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Time-course microarrays reveal early activation of the immune transcriptome in a choline-deficient mouse model of liver injury

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ABSTRACT

Aims: Choline-deficient diet is extensively used as a model of nonalcoholic fatty liver disease (NAFLD). In this study, we explored genes in the liver for which the expression changed in response to the choline-deficient (CD) diet.
Main methods: Male CD-1 mice were divided into two groups and fed a CD diet with or without 0.2% choline bitartrate for one or three weeks. Hepatic levels of choline metabolites were analyzed by using liquid chromatography mass spectrometry and hepatic gene expression profiles were examined by DNA microarray analysis.
Key findings: The CD diet lowered liver choline metabolites after one week and exacerbated fatty liver between one and three weeks. We identified >300 genes whose expression was significantly altered in the livers of mice after consumption of this CD diet for one week and showed that liver gene expression profiles could be classified into six distinct groups. This study showed that STAT1 and interferon-regulated genes was up-regulated after the CD diet consumption and that the Stat1 mRNA level was negatively correlated with liver phosphatidylcholine level. Stat1 mRNA expression was actually up-regulated in isolated hepatocytes from the mouse liver with the CD diet.
Significance: This study provides insight into the genomic effects of the CD diet through the Stat1 expression, which might be involved in NAFLD development.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases in Western countries, the incidence of which is strongly associated with obesity [1]. NAFLD encompasses a series of hepatic alterations that begin with steatosis, subsequently progressing to advanced and clinical states such as nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [2]. Genetic and nutritional rodent models for liver diseases have provided information on the molecular mechanisms involved, which include oxidative stress [3], mitochondrial dysfunction [4], and cytokine expression [5]. However, the pathological changes during NASH development in the context of the two-hit hypothesis [6] remain incompletely understood, particularly the molecular mechanisms controlling the onset of NAFLD/NASH.

A nutritional model produced by feeding on a diet deficient in choline is one of the most commonly used models in NAFLD/NASH research, and it is well characterized by an increase in triglyceride, steatosis, hepatocellular death, inflammation, oxidative stress, and fibrosis. Liver choline is crucial for the *de novo* synthesis of phosphatidylcholine (PC), which is essential for the secretion of triglyceride as very low-density lipoproteins [7,8]. On the other hand, intracellular choline is oxidized to betaine, which functions as a methyl donor for the conversion of homocysteine to methionine [9,10]. Methionine is an amino acid that plays essential roles in many cellular functions because it is used for protein synthesis and as an intermediate of *S*-adenosylmethionine [11], which is a universal methyl donor essential for DNA methylation. These factors indicate that choline is also an important nutrient, which is involved in cellular signaling pathways as an epigenetic modulator. Choline is widely distributed in various types of food; however, evidence has shown that many adults are not obtaining adequate amounts of this nutrient from their diet [12], suggesting that the nutritional importance of choline is under-recognized. Most animal studies have been conducted by feeding on a diet deficient in choline and methionine (MCD) [13,14] in order to induce NASH. However, with the exception of a few reported studies that compared the MCD diet with the choline-deficient (CD) diet, the individual contribution of choline deficiency to NASH progression has remained unclear.

As mentioned above, because dietary choline is essential in the maintenance of PC synthesis in the liver, choline deficiency interrupts

Abbreviations: CD, choline deficient; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; VLDL, very low density lipoprotein; PC, phosphatidylcholine; Stat1, signal transducer and activator of transcription 1; FADS, fatty acid desaturases; ELOVL, elongation of very long chain fatty acids; IFN, interferon.

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the secretion of triglyceride from hepatocytes, contributing to the macrovesicular steatosis characterized by TG and free fatty acids. However, the onset and development of NAFLD in the liver with the CD diet are not well understood because of the complexity of genetic and epigenetic events that occur in hepatocytes in response to the CD diet. In addition, importantly, alteration in the cell type in the liver has indicated a pathological role for immune cells as contributors to chronic inflammation, including macrophages, T lymphocytes, and mast cells, strongly suggesting that the crosstalk between hepatocytes and immune cells is crucial for chronic inflammation and tissue remodeling.

