Cide-a and Cide-c are induced in the progression of hepatic steatosis and inhibited by eicosapentaenoic acid

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Abstract

Cide-a and Cide-c belong to the cell death-inducing DNA fragmentation factor-α-like effector family. Recent evidences suggest that these proteins may be involved in lipid accumulation in liver and adipose tissues. We confirmed that in the high-fat/high-sucrose diet-induced murine model of hepatic steatosis, the expression levels of the Cide-a and Cide-c genes were markedly and time-dependently increased, but returned to normal levels following improvement of hepatic steatosis by eicosapentaenoic acid (EPA) administration. Levels of expression of the Cide-a and Cide-c genes correlated well with plasma ALT. EPA inhibited the promoter activity of the Cide-a gene *in vitro*. Sterol regulatory element-binding protein-1 (SREBP-1) markedly enhanced the promoter activity of Cide-a, and EPA inhibited the expression of Cide-a mRNA. SREBP-1 and EPA didn't affect those of Cide-c. These findings indicate that Cide-a and Cide-c are closely involved in the progression of hepatic steatosis, and that EPA inhibits Cide-a gene expression through SREBP-1 regulation.

Introduction

Cide-a and Cide-c are cell death-inducing DNA fragmentation factor-α-like effector (CIDE) family proteins, and are well known as apoptosis-inducing factors for cultured cells [1, 2]. Recent functional evidence has indicated that Cide-c is localized in lipid droplets of white adipose tissues, and plays a key role in the formation of lipid droplets [3-5]. Cide-a is also involved in the formation of lipid droplet, like Cide-c [6, 7], and deficiency of Cide-a has been reported to contribute to enhancement of lipolysis in brown adipose tissues [8]. Furthermore, Cide-a knock-out mice exhibit resistance to obesity [8]. Protein expression of Cide-a and Cide-c has been reported to increase in the liver of obese mice, although it is minimal in normal liver tissues [9]. In addition to their effects on induction of apoptosis, these factors have received considerable attention with regard to their relationship to energy homeostasis and lifestyle-related metabolic disorders through regulation of lipid accumulation [10, 11].

A number of epidemiological studies as well as clinical studies have demonstrated that intake of fish oil or n-3 polyunsaturated fatty acids (PUFA) contributes to reduction of risk for cardiovascular events (CVE) [12]. EPA is one of the major n-3 PUFAs contained in fish oil, and is known to variety of pharmacological effects, such as serum lipid-lowering activity [13], anti-platelet activity [14, 15], anti-inflammatory activity [16, 17], and inhibitory effects on the progression of arteriosclerosis [18, 19]. In addition to these activities, Japan EPA Lipid Intervention Study (JELIS), a large-scale clinical trial conducted in Japan using highly purified EPA, has revealed that EPA suppressed the onset of CVE [20].

EPA inhibits the expression of mRNA encoding sterol regulatory element-binding protein-1 (SREBP-1), a transcription factor regulating the biosynthesis of fatty acids and triglyceride, as well as nuclear protein expression [21], and at least some of the pharmacological activities of EPA are mediated through its inhibitory effects on SREBP-1c [22]. For example, Sekiya and associates have reported that EPA suppressed expression of SREBP-1 in the liver of leptin-deficient *ob/ob* mouse model of obesity, and ameliorated hepatic steatosis [23]. Moreover, it has been reported that EPA ameliorated signs of nonalcoholic steatohepatitis (NASH) not only in a mouse model [24] but also a clinical trial [25], suggesting the possibility that EPA might serve as a useful modality for treatment of liver disorders associated with lipid accumulation.

In this study, we demonstrate that Cide-a and Cide-c are closely related to progression of hepatic steatosis with elevation of alanine aminotransferase (ALT), a plasma marker of hepatic injury. We also demonstrate the possibility that expression of Cide-a is regulated by SREBP-1 and EPA.

Materials and Methods

Materials. Highly purified EPA ethyl ester (purity: >98%) was obtained from Nippon Suisan Kaisha. Ethyl palmitate (purity: >95%) was purchased from Wako (Tokyo). Sodium oleate was from Sigma Aldrich (St. Louis, MO) and eicosapentaenoic acid sodium salt was purchased from Nu-Chek-Prep. (Elysian, MN).

