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Eicosapentaenoic acid ameliorates steatohepatitis and hepatocellular carcinoma in hepatocyte-specific Pten-deficient mice☆

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***Background/Aims*: Eicosapentaenoic acid (EPA) has been known as a reagent for improving lipid metabolism and inflammation. Hepatocyte-specific Pten-deficient mice exhibit hepatic lesions analogous to non-alcoholic steatohepatitis (NASH). Therefore, we administered EPA to Pten-deficient mice to investigate the mechanisms of NASH.**

***Methods*: Pten-deficient mice were assigned to a control group fed with a standard chow or an EPA group fed with a 5% EPA-supplemented standard chow. At 40 weeks, livers from each group were processed to measure triglyceride content, gene expression analysis, Western blotting analysis, and histological examination. Level of serum reactive oxygen species (ROS) was also determined. Forty- and 76-week-old mice were used in tumor burden experiments.**

***Results*: EPA-ameliorated hepatic steatosis in Pten-deficient mice was based on decreased expression of AMPKα1-mediated SREBP-1c and increased PPARα expression. The EPA group exhibited less severe chronic hepatic inflammation compared to the control group, resulting from decreased ROS formation and a dramatically low ratio of arachidonic acid to EPA. Moreover, EPA inhibited development of hepatocellular carcinoma (HCC) in Pten-deficient mice based on an inhibition of MAPK activity and a low ratio of oleic to stealic acid, and a reduction in ROS formation.**

***Conclusions*: EPA ameliorated steatohepatitis and development of HCC in Pten-deficient mice.**

***Keywords*: Omega-3 fatty acids; NASH; Hepatocellular carcinoma; Pten; Reactive oxygen species**

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Abbreviations: Pten KO, Pten-deficient; EPA, eicosapentaenoic acid; ROS, reactive oxygen species; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease; TG, triglyceride; PUFA, polyunsaturated fatty acid; SREBP-1, sterol regulatory element binding protein-1; PPARα, peroxisome proliferator-activated receptor α; Nrf2, NF-E2-related factor 2; ALT, alanine aminotransferase; AMPKα1, AMP-activated protein kinase-α1; Gclc, glutamate-cysteine ligase, catalytic subunit; GST, glutathione *S*-transferase; PKB/Akt, protein kinase B; MAPK, mitogen-activated protein kinase; PCNA, proliferation cell nuclear antigen.

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1. Introduction

Non-alcoholic steatohepatitis (NASH) is currently receiving serious attention as a public health problem worldwide because the number of people with obesity, diabetes, and hyperlipidemia, which are considered to be causes of NASH, is increasing in Western countries and Japan. Recent evidence has clearly shown that NASH is a progressive liver disease followed by liver cirrhosis and even hepatocellular carcinoma (HCC) [1]. The two-hit hypothesis, in which steatosis caused by the first hit makes the liver vulnerable to the second hit leading to inflammation and fibrosis, has been proposed for the mechanism of NASH [2]. The first hit is an excessive triglyceride (TG) accumulation caused by a discrepancy between the influx and synthesis of hepatic lipids on one side and their β-oxidation and export on the other [1]. Following hepatic steatosis, oxidative stress caused by reactive oxygen species (ROS), proinflammatory cytokines, and adipocytokines as second hits induces necroinflammation in the liver [1]. However, the mechanism by which simple steatosis progresses to steatohepatitis, which is critical for the prognosis of NASH, has not been fully clarified. Accordingly, there has been no definitive reagent for inhibiting onset or exacerbation of NASH.

Eicosapentaenoic acid (EPA) is a typical dietary *n*-3 polyunsaturated fatty acid (PUFA) present in fish oil and is known as a reagent for improving lipid metabolism [3]. EPA or fish oil inhibits hepatic lipogenesis by decreasing the expression of sterol regulatory element binding protein-1 (SREBP-1), a key transcriptional activator for lipid synthesis [4], as well as increasing fatty acid degradation based on the activation of peroxisome proliferator-activated receptor α (PPARα) [5,6], which is a transcriptional activator of peroxisomal, microsomal, and mitochondrial fatty acid oxidation [7]. Recently, it has been reported that EPA induces NF-E2-related factor 2 (Nrf2), which is a master transcription factor that regulates expression of numerous detoxifying and antioxidant genes via the antioxidant response element [8]. Moreover, it is known that dietary EPA partially replaces the *n*-6 PUFAs from various cells including hepatocytes [9], and inhibits inflammation by decreasing the *n*-6/*n*-3 polyunsaturated fatty acids ratio [10]. Thus, we considered EPA as an anti-inflammatory as well as an anti-lipogenic reagent to inhibit onset or exacerbation of NASH.