Here, in order to identify key phenomena during the onset and development of NAFLD induced by choline deficiency, we gathered basic information on the liver concentration of choline metabolites and on gene expression in the liver of mice in response to the CD diet. We isolated candidate genes that were markedly regulated during the onset and development of NAFLD in the liver upon consumption of the CD diet and that showed early responses to such consumption. We considered the expression levels of these genes as early markers for the CD-induced liver pathology. The observations in this study provide novel insight into the biological roles of dietary choline, which may be protective against liver diseases.

2. Materials and methods

2.1. Animals, diets and histological analysis

Male CD-1 (ICR): Crj mice (4 weeks old, Charles River Japan, Hino, Japan) were housed in groups of 2 or 3 in metal cages in a room with controlled temperature (24 ± 1 °C) and a 12 h light/dark cycle; light from 0800 to 2000, daily. They had free access to stock diet and deionized water. After consuming a commercial stock diet (MF, Oriental Yeast, Tokyo, Japan, Supplemental Table 1) for 1 week, mice had free access to diets and deionized water. Mice (5 weeks) were fed either a choline-deficient diet (F2CDD, Oriental Yeast, Supplemental Table 2) or F2CDD plus 0.2% choline bitartrate for 3, 5, 7 and 21 days (n = 4–6). The animal study was approved by the Hiroshima University Animal Committee (Permit Number: C13-3), and the mice were maintained in accordance with the Hiroshima University Guidelines for the Care and Use of Laboratory Animals. Livers of mice consuming F2CDD or F2CDD plus 0.2% choline for three weeks were fixed with neutral buffered formalin and embedded in paraffin. Hematoxylin and eosin staining was carried out using 4-μm-thick paraffin-embedded sections.

2.2. DNA microarray

Total RNAs from livers were isolated using RNeasy lipid tissue kit (Qiagen Sciences, Germantown, MD), and pooled RNAs were subjected to cRNA synthesis for a DNA microarray analysis according to the manufacturer's instructions (44K whole mouse genome 60-mer oligo microarray, Agilent Technologies, Palo Alto, CA). All procedures of fluorescence labeling, hybridization, slide, and image processing were carried out according to the manufacturer's instructions. In this experiment, each comparison was hybridized to two arrays employing a DyeSwap method. DyeSwap method was carried out in order to eliminate the bias between dyes because the difference between Cyanine 3-CTP and Cyanine 5-CTP was altered the efficiency of hybridization in case of competitive DyeCoupling assay. Gene expression data were obtained using Agilent Feature Extraction software, using defaults for all parameters except ratio terms, which were changed according to the Agilent protocol to fit the direct labeling procedure. Files and images, including error values and *p* values, were exported from the Agilent Feature Extraction Program (version 9.5). The microarray data are also deposited in the NCBI GEO data base (available on the World Wide Web at www.ncbi.nlm.nih.gov/geo) under accession number GSE78053.

2.3. RNA analyses

The reverse transcriptase reaction was carried out with 1 μg total RNA as a template to synthesize cDNA using ReverTra Ace (TOYOBO, Osaka, Japan) and random hexamers (TaKaRa Bio, Kyoto, Japan), according to the manufacturer's instructions. Quantitative PCR analysis was performed by the SYBR Green method using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and StepOnePlus™ (Applied Biosystems, Foster City, CA). Conditions were set to the following parameters: 2 min at 95 °C, followed by 40 cycles each of 15 s at 95 °C and 1 min at 60 °C. The primers used for PCR analyses were as follows: mouse Stat1, forward, 5′-CTGGTTCACCATTGTTGCAG-3′, and reverse, 5′-GTTCGATCTGA CAACACCTG-3′; mouse IFIT1, forward, 5′-CCTGCAGAGCTTGAAAGAAG-3′, and reverse, 5′-CACAGTCCATCTCAGCACAC-3′; mouse RSAD2, forward, 5′-GCTTCAACGTGGACGAAGAC-3′, and reverse, 5′-CACCAAA CAGGACACCTCTT-3′; mouse TLR2, forward, 5′-CAGGTTCCAGTTTT CACCAC-3′, and reverse, 5′-GTACAGTCGTCGAACTCTAC-3′; mouse TLR4, forward, 5′-AGGAACAGAAGCAGTTCTTG-3′, and reverse, 5′-CACAATCACACTGACCACTG-3′; mouse L19, forward, 5′-GGCATAG GGAAGAGGAAGG-3′, and reverse, 5′-GGATGTGCTCCATGAGGATGC-3′.

