Deficiency. A family history of liver disease. 2007.

Barlett GR. Phosphorous assay in column chromatography. J. Biol. Chem.1959, 234: 466.

Buang Y, Wang YM, Cha JY, Nagao K, Yanagita T. Dietary phosphatidylcholine alleviates fatty liver induced by orotic acid. Nutrition 2005, 21: 867.

Burns KA , Heuvel JPV. Review: Modulation of PPAR activity via phosphorylation. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 2007, 1771: 952.

Conway JG, Tomaszewski KE, Olson MJ, Cattley RC, Marsman DS, Popp JA. Relationship of oxidative damage to the

hepatocarcinogenicity of the peroxisome proliferators DEHP and Wy-14, 643. Carcinogenesis 1989, 10: 513.

Cotrim HP, Parana R, Braga E. Nonalcoholic steatohepatitis and hepatocellular carcinoma: natural history. Am J Gastroenterol. 2000, 95:3018.

Dixon JB, Bhathal PS, O'Brien PE. Nonalcoholic fatty liver disease: Predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. Gastroenterology 2001, 121: 91.

Fletcher MM. A colorimetric method for estimating serum triglycerides. Clin. Chim. Acta. 1968, 22: 393.

Folch J, Lees M, Sloane-Starley GH. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem.1957, 226: 497.

Harrison SA, Diehl AM. Fat and liver; a molecular overview. Semin Gastrointest Dis 2002, 13: 3.

Hasmall SC, James NC, Macdonald N, Soames AR, Roberts RA. Species differencies in response to diethylhexyl phthalate: Supression of apoptosis, induction of DNA synthesis and peroxysome proliferator activated receptor alfa-mediated gene expression. Arch Toxicol. 2000, 74: 85.

Howarth JA, Price SC, Dobrota M, Kentish PA, Hinton RA. Effects on male rats of di (2-ethyl hexyl) phthalate and di-n hexylphthalate administered a lone or in combination. Toxicol. Letters 2001: 35.

Latruffe NM, Malki C, Nicholas-Frances V, Jannin B, Clemencet MC, Hansmannel F, Passilly-Degrace P, Berlot JP. Peroxisome proliferator-activated receptors as physiological sensors of fatty acid metabolism: molecular regulation in peroxysomes. Biochem. Soc. Transac. 2000, 29: 305.

Lowry OH, Rosebrough NJ. Farr AL. Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951, 193: 265.

Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clinic Proc 1990, 55: 434.

Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathology severity. Gastroenterology 1999, 116: 1413.

Nagao K, Wang YM, Inoue N, Han SY, Buang Y, Noda T, Kouda N, Okamtsu H, Yanagita T. The 10trans, 12cis Isomer of Cunjugated Linoleic Acid Promotes Energy Metabolism in OLETF Rats. Nutrition. 2003, 19 (7-8): 652.

Raubenheimer PJ, Nyirenda MJ, Walker BR. A Choline-Deficient Diet Exacerbates Fatty Liver but Attenuates Insulin Resistance and Glucose Intolerance in Mice Fed a High-Fat Diet. Diabetes 2006, 55 (7): 2015.

Roberts RA, James NH, Hasmall SC, Holden PR, Lambe K, Macdonald N, West D, Woodyatt NJ, Whitcome D. Apoptosis and proliferation in nongenotoxic carcinogenesis: species differences and role of PPRα. Toxicol. Letters 2000, 112: 49.

Sperry WM, Webb M. A revision of the schoenheimer-sperry method for cholesterol determination. J. Biol. Chem. 1950, 187: 97.

Starkel P, Sempaoux C, Leclercq I, Herin M, Debby C, Desager JP, Horsmans Y. Oxidative stress, KLF6 and transforming growth factor-β upregulation differentiate non-alcoholic steatohepatitis progressing to fibrosis from uncomplicated steatosis in rats. Journal of Hepatology 2003, 39: 538.

Suvorava T, Kojda G. Reactive oxygen species as cardiovascular mediators: lessons from endothelial-specific protein overexpression mouse models. Biochim Biophys Acta. 2009, 178 (7): 802.

Takagi A, Sai K, Umemura T. Hepatomegaly is an early biomarker for hepatocarcinogenesis induced by peroxisome proliferators. J Environ Pathol Toxicol Oncol. 1992, 11: 149.

Tiku ML, Narla H, Jain M, Yalamanchili P. Glucosamine prevents in vitro collagen degradation in chondrocytes by inhibiting advanced lipoxidation reactions and protein oxidation. Arthritis Research & Therapy 2007, 9: R76.

Yanagita T, Satoh M, Enomoto N, Sugano M. Di (2-ethyl hexyl) phthalate enhances hepatic phospholipids synthesis in rats. Bioch. Biophys. Acta 1987, 919: 64.