Animals and diets. Male C57BL/6J mice (Clea Japan, Tokyo) at 7-9 weeks of age were given free access to water and one of the following three diets. The control group was fed control diet (fish meal-free F1; Funabashi farm, Chiba, Japan). The high-fat/high-sucrose diet (HF/HS) group was fed TD88137 (21.2 wt% fat, 34.1 wt% sucrose; Harlan TEKLAD, Madison, WI) plus 5 wt% ethyl palmitate. The HF/HS+EPA group was fed HF/HS in which 5 wt% ethyl palmitate replaced EPA ethyl ester. KK-A^y/Ta Jcl (Clea Japan) and ob/ob mice (Charles River Laboratories, Yokohama, Japan) at 7-9 weeks of age were given free access to water and diet (CE2, Oriental Yeast, Tokyo). Mice had withdrawn blood under anesthesia or were sacrificed by euthanasia at the indicated timepoint without fasting. All experiments were carried out in accordance with the guidelines for the use and care of laboratory animals of Mochida Pharmaceutical.

Measurement of liver parameters and DNA micro-array analysis. Plasma ALT, liver triglyceride, and liver total cholesterol were measured using commercially available kits. For gene expression analysis, total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA) and purified with the Purelink Micro-to-Midi total RNA purification system (Invitrogen) or RNeasy mini kit (QIAGEN, Hilden, DE) according to the manufacturer’s instructions. cDNA was generated using a SuperScript™ III first-strand synthesis system (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was performed using an ABI Prism® 7000 or 7500 (Applied Biosystems, Foster City, CA). Levels of gene expression were measured using primers and probes shown in Supplementary Table 1. For Cide-a and Cide-c gene expression assay, TaqMan® gene expression assay kits (code nos. Mm00432554-ml and Mm00617672-ml, respectively, Applied Biosystems) were used. 18s rRNA was measured using TaqMan® ribosomal RNA control reagents (Applied Biosystems) as an internal control. DNA micro-array analysis was performed with the Gene Chip® mouse genome 430 2.0 array kit (Affymetrix, Santa Clara, CA).

Cell culture and construction of expression plasmids. McA-RH7777 (rat hepatoma) and Cos-1 cells were purchased from American Type Culture Collection (Manassas, VA). Culture medium was Dulbecco's modified Eagle Medium (DMEM, Sigma Aldrich) supplemented with inactivated 10 % (v/v) fetal bovine serum (Nichirei, Tokyo) and 1% (v/v) penicillin /streptomycin. All cells were maintained at 37 ºC, 95 % air plus 5 % CO_2. cDNAs coding the full lengths of mouse Cide-a and Cide-c which were amplified by PCR were cloned into ptk2435 vector to produce mammalian expression plasmids under the control of elongation factor promoter. Human SREBP-1a and SREBP-1c active form expression plasmids were prepared by subcloning into pCI expression

plasmid (Promega, WI) by PCR amplification.

Reporter plasmid construction for promoter analysis of mouse Cide-a and Cide-c. From mouse Cide-a and Cide-c promoter sequence data and transcription start site (+1) as previously described [26, 27], we amplified the Cide-a promoter (from -2179 bp to +738 bp) and Cide-c promoter (from -1629 bp to +1269 bp) by PCR and cloned them into pGL4.17 basic plasmid (Promega) for the construction of Cide promoter luciferase plasmid, and performed to ensure fidelity of PCR sequencing amplification.

Statistical analysis. All data were assessed by Student's *t*-test. The relationships of ALT as an index of liver injury with other liver parameters (triglyceride, total cholesterol, and mRNA expressions of Cide-a, Cide-c, SREBP-1a, SREBP-1c, and PPARγ) were examined by multiple regression analysis using a stepwise method.

