Archives of Biochemistry and Biophysics 555–556 (2014) 55–65

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Metabolomics-based search for therapeutic agents for non-alcoholic steatohepatitis

Yoshihiko Terashima [a], Shin Nishiumi [a,*], Akihiro Minami [a], Yuki Kawano [a], Namiko Hoshi [a], Takeshi Azuma [a], Masaru Yoshida [a,b,c,*]

[a] Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chu-o-ku, Kobe, Hyogo 650-0017, Japan
[b] The Integrated Center for Mass Spectrometry, Kobe University Graduate School of Medicine, 7-5-1 Chu-o-ku, Kusunoki-cho, Kobe, Hyogo 650-0017, Japan
[c] Division of Metabolomics Research, Kobe University Graduate School of Medicine, 7-5-1 Chu-o-ku, Kusunoki-cho, Kobe, Hyogo 650-0017, Japan

ARTICLE INFO

Article history:
Received 30 January 2014
and in revised form 29 April 2014
Available online 22 May 2014

Keywords:
NASH
Hypotaurine
GC/MS
Metabolome analysis

ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is the commonest form of chronic liver disease in developed countries. Non-alcoholic steatohepatitis (NASH), which represents advanced stage NAFLD, is increasingly being recognized as a major cause of liver-related morbidity and mortality. However, no effective drugs against NASH have yet been developed. Therefore, we searched for candidate therapeutic agents based on the changes in levels of hepatic metabolites via gas chromatography mass spectrometry (GC/MS)-based metabolome analysis of livers from methionine-choline deficient (MCD) diet-fed mice, a mouse model of NASH.
Methods: The metabolites were extracted from the livers of the MCD diet-fed mice and then analyzed using GC/MS. Subsequently, the MCD diet-fed mice were supplemented with hypotaurine, and the therapeutic effects of hypotaurine against steatohepatitis were evaluated.
Results: Ninety-nine metabolites were detected in the livers of the MCD diet-induced steatohepatitis model mice. Among these metabolites, hypotaurine exhibited the greatest decrease in its concentration in the mice. Supplementation with 2 mmol/kg BW hypotaurine attenuated liver injuries and fat accumulation caused by the MCD diet-induced steatohepatitis. Furthermore, 10 mmol/kg BW hypotaurine supplementation ameliorated fibrosis and oxidative stress induced by the MCD diet.
Conclusion: The present metabolome analysis-based study demonstrated that hypotaurine is a novel candidate therapeutic agent for NASH.

Introduction

Non-alcoholic fatty liver disease (NAFLD)[1] is becoming increasingly prevalent in developed countries and is commonly recognized as a hepatic manifestation of metabolic syndrome. [1–3] NAFLD is a spectrum of liver diseases involving fat accumulation, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) [2,3] which can progress to fatal conditions such as liver cirrhosis and hepatocellular carcinoma [1–3]. The mechanisms responsible for the progression of NASH are entirely unknown, but lipotoxicity, adipose tissue-derived factors and gut-derived factors are being commonly recognized as major factors to develop steatohepatitis [4,5]. Steatosis involves hepatic fat accumulation caused by increased free fatty acids supply due to increased lipolysis from adipose tissue, decreased hepatic free fatty acid oxidation, increased hepatic de novo lipogenesis [1,2,4,6], and decreased lipid export from the liver [1,4]. Triglyceride accumulation in the liver seems to be regarded as the protective actions against FFA overload [4–6]. However, when the hepatic capacity to utilize FFA is overwhelmed, lipotoxicity is considered to be induced via oxygen species formation, ER stress, mitochondria dysfunction and TLR signaling activation, resulting in hepatocellular injury, cell death and inflammation [4–6]. Oxidative stress can be raised from excessive β-oxidation and ω-oxidation of FFA in hepatocytes, and promotes pro-inflammatory cytokine

* Corresponding authors. Address: Division of Gastroenterology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chu-o-ku, Kobe, Hyogo 650-0017, Japan. Fax: +81 78 382 6309.
E-mail addresses: nishiums@med.kobe-u.ac.jp (S. Nishiumi), myoshida@med.kobe-u.ac.jp (M. Yoshida).

[1] *Abbreviations used:* AST, aspartate aminotransferase; ALT, alanine aminotransferase; BW, body weight; CAT, catalase; ER, endoplasmic reticulum ; FFA, free fatty acid; GC/MS, gas chromatography/mass spectrometry; GPX, glutathione peroxidase; H&E, hematoxylin and eosin; NAFLD , non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis, MCD, methionine choline deficient; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TLR, toll-like receptor; UPLC-ESI-QTOFMS, ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry.

http://dx.doi.org/10.1016/j.abb.2014.05.013
0003-9861/

expressions [1,2] and hepatic stellate cell activation, leading to fibrosis [1]. In addition, gut derived endotoxins are considered to activate the TLR signaling of hepatocytes and hepatic Kupffer cells which also contributes to oxidative stress, liver inflammation and fibrogenesis [4,5].

Various therapies have been used to try and control the medical conditions associated with NASH [1–3]. The current pharmacological therapies for NASH include anti-oxidants, such as vitamin E [1–3,7]; insulin-sensitizing agents [1–3,8]; lipid-lowering drugs [1–3,9]; renin–angiotensin system-suppressing agents [1–3,10,11]; and ursodeoxycholic acid [1–3]. These agents induce modest improvements in pathological conditions derived from NASH, but there are no effective therapies for NASH itself [1]; therefore, attempts to elucidate the pathogenesis of NASH and to discover therapies for it are ongoing. So far, various animal and human studies have examined the potential therapeutic effects of anti-pro-inflammatory cytokines, such as pentoxifylline [2,3,12,13]; PPAR α/γ agonists [2,14]; farsenoid X receptor agonist [2,15]; and novel anti-oxidant agents such as cysteamine bitartrate [2,16].

