主論文の要旨

The modulation of hepatic adenosine triphosphate and inflammation by eicosapentaenoic acid during severe fibrotic progression in the SHRSP5/Dmcr rat model

（エイコサペンタエン酸による重症肝線維化進展モデルラット SHRSP5/Dmcr のアデノシントリホスフェイトと炎症の調節）

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【Introduction】

Non-alcoholic steatohepatitis (NASH) is characterized by steatosis, inflammation and fibrosis, which can progress to cirrhosis and hepatocellular carcinoma. NASH treatment is always a focus of researches. However, the exact treatment strategy remains poorly established.

Eicosapentaenoic acid (EPA) has been found to be a promising therapy to prevent or ameliorate NASH in NASH patients. In rodents, EPA was reported to ameliorate steatosis by decreasing *de novo* lipogenesis and increasing fatty acid oxidation, and to improve inflammation and fibrosis progression. However, the precise efficacy of EPA supplementation under various conditions must still be confirmed. Thus, this study aimed to evaluate the therapeutic functions and underlying mechanisms of EPA supplementation using a rat model with steatohepatitis and severe fibrosis.

【Methods】

Male stroke-prone spontaneously hypertensive 5/Dmcr (SHRSP5/Dmcr) rats were randomly assigned to three groups, and fed with either the stroke-prone (SP) control diet or high fat-cholesterol (HFC) diet with or without EPA (1mg/g body weight/day) for 2, 8 and 14 weeks, respectively. The liver histopathology, serum and hepatic biochemical features, mRNA and protein levels of related markers, and NF-κB DNA binding activity were determined.

【Results】

1. The effects of EPA on body weight, absolute and relative liver weight

The rats fed HFC diet with or without EPA developed lower body weights and higher absolute and relative liver weights than SP-fed control rats. Unexpectedly, EPA treatment decreased body weight at 2 weeks and increased absolute and relative liver weights at 14 weeks compared to HFC diet alone (Table 1).

2. The effects of EPA on serum or liver parameters

As shown in Table 1, EPA treatment inhibited HFC-diet induced increase in the levels of γ-GTP and IL-6 at 8 weeks. Unlike HFC-diet alone, EPA supplementation substantially reduced pro-inflammatory TNF-α and elevated anti-inflammatory adiponectin levels at 2 and 8 weeks, respectively, followed by lower TNF-α/adiponectin. Moreover, EPA supplementation significantly decreased serum and hepatic TG levels at 2 and 8 weeks, respectively.

3. The effects of EPA on histopathology

The control rats exhibited normal livers throughout the entire study (Fig. 1A and B). The H&E staining revealed that HFC-fed rats exhibited mild steatosis with foci of inflammatory infiltration at 2 weeks, which progressed to extensive steatosis with large

vacuoles, accompanied by inflammation and ballooning degeneration at 8 and 14 weeks (Fig. 1A). EPA supplementation for 2 weeks appeared to reduce inflammatory infiltration, and to improve macrovesicular steatosis at 8 weeks, as well as hepatocyte ballooning at 14 weeks. The histopathological scoring (Table 2) confirmed that EPA significantly improved the mean scores of inflammation, macrovesicular steatosis, and ballooning degeneration at 2, 8 and 14 weeks, respectively.

The EVG staining showed that rats fed HFC diet with or without EPA developed severe fibrosis at 8 and 14 weeks (Fig. 1B). Evaluation of the fibrotic areas revealed that no attenuation in fibrotic progression was detected after EPA supplementation.

4. The effects of EPA on hepatic ATP levels

EPA treatment sharply increased the ATP levels at 14 weeks relative to HFC diet alone or control diet, although a decreased level was detected at 2 weeks (Fig. 1A).

5. The effects of EPA on fatty acid metabolism

Although EPA decreased SREBP-1, FAS, SCD1, and DGAT2 at the mRNA levels (Fig. 2A), it did not significantly affect their protein levels (Fig. 2B). In fatty acid oxidation pathway, relative to HFC-diet alone, EPA decreased PPARα mRNA at 14 weeks (Fig. 3A), but increased the protein expression at 2 weeks (Fig. 3B). Importantly, EPA inhibited HFC diet-induced decrease in protein levels of representative enzymes for fatty acid oxidation including CPT1, VLCAD and PH at 8 weeks.