Results and Discussion

Following HF/HS diet for 20 weeks, mouse liver exhibited remarkable hepatic steatosis, TG content significantly increased from the control group level of 14 ± 1 mg/g tissue (mean ± S.E.) to 350 ± 50 mg/g tissue in the HF/HS group, and plasma level of ALT rose by approximately 4.2-fold (HF/HS group) (Fig. 1). Concomitant administration of EPA with HF/HS significantly suppressed the elevation of hepatic triglyceride level (19 ± 3 mg/g tissue) as well as plasma levels of ALT to almost normal levels (HF/HS+EPA group) (Fig. 1). The results of DNA micro-array analysis of mRNA expression in liver are shown in Table 1. In this table, genes which meet the following two criteria are listed in order of magnitude of criterion; (1) the level of expression in the HF/HS group was at least two-fold in the control group, and (2) the level of expression in the HF/HS+EPA group was at least by half that in the HF/HS group. Among genes, the Cide-a gene was up-regulated to the greatest extent in the HF/HS group. Its level of expression in the HF/HS group was approximately 500-fold that in the control group, while its level of expression in the HF/HS+EPA group was close to that in the control group. Gene expression of Cide-c, another CIDE family protein, was modulated in similar fashion to Cide-a. On the other hand, gene expression of Cide-b was affected by neither HF/HS nor EPA (data not shown). These behaviors of Cide-a and Cide-c were supported by quantitative PCR analysis (Fig. 1). These findings demonstrated that Cide-a and Cide-c were drastically induced by hepatic steatosis. As shown in Fig. 1, HF/HS induced up-regulation of SREBP-1c, a transcription factor regulating lipogenesis, as well as PPARγ, whereas EPA counteracted these effects of HF/HS. Subsequently, we examined the correlation between gene expression of Cide-a/Cide-c and the progression of hepatic steatosis. HF/HS induced elevation of

triglyceride levels in the liver as a function of duration of HF/HS. Plasma level of ALT rose to a significant extent at week 14 following initiation of HF/HS feeding. At week 4 after initiation of HF/HS feeding, Cide-a mRNA level rose to a significant extent. Cide-c mRNA level rose to a significant extent at week 1, though only modestly, and to a markedly high level at week 14 after the initiation of HF/HS feeding. Similarly, mRNA expression of SREBP-1c and PPARγ increased as a function of duration of HF/HS feeding (Fig. 2A). These findings demonstrated that level of Cide-a and Cide-c gene expression rose as a function of progression of hepatic steatosis and accompanied liver injury. Table 2 shows the results of multiple regression analysis between indicated parameters and plasma ALT. The contribution of hepatic expression of Cide-a or Cide-c to plasma ALT was greater than that of hepatic triglyceride content. The contribution of hepatic expression of PPARγ to plasma ALT was not significant.

Next, we investigated triglyceride content in the liver and plasma levels of ALT of genetically obese mouse, as well as levels of liver Cide-a and Cide-c gene expression. Triglyceride contents in the liver of KK-A^y/Ta Jcl mice and ob/ob mice were approximately 6.0-fold and 12.3-fold higher than those in C57BL/6J mice, respectively. Plasma levels of ALT were increased to a significant extent in KK-A^y/Ta Jcl mice, though rather modestly, while plasma levels of ALT rose to a markedly high level in ob/ob mice. Levels of Cide-a and Cide-c gene expression were approximately 34.7-fold and 10.7-fold higher in KK-A^y/Ta Jcl mice than in C57BL/6J mice, respectively. Similarly, levels of Cide-a and Cide-c gene expression were approximately 921-fold and 115-fold higher in ob/ob mice than that of C57BL/6J mice, respectively, in excellent agreement with patterns of changes in triglyceride content in the liver and plasma levels of ALT (Fig. 2B).

