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Synthetic triglyceride containing an arachidonic acid branch (8A8) prevents lipopolysaccharide-induced liver injury

Nanzhe Piao, Kenichi Ikejima *, Kazuyoshi Kon, Tomonori Aoyama, Taro Osada, Yoshiyuki Takei [1], Nobuhiro Sato, Sumio Watanabe

Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan

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ABSTRACT

Aims: In this study, we investigated the effect of synthetic triglyceride containing an arachidonic acid branch (8A8) on lipopolysaccharide (LPS)-induced production of tumor necrosis factor (TNF)-α and nitric oxide (NO) in macrophages, and LPS-induced liver injury in the rat.
Main methods: RAW264.7 macrophages were co-incubated with 8A8 and LPS (100 ng/ml), and TNF-α mRNA/protein levels, nuclear factor (NF)-κB DNA binding activity, expression of inducible-type NO synthase (NOS2), and NO_2 production were measured. Male Wistar rats were given a single intraperitoneal injection of 8A8 prior to an intravenous injection of LPS (5 mg/kg), and liver histology, apoptotic cell death, serum TNF-α levels, and hepatic TNF-α mRNA were then evaluated.
Key findings: LPS-induced increases in TNF-α production in RAW264.7 macrophages were blunted by 8A8 in a dose-dependent manner, with 40% inhibition at 100 ppm. Further, 8A8 dose-dependently prevented LPS-induced increases in TNF-α mRNA levels, as well as NF-κB DNA binding activities, in RAW264.7 macrophages. LPS-induction of NOS2 and NO_2 release from these cells was also decreased by 8A8 in a dose-dependent manner. *In vivo*, LPS-induced liver injury, including hepatocyte apoptosis, was largely prevented when 8A8 (100 μl/kg) was given 30 min prior to LPS. Indeed, 8A8 blunted increases in both serum TNF-α and hepatic TNF-α mRNA levels significantly.
Significance: LPS-induced liver injury was prevented by 8A8 most likely through the inhibition of TNF-α and NO production from hepatic macrophages, suggesting a potential usefulness of 8A8 as an immunomodulating nutrient for prevention/treatment of endotoxin-related organ injuries including alcoholic liver disease and non-alcoholic steatohepatitis (NASH).

Introduction

Endotoxin (lipopolysaccharide; LPS) is a component of the outer wall of gram-negative bacillus, which plays a pivotal role in a broad spectrum of pathophysiological conditions comprising from local inflammatory responses to septic shock. Since the majority of bacterial flora in the gut consists from gram-negative rods, impairment of intestinal mucosal barrier results in the increase in endotoxin levels in the portal blood, triggering inflammatory responses in the liver. Lines of evidence clearly indicate that gut-derived LPS activates hepatic macrophages (Kupffer cells), which produce toxic mediators such as tumor necrosis factor (TNF)-α, eicosanoids, and free radicals, thereby causing hepatocellular injury in alcoholic liver diseases (Enomoto et al. 2000; Thurman 1998). More recently, the similar hypothesis is implicated in non-alcoholic steatohepatitis (NASH) that quite resembles pathological features of alcoholic liver disease despite the lack of heavy drinking (Solga and Diehl 2003). A neutralizing antibody to TNF-α ameliorates both alcoholic and non-alcoholic models of steatohepatitis in rodents (Iimuro et al. 1997; Li et al. 2003). However, clinical application of an anti-TNF antibody and a TNF receptor chimera are limited for severe alcoholic hepatitis with indefinite efficacy, and the long-term outcome has been reported to be even worse when used in combination with corticosteroid (Naveau et al. 2004). Anti-TNF strategies with safer and more efficient ways are theoretically promising for the prevention and treatment of steatohepatitis both in alcoholics and non-alcoholics.

Triglyceride, one of the major nutrients, consists of glycerol and three fatty acid chains with various lengths and combinations. Triglyceride and related compounds are not only the source of free fatty acids and their metabolites, but exerts various physiological functions by themselves. Recently, 2-arachidonoyl glycerol (2-AG) has been recognized as an endogenous cannabinoid receptor agonist (Mechoulam et al. 1995; Sugiura et al. 1995). Since cannabinoid receptors (CB1 and CB2) are

* Corresponding author. Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan. Tel.: +81 3 5802 1060; fax: +81 3 3813 8862.
E-mail address: ikejima@juntendo.ac.jp (K. Ikejima).
[1] Current affiliation: Department of Gastroenterology and Hepatology, Mie University Graduate School of Medicine, Mie, Japan.

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widely expressed in peripheral tissues including liver, 2-AG is postulated to act as an intrinsic regulator of hepatic inflammation and fibrogenesis through the cannabinoid receptor-mediated pathway (Julien et al. 2005; Mallat and Lotersztajn 2008). Here in this study, we investigated the effect of synthetic triglycerides containing one arachidonic acid and two caprylic acid branches (8A8 and 88A; Fig. 1A), which are potent cannabinoid receptor agonists, on LPS-induced production of TNF-α and nitric oxide (NO) in RAW264.7 macrophages. Further, we evaluated the preventive effect of 8A8 on LPS-induced liver injury in the rat.

Materials and methods

Materials

Synthetic triglycerides containing one arachidonic acid and two caprylic acid branches (SUN8A8-E and 88A; molecular weight 631 Da, Fig. 1A) were prepared enzymatically, and kindly provided by Suntory Co. Ltd. (Osaka, Japan). Lipopolysaccharide (LPS, from E. coli 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of analytical grade unless otherwise specified.