6. The effects of EPA on inflammation

HFC diet stimulated NF-κB activation pathway, and induced nuclear accumulations of p50 protein during the study and p65 at 2 weeks (Fig. 4). EPA treatment decreased nuclear p50 and p65 proteins at 2 and 8 weeks by inhibiting IκBα phosphorylation. Interestingly, EPA did not improve p65 DNA binding activity at 2 weeks, but rather elevated it at 8 and 14 weeks. HFC diet alone also induced MCP-1, an NF-κB downstream target. However, EPA did not alter MCP-1.

The MAPK signal transduction pathways were further assessed (Fig. 5). EPA treatment did not improve the activated ERK1/2, JNK p54 and p46.

【Discussion】

In this study, histopathological evaluation revealed that EPA co-administration with an HFC diet partially ameliorated diet-induced inflammation and macrovesicular steatosis at 2 and 8 weeks, respectively, which is consistent with previous reports. EPA administration also improved ballooning degeneration at 14 weeks, which corresponded to EPA-induced significant elevation in hepatic ATP levels. All of these features encouraged us to unravel the potential mechanisms of the roles of EPA in fibrotic steatohepatitis.

ATP depletion may be an important cause of hepatocellular injury in the steatotic liver.

EPA improved hepatocellular abnormal mitochondrial morphology and increased the levels of ATP *in vitro*. Here, EPA administration sharply elevated hepatic ATP content at 14 weeks concurrently with a pathological inhibition of hepatocyte ballooning. These functions have not been reported yet in the liver under severe fibrosis, and suggested that EPA treatment might reduce HFC diet-induced mitochondrial dysfunction and restore hepatic ATP stores, thereby protecting hepatocytes from necrosis.

Steatosis is the excessive accumulation of TG in the liver. The imbalance of lipid availability and lipid disposal is a critical determinant in the pathogenesis of steatosis. We investigated two aspects of the underlying mechanisms: *de novo* lipogenesis and fatty acid oxidation. EPA treatment stimulated β-oxidation in both mitochondria and peroxisomes at 8 weeks by inhibiting HFC-induced decrease in protein expression of enzymes including CPT1, VLCAD and PH, which have been demonstrated by several studies, especially in a NASH model using medaka. However, we failed to observe a significant role of EPA in lipogenic enzymes at the protein levels. Therefore, EPA-mediated reduction of hepatic TG and attenuation of macrovesicular steatosis at 8 weeks may be attributed to the enhanced mitochondrial and peroxisomal β-oxidation.

EPA has been identified as an anti-inflammatory PUFA. Here, the inhibition of inflammation by EPA was histologically detected as early as 2 weeks, with a concomitant decrease in TNF-α and increase in adiponectin levels in serum. The NF-κB signaling pathway plays a role in the pathogenesis of steatohepatitis. EPA reportedly inhibits NF-κB activation in various cells. EPA may predominantly affect the abundant pro-inflammatory components of p65:p50 heterodimers in the NF-κB family, accounting for the suppression of nuclear p65 and p50 subunits at 2 and 8 weeks. This inhibition was a phospho-IκBα-dependent event. Unexpectedly, EPA did not affect p65 DNA binding activity at 2 weeks. The delayed action on p65 binding activity might occur between 2 and 8 weeks, as the duration of NF-κB activation was prolonged after exposure to EPA. The increased activity at 8 and 14 weeks after EPA treatment may indicate a possible involvement of NF-κB in liver regeneration following liver injury. Therefore, EPA treatment for 2 weeks ameliorated inflammation mainly by preventing HFC diet-induced increase in serum TNF-α, TNF-α/adiponectin and NF-κB activation pathway.

【Conclusion】

Initial amelioration of the inflammation and steatosis in rats after EPA supplementation indicates a possibility to treat steatohepatitis. Additionally, this study provides new insights into the roles of EPA in hepatic ATP depletion and subsequent hepatocellular injury during severe fibrosis.