In mice, mRNA of Cide-a and Cide-c is highly expressed in brown adipose tissue and white adipose tissue, respectively, whereas Cide-a/Cide-c genes are minimally expressed in normal liver tissue [2, 5, 9]. It has been reported that Cide-a/Cide-c genes are expressed in the liver of aged mice, of type 2 diabetic mice exhibiting steatosis [28], and of leptin-deficient ob/ob mice [9]. It has also been reported that Cide-a and Cide-c play important roles in the formation of lipid droplets in adipocyte [3-6], and that Cide-c might contribute to lipid accumulation in the liver [9]. However, no report has documented a correlation between the onset/progression of hepatic steatosis and Cide-a/Cide-c. In the present study, levels of Cide-a and Cide-c gene expression were increased to a greater extent as a function of duration of HF/HS, in association with increases in triglyceride content in the liver and elevation of plasma levels of ALT. At the same time, EPA counteracted Cide-a/Cide-c gene expression, concurrently with improvement of hepatic steatosis. Although both

KK-A^y/Ta Jcl mice and ob/ob mice exhibited hepatic steatosis, triglyceride content in the liver, plasma levels of ALT, and levels of Cide-a/Cide-c gene expression were higher in ob/ob mice than in KK-A^y/Ta Jcl mice. These findings suggest that mRNA expression of Cide-a and Cide-c is closely related to the magnitude of hepatic steatosis.

Cide-a/Cide-c were originally identified as factors regarding apoptosis, but the relationship between ectopic expression of Cide-a/Cide-c in hepatic steatosis and cell injury remained unclear. It has been reported that Cide-a mRNA in mouse liver was experimentally induced by treatment with Wy-14643, a PPARα agonist, though without increase in apoptosis [26]. However, in the present study, there were excellent correlations between plasma level of ALT, a marker of liver injury, and levels of Cide-a/Cide-c gene expression, and the results of multiple regression analysis revealed that mRNA expression of Cide-a/Cide-c significantly contributed to plasma levels of ALT (Table 2). Taken together, these findings suggest that it may be possible that Cide-a and Cide-c contribute to hepatic injuries associated with hepatic steatosis, in addition to accumulation of triglyceride.

We also investigated whether ectopic Cide-a and Cide-c expression affects features of the lipid droplets formed by the addition of 200 μM oleic acid. Some large lipid droplets were found in McA-RH7777 hepatoma cells and Cos-1 cells in which Cide-a and Cide-c gene expression had been induced (Fig. 3A). This is the first report that Cide-a was indeed involved in formation of lipid droplet in liver-derived cells. Cell numbers and LDH levels in culture media examined in the same experiment indicated that Cide-a and Cide-c gene expression induced cell death at the same time (Fig. 3B). These *in vitro* findings supported the *in vivo* result that Cide-a and Cide-c were involved in the progression of hepatic steatosis and liver injuries associated with hepatic steatosis.

It has been reported that Cide-a and Cide-c gene expression is controlled by PPARα [26] or PPARγ [9]. In the present study, Cide-a and Cide-c gene expression was modulated in parallel with PPARγ gene expression. On the other hand, EPA inhibited mRNA expression of Cide-a, Cide-c, and PPARγ induced by HF/HS (Fig. 1). Since EPA activates PPARα [29] and PPARγ [30, 31], these findings are contradictory. Thus, the inhibitory effect of EPA on Cide–a and Cide–c expression was not mediated through activation of PPARα [26] or PPARγ [9]. We therefore investigated direct effects of EPA on Cide-a and Cide-c gene expression *in vitro*.

EPA inhibited Cide-a promoter activities but not Cide-c promoter activities in the luciferase assay (Fig. 4A), suggesting that EPA may have different mechanisms of manifestation of inhibitory effects on Cide-a and Cide-c gene expression. SREBP-1 mRNA expression and nuclear protein expression are known to be inhibited by EPA [21]. In the present study, SREBP-1c and Cide-a and

Cide-c in the liver were modulated in similar fashion (Fig. 1). We then examined the effects of SREBP-1 on Cide-a and Cide-c promoter activities. When SREBP-1a or SREBP-1c was co-transfected, Cide-a promoter activities were markedly increased, whereas Cide-c promoter activity was only slightly affected by SREBP-1s (Fig. 4B). These results are in agreement with the effects of EPA on Cide-a and Cide-c promoter activities, strongly suggesting that the inhibitory effect of EPA on Cide-a promoter activity is mediated through SREBP-1.