Cell culture and treatment in vitro

RAW264.7 macrophages, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were cultured in Dulbecco's modified essential medium (D'MEM, GIBCO®, Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, GIBCO®, Invitrogen Corp.) and antibiotics (100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate) in a humidified air containing 5% CO_2 at 37 °C. Cells were cultured on regular 60 mm polystyrene dishes unless otherwise specified. Every experiment was performed in duplicate, and then repeated for 6 times to obtain the average data. Cells were incubated with LPS (100 ng/ml) in the presence/absence of 8A8 and 88A [dissolved in 2 volumes of dimetylsulfoxide (DMSO)] at various concentrations ranging from 10 to 1000 ppm (vol/vol). The final concentration of DMSO in the media did not exceed 0.2%.

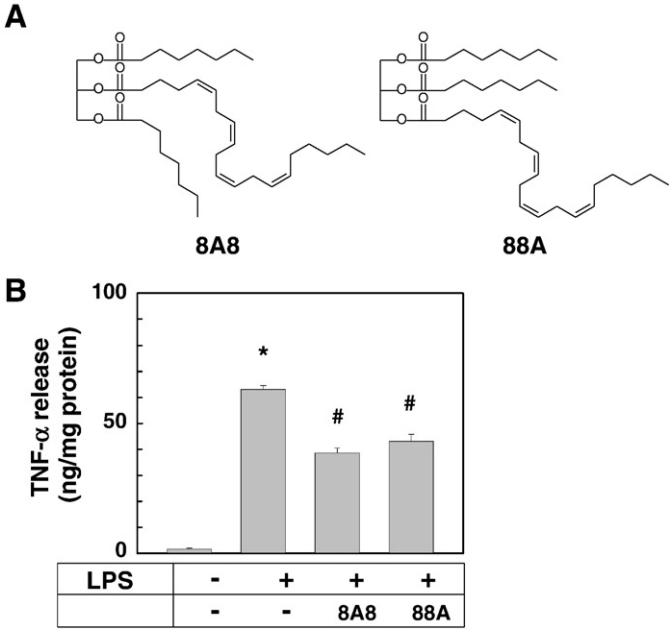

Fig. 1. The effect of 8A8 and 88A on LPS-induced production of TNF-α in cultured macrophages. Chemical structures of 8A8 and 88A, the synthetic triglycerides containing one arachidonic acid and two caprylic acid branches, are shown (A). RAW264.7 macrophages were incubated with 100 ng/ml LPS in the presence of 8A8 and 88A (100 ppm) for 6 h, and concentrations of TNF-α in the media were measured by ELISA (B). Data express mean ± SEM from 6 individual samples (*; $p<0.05$ vs. controls, #; $p<0.05$ vs. LPS alone by ANOVA on ranks and Student–Newman–Keuls post hoc-test).

Animal experiments

Specific pathogen-free female Wistar rats were purchased from Charles River Japan Inc. (Saitama, Japan). All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to institutional guidelines. All animals were allowed free access of water and laboratory chow diet and housed for several days prior to experiments. Rats weighing 200–250 g were given a single intraperitoneal injection of 8A8 [10–100 μl/kg body weight (BW), dissolved in olive oil] 30 min prior to a single intravenous injection of LPS (5 mg/kg BW). Animals were sacrificed under light ether anesthesia 90 min and 24 h after injection of LPS by exsanguinations from inferior vena cava to obtain liver and serum samples. Serum and liver tissue samples were kept frozen at −80 °C until assayed. For histological analysis, liver specimens were fixed with 10% buffered formalin, embedded with paraffin, and hematoxylin–eosin (H-E) staining and esterase staining were performed. Specimens were photographed using a microscope (BH-2, Olympus Corp., Tokyo, Japan) equipped with a digital imaging system (VB-6010, Keyence Corp., Osaka, Japan). Pathological evaluation was performed by one of the co-authors by a blinded fashion.

Measurement of aminotransferases, TNF-α and NO_2 levels

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured spectrophotometrically by a standard enzymatic method (KAINOS Laboratories Inc., Tokyo, Japan). Tumor necrosis factor (TNF)-α levels in the culture media and serum were determined using enzyme-linked immunosorbent assay (ELISA) kits (BioSource International Inc., Camarillio, CA). NO_2 in the culture media was quantified colorimetrically by Griess reaction using a commercial kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

Cell viability assay and detection of apoptotic cell death

Cells were plated in 96-well microplates (Sumitomo Bakelite Co., Tokyo, Japan) at a density of 5×10^4 cells/well in the complete culture medium. Number of viable cells was measured spectrophotometrically using a commercial kit (Cell counting Kit-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Experiments were performed in duplicated wells for each condition, and repeated for 6 times.

Apoptotic cell death was visualized by Hoechst 33342 fluorescent-dye staining. Briefly, the whole cells in the culture were harvested and collected by centrifugation at 200×*g* for 10 min. Cells were fixed with 1% glutaraldehyde solution overnight, re-suspended in phosphate buffer solution, and then stained with 167 μM Hoechst 33342 (Dojindo Molecular Technologies, Inc.). Specimens were observed and photographed under a fluorescent microscope (Axiovision 3.1/Axioplan 2 imaging, Carl Zeiss Microimaging GmbH, Germany).

RNA preparation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from cultured cells and frozen tissue samples were prepared using Trizol reagent (Invitrogen Corp.) and Quick Prep™ total RNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ), respectively. The concentration and purity of isolated RNA were determined by measuring optical density at 260 and 280 nm. Further, the integrity of RNA was verified by electrophoresis on formaldehyde-denaturing agarose gels.