2.4. Analyses of liver metabolites

The extraction method of Bligh and Dyer [15] was used. Liver tissues were homogenized using the mixture of water and methanol with a ratio of 1:4 (v/v) and transferred to a screw capped glass tube. The mixture of water and methanol (1:4, v/v) was added to the tube to bring the volume up to 400 μl. Chloroform was added to yield a water/methanol/chloroform ratio of 1:4:2 (v/v/v). Samples were shaken for 1 h. 160 μl of chloroform and 160 μl of water were added to achieve the phase separation. After vortexing for 1 min, the samples were centrifuged at 1800 × *g* for 15 min. The upper aqueous layer was dried, dissolved in MeOH, and subjected to UPLC-MS analysis. The UPLC-MS analysis was carried out using an Acquity UPLC system (Waters, Milford, MA, USA) coupled to Acquity TQD tandem quadrupole mass spectrometer (Waters) with electrospray ionization (ESI) in the positive ion mode electrospray ionization. Samples were injected onto a BEH HILIC column (Waters; 2.1_50 mm, 1.7_m) at a flow rate of 0.4 ml/min using gradient elution with 3 mM ammonium acetate (pH 8.0) in 80% acetonitrile (A) and 2.3 mM ammonium acetate (pH 8.0) in 5% acetonitrile (B) as follows: 0–0.1 min 100% A, 0.1–2.75 min 0% B, 2.75–5.0 min 100% B. Analyte detection was performed using single ion recording: *m/z* 104 for choline, *m/z* 258 for glycerophosphocholine and *m/z* 118 for betaine. Cone voltage was 25 V. Dwell time was 50. The lower layer was dried, dissolved in MeOH, and subjected to UPLC-MS analysis with ESI in the positive modes. Sample solution was injected onto a reversed-phase BEH C18 column (Waters, 2.1 × 50 mm, 1.7 μm) at a flow rate of 0.4 ml/min using gradient elution with 10 mM ammonium acetate (pH 5.0) in 40% acetonitrile (A) and 10 mM ammonium acetate (pH 5.0) in acetonitrile and isopropanol with a ratio of 1:9 (v/v) (B) as follows: 0–3.0 min 20% B → 100% B, 3.0–4.0 min 100% B. Column temperature was maintained at 40 °C. Analyte detection was performed using multiple reaction monitoring (MRM) with the following transitions: *m/z* 758.7 → 184.2 for 16:0/18:2 PC and *m/z* 496.4 → 184.2 for 16:0 lysoPC. Cone voltage and collision energy were 60 V and 5 V for PC and 30 V and 25 V for lysoPC, respectively. Dwell time was 200 ms. Peak area ratios of the analyte to the IS were calculated as a function of the concentration ratios of the analyte (QuanLynx, Waters). The lower layer was dried, dissolved in MeOH, and also subjected to triglyceride (TG) assay kit (Wako, Tsukuba, Japan), according to the manufacturer's instructions. Total cellular lipids were separated by one-dimensional thin-layer chromatography on silica gel 60 plates (Merck Millipore, Germany) in methyl acetate/chloroform/1-propanol/methanol/0.25% aqueous KCl (25:25:25:10:9, v/v/v/v/v). The separated lipids were stained with primulin, subjected to