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Quantitative analysis of lipid hydroperoxides levels in peripheral organs of Juvenile Visceral Steatosis (JVS) Mice at 1 month of age

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Juvenile visceral steatosis (JVS) mouse is an animal model of the systemic carnitine deficiency. JVS mice first develop fatty liver following cardiac hypertrophy, hyperammonemia, etc. To clarify the relationship between fatty liver and other symptoms, lipid hydroperoxides levels of peripheral oragans in JVS mice at 1 month were determined by the use of phosphine derivatives. We also report here a new method to quantitate the lipid components level in fatty liver of JVS mice.

Key words: Juvenile Visceral Steatosis (JVS) mice, fatty liver, lipid hydroperoxides, phosphine derivatives

INTRODUCTION

In 1988, Koizumi *et al* reported the homozygous mutant mice, named Juvenile visceral steatosis (JVS) mice, which have systemic L-carnitine deficiency and develop the fatty liver following hyperammonemia, hypoglycemia, cardiac haypertrophy and growth retardation.[1] Seventy % of JVS mice were died within 30~40 days after birth. Among these symptoms, only the fatty liver directly relates to the systemic L-carnitine deficiency in JVS mice. So, it is quite reasonable to consider that the secondary symptoms were caused by abnormal lipid accumulation in the liver of JVS mice. In this meaning, it is necessary to clarify the relationship between the lipid and lipid hydroperoxides associated with various symptoms.

The most commonly used method for the quantitation of lipid hydroperoxides is thiobarbituric acid-reactive substance (TBA-RS) assay system. The values obtained by TBA-RS are dependent on the secondary oxidized products produced from the decomposition of lipid peroxides. On the other hand, the analysis of the lipid is performed using thin layer chromatography (TLC) fractionation and chemical reaction methods. However these methods have serious problems such as low sensitivity and requiring large amount of lipid samples.

In this study we used the direct methods for the lipid hydroperoxides determination from the lipid and also developed the small scale and sensitive methods of the lipid

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analysis of tissue homogenates.

MATERIALS AND METHODS

Animals Homozygous mutants (jvs/jvs) mice were used as affected mice and wild (+/+) mice and heterozygous (+/jvs) mice were used as controls. The JVS mice and the control mice were studied at 25 days after birth. All these experiments were carried out according to the Guide lines for Animal Experimentation, Faculty of Medicine, Kagoshima University.

Reagents Triglyceride G-test kit, NEFA C-test kit, Cholesterol E-test kit and P test kit were purchased from Wako pure chemical Industries, Ltd (Japan).

Determination of lipid hydroperoxides in mouse tissues

Lipid hydroperoxides assay was performoed as previously described [2]. Briefly, the tissue removed from mice were homogenaized in PBS(-). The lipid in the homogenate was extracted with a mixture (2:1) of chloroform and methanol containing 0.01% (w/v) of butylated hydroxytoluene. The collected chloroform layers were evaporated at room temperature. Then the resulting lipid extract was dissolved in 300 μ l of chloroform. A hundred μ l aliquot of the lipid solution and 100 μ l of the 2mM NDPP (1-napthyldiphenylphosphine) solution were mixed and reacted at 60℃ for 1 h. The reaction mixer was injected to HPLC system. The separation of NDPP and its oxide-NDPPO (1-napthyldiphenylphosphineoxide) was performed by using a Cosmosil $5C_{18}$, 150×4.6mm, Nacalai Tesque. The mobile phase was 85% (v/v) methanol. The flow rate was 1.0 ml/min and UV detection was set up at 300 nm.

Lipid analysis The lipid in the homogenate was extracted and evaporated by similar methods as described above. Then the residual lipid was dissolved in 3ml of chloroform. Triacylglycerol [3], free fatty acid and total cholesterol in chloroform solution were determined by the kits for serum diagnosis using enzymatic methods. After hydrolysis with perchloric acid and hydrogen peroxide, inorganic phosporus in the phosholipids was measured by the kit according to molybdenum blue methods.

1) Determination of triacylglycerol and free fatty acid

Lipid solution was evacuated with a vacuum drying oven. Then the reagent of Triglyceride G-test kit (glycerol-3-phosphate oxidase・p- chlorophenol method) or NEFA C-test kit (acyl -CoA synthetase・acyl CoA- oxidase method) was added to the residual lipid, the reaction mixer was measured absorbance of red quinone pigment at 550 nm.

2) Determination of total cholesterol

In order to protect cholesterol from overdrying, 40 μ l of distilled water was add to 100 μ l aliquot of lipid solution. After evacuated, the residual lipid and remained distilled water were incubated at 37℃ for 60 min. Then, the reagent of Cholesterol E-test kit (cholesterol oxidase・3,5-dimethoxy-N-ethyl-N- (2-hydroxy-3-sulfopropyl) –aniline sodium method) was added to the solution. The reaction mixture was kept 37℃ for 4 h and the absorbance of blue quinone pigment at 600 nm was measured. In this case triacylglycerol inhibited color reaction of cholesterol and color formers, lipase was added to homogenate to avoid side reactions and incubated 37℃. After 60 min, the lipid in the reaction mixture was extracted.

3) Determination of phospholipid

Five hundred μ l aliquot of the lipid solution was evacuated. A hundred μ l of distilled water and 100 μ l of 60% (w/w) perchloric acid was added to the residue, and heated 110℃ for 1 h. Twe hundred μ l of 30% (w/w) hydrogen peroxide was added to the reaction solution and the solution heated for 6h continuously. Then, the reagent of P test kit was added to the lipid solution and measured the absorbance of molybdenum blue at 750 nm.

4) Determination of total lipid

The amount of total lipid was measured gravimetrically. [3]

Protein assay Protein concentrations were determined according to the method of Bradford.

RESULTS AND DISUCUSSION

In the previous studies, the most commonly used method to evaluate the lipid peroxidation level is the determination thiobarbituric acid-reactive substance (TBA-RS) produced in the decomposition of the lipid hydroperoxides. This method has several drawbacks because it needs severe reaction conditions for the formation of TBA-RS. The prolonged thermolysis of the lipid hydroperoxides also provides various decomposition products which also react with TBA. In this meaning, the TBA-RS values obtained are quite suspicious from the chemical standpoint of view. Therefore, we used another method that is based on the direct chemical reaction between lipid hydroperoxides and trivalent phosphine derivative NDPP (1-napthyl-diphenylphosphine). The lipid hydroperoxide react smoothly with NDPP to afford NDPPO and the formation of NDPPO can easily be quantitated by HPLC. So, we could evaluate the levels of lipid hydroperoxides in the tissue of JVS mice and the control mice by use of this method. The levels of lipid hydroperoxides in the liver of JVS mice were much higher than those of the control mice.

The widely used TLC method for the quantitative analysis of the lipids requires a large amount of lipids. It is quite difficult to get a large amount of lipids from some organs of mice. It is desirable to improve the new method for the quantitation of lipids in small amounts. The enzymatic methods using in this work did not need fractionation of the lipids and these methods are the direct measurement of lipid components. These methods are applicable to the small amount of lipid. The results by use of these methods showed similar values to those of the conventional methods by Kuwajima *et al* [4]. The main lipid component in fatty liver was triacylglycerol, which suggested the increased lipid hydroperoxides levels were due to the increased amount of triacylglycerol.

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