We established hepatocyte-specific Pten-deficient (Pten KO) mice to investigate the role of Pten in the liver, reporting that these mice suffered from steatohepatitis with ballooned hepatocytes, Mallory's hyaline, lobular inflammation, and ultimately pericellular fibrosis followed by HCC [11]. In the liver of Pten KO mice, it has been verified that uptake of fatty acids to the hepatocytes is increased by the enhanced expression of SREBP-1c, and inflammatory cell infiltration is induced by increased ROS based on the enhanced expression of peroxisome proliferator-activated receptor γ (PPARγ) [12]. Moreover, Pten KO mice are more physiologically representative of NASH than *ob*/*ob* mice and mice fed a methionine-choline-deficient diet, which are often used as model animals of this pathological condition, in the respect that Pten KO mice not only spontaneously develop steatohepatitis and hepatic fibrosis but also hepatocellular carcinoma, and the substantial mechanism underlying hepatic steatosis is ascribed to the enhanced expression of SREBP-1c, as is the case in non-alcoholic fatty liver disease NAFLD or NASH.

2. Materials and methods

2.1. Generation of Pten KO mice

Pten$^{flox/flox}$ mice (129Ola×C57BL6/J F_2), generated as previously described [13], were mated to *AlbCre* transgenic mice (C57BL6/J background; The Jackson Laboratory, Bar Harbor, ME, USA) [14], in which expression of Cre is controlled by the promoter of the hepatocyte-specific gene *Albumin*. Offspring carrying AlbCre and two copies of the floxed Pten allele (*AlbCrePten*$^{flox/flox}$) were used in this study as homozygous mutant (Pten KO) mice.

2.2. Experimental procedure

Just after weaning, 32 Pten KO mice were fed with a standard chow (control group; 8 males and 8 females) or a 5% EPA-supplemented standard chow (EPA group; 8 males and 8 females) at random. All animals had free access to food and water until the experiment was terminated. Eight (4 males and 4 females) mice each from both groups were sacrificed at 40 and 76 weeks of age, respectively. When mice were sacrificed at 40 weeks, eight control and eight EPA group mice were subjected to scoring of steatosis, lobular inflammation, and ballooning hepatocytes in the liver according to the modified NAFLD activity score [15], and tumor burden experiments in which we performed microscopic and macroscopic analyses. Then, excised livers were utilized in biochemical assays and sera were collected in four (2 males and 2 females) control and four (2 males and 2 females) EPA group mice. Total lipids were extracted from a portion of the excised liver, as described previously [16]. Another portion of the excised liver was used to isolate total RNA and protein for real-time PCR and Western blotting analysis, respectively. Eight mice, each from both groups scarified at 76 weeks of age, were used in tumor burden experiments.

All animal experiments were approved by the Institutional Review Board of Akita University School of Medicine.

2.3. Biochemical analyses of liver extracts and serum

Levels of TG in total lipids extract and alanine aminotransferase (ALT) in serum were determined by colorimetric, UV kinetic, or enzymatic assays. The fatty acid composition of hepatic lipid fraction was analyzed by gas chromatography [17,18]. The concentration of each fatty acid was calculated as described previously [19]. Levels of total free radical derived from ROS in serum were determined in a total ROS assay system using Fenton reaction [20].

2.4. Real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using a TaqMan SuperScript First Strand Synthesis System for RT-PCR according to the protocol recommended by the manufacturer (Invitrogen). PCR for SREBP-1c (GenBank Accession No. NM011480), AMP-activated protein kinase-α1 (AMPKα1) (GenBank Accession No. NM001013367),