In the present study, we performed metabolome analysis using gas chromatography mass spectrometry (GC/MS) to evaluate the metabolite alterations that occur in the NASH mouse model evoked by the methionine-choline deficient (MCD) diet, which results in liver injuries similar to those seen in human NASH, and then searched for candidate therapeutic agents for treating NAFLD/NASH. In metabolome analysis, which is the comprehensive study of low molecular weight metabolites, the characteristics and interactions of low molecular weight metabolites under specific conditions are evaluated [17–19]. It is possible that alterations in the levels of metabolites are better indicators of the functional status of a cell than the changes in the levels of RNA or proteins because metabolite alterations are located downstream of DNA, RNA and proteins, and furthermore, reflect not only genetic conditions but also environmental factors [17,20,21]. In addition, the profiles of these metabolites represent a summary of the metabolic processes that occur in the target cells, such as anabolism, catabolism, absorption, resource distribution, detoxification, and energy utilization [17,20,21]. Recently, metabolome analysis has been applied to biomarker discovery and disease diagnosis in the medical research field. [17,19–23] Furthermore, reversing metabolite alterations, e.g., by supplementing individuals with some of the metabolites that they lack, might lead to improvements in disease conditions [20,21]. Therefore, metabolome analysis could increase our understanding of NASH.

Materials and methods

Reagents

Hypotaurine and taurine were purchased from Sigma–Aldrich (St Louis, MO), and dissolved in 0.9% saline for the intraperitoneal injections.

Animals

All of the animal experiments performed in this study were approved by the institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations. Seven-week-old male C57BL/6J mice and ob/ob mice were purchased from CLEA Japan (Tokyo, Japan) and Oriental Yeast Co. (Tokyo, Japan), respectively. All mice were housed and bred at the animal unit of the Kobe University School of Medicine in a specific pathogen-free facility under an approved experimental protocol. The MCD diet was purchased from Oriental Yeast Co. (Tokyo, Japan). The constituent of MCD diet was shown in Supplemental Table 1. One week before the start of the experiment, all of the mice were fed the standard diet to allow them to acclimatize to their surroundings.

Study groups and experimental design

Four experiments were conducted in this study. In the first experiment, male C57BL/6J mice ($n = 16$) were randomly divided into 4 groups, and 2 of these groups were fed the MCD diet. The remaining 2 groups were fed the regular diet. The mice were sacrificed at 2 or 4 weeks after the start of the experiment. In the second experiment, male C57BL/6J mice ($n = 12$) were randomly divided into 3 groups after they had been consuming the MCD diet for 1 week. Then, the mice were intraperitoneally injected with 2 mmol/kg BW taurine, 2 mmol/kg BW hypotaurine, or saline as a control. All of the mice received 5 injections over a 2-week period (Supplemental Fig. 1A). In the third experiment, male C57BL/6J mice ($n = 12$) were randomly divided into 3 groups after they had been consuming the MCD diet for 1 week. Then, the mice were intraperitoneally injected with 2 mmol/kg BW or 10 mmol/kg BW hypotaurine, or saline as a control. All of the mice received 5 injections over a 2-week period (Supplemental Fig. 1B). In the fourth experiment, to evaluate other NAFLD model accompanied with insulin resistance, spontaneous mutation leptin deficient ob/ob mice were utilized. Ob/ob mice ($n = 15$) were randomly divided into three groups. During all experimental period, ob/ob mice (each $n = 5$) and C57BL/6J mice ($n = 5$) were fed normal diet. The ob/ob mice were intraperitoneally injected with 2 mmol/kg BW taurine, 2 mmol/kg BW hypotaurine or saline as a control. The C57BL/6J mice were also intraperitoneally injected with saline as a control. All of the mice received 9 injections over a 5-week period (Supplemental Fig. 1C). At the end of the experiments, the mice were sacrificed, and their blood was collected by cardiac puncture. The blood samples were centrifuged at 8000*g* for 10 min at 4 °C to obtain plasma. In addition, the liver was perfused with 0.9% saline through the inferior vena cava and then removed. A portion of the liver was fixed in 10% formalin neutral buffer solution for histological evaluation, and another portion of the liver was frozen in OCT compound (Sakura Finetek Japan, Tokyo, Japan) for oil red staining. The plasma samples and the remaining portion of the liver were stored at −80 °C until use.

GC/MS analysis

For GC/MS analysis, the extraction of metabolites from the liver and plasma was performed according to the methods described in our previous report [19]. The GC/MS measurements were performed using a GCMS-QP2010 Ultra system (Shimadzu, Kyoto, Japan) with a fused silica capillary column (CP-SIL 8 CB low bleeds/MS; inner diameter: 30 m × 0.25 mm, film thickness: 0.25 μm; Agilent Co., Palo Alto, CA), and the data analysis for the semi-quantitative evaluation was carried out according to the method described in a previous report [24,25].

Measurement of plasma biochemical markers

The plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glucose were measured using commercial kits purchased from Wako (Tokyo, Japan).

Measurement of the hepatic triglyceride level

The liver tissue of the mice was homogenized, and then lipids were extracted by a modified version of the method developed by Bligh and Dyer [26,27]. The chloroform layer obtained was evaporated just before use, and then the remaining lipids were