In summary, we have demonstrated that the expression of Cide-a and Cide-c, which are apoptosis-inducing factors, was induced by accumulation of lipids in liver, and returned to normal level following improvement of hepatic steatosis by treatment of EPA. Our findings suggest the possibility that Cide-a and Cide-c expression might trigger or cause the formation of large lipid droplets and hepatic injury. We also demonstrated that SREBP-1 enhanced Cide-a gene expression, and EPA may have contributed to successful treatment of hepatic steatosis and liver injury associated with hepatic steatosis possibly through inhibition of Cide-a gene expression via inhibition of SREBP-1. Further studies are needed to clarify the mechanisms and roles of EPA inhibition of Cide-c.

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Legends

Fig. 1 Suppressive effects of EPA on hepatic steatosis, plasma ALT, and expression of lipogenic genes induced by HF/HS feeding. Ten-week-old C57BL/6J male mice (n=7-10) in each group were fed the experimental diets described in the text. After 20 weeks of treatment, mice were sacrificed to obtain liver and blood samples. Plasma ALT activities and levels of mRNA expression in liver were determined. Each column represents mean ± S.E. **P*<0.05; ***P*<0.01, compared with control by Student's *t*-test.

Fig. 2 Hepatic triglyceride content, plasma ALT, and hepatic mRNA levels in various models of hepatic steatosis. (A) Ten-week-old of C57BL/6J male mice (n=7-10) in each group were fed the experimental diets described in the text. After the indicated periods of feeding, mice were sacrificed and plasma ALT, hepatic triglyceride contents, and mRNA levels were determined. (B) Genetically obese mice (male, 9-11 weeks old, n=5-7) were fed CE2 diet and sacrificed. (mean ± S.E.). **P*<0.05; ***P*<0.01, compared with control by Student's *t*-test.

Fig. 3 Functions of Cide-a and Cide-c in lipid droplet formation and injury in cultured cells. (A) To estimate lipid droplet formation by Cide-a and Cide-c expression, 2.5×10^4 cells/well of Cos-1 and McA-RH7777 cells were seeded in 24-well plates. After overnight incubation, all cells were transfected with 0.5 µg of Cide-a or Cide-c expression plasmids using Fugene-6 Transfection Reagents according to the manufacturer's instructions, and 4 hr after transfection the medium was exchanged with fresh DMEM medium containing 200 µM sodium oleate/0.5% BSA mixture. After 1- day incubation, cells were stained by Oil-red O and Mayer hematoxylin.
In the Cide-a and Cide-c gene-transfected groups, large lipid droplets were observed, as shown by arrows. (B) Cos-1 cells were seeded at 8×10^4 cells/well in 12-well plate, and the next day co-transfected with 1.0 µg of Cide-a or Cide-c expression plasmids. Two days later, LDH activities were measured as leakage from cells into culture medium with the Cytotoxicity Detection kit (Roche Diagnostics, Mannheim, GE), and numbers of viable cells were counted with hemacytometer (n=3, mean ± S.E.). **P*<0.05; ***P*<0.01, compared with control by Student's *t*-test.

Fig. 4 Effects of EPA and SREBP-1 on the promoter activities of Cide-a and Cide-c genes. (A) A total of 2.5×10^4 cells/well of Cos-1 or McA-RH7777 cells were seeded in 24-well-plates and incubated for 1 day. Then cells were co-transfected with 0.5 µg of Cide-a or Cide-c promoter luciferase plasmid. pRL-SV40 plasmids (Promega) expressing Renilla luciferase were used as an internal control. After 4 hr, all medium was exchanged with fresh medium containing 50 µM EPA/0.5% BSA mixture or vehicle and 10 % delipidated serum instead of fetal bovine serum.

Delipidated serum was prepared from immobilized fetal bovine serum according to a reference [32]. Luciferase activity was measured at 1 day after transfection using the Dual Glo Luciferase assay kit (Promega) according to the manufacturer's instructions (n=3-4, mean ± S.E.). *P<0.05; **P<0.01, compared with control by Student's *t*-test. (B) To determine the effects of SREBP-1 on Cide promoter activity, human SREBP-1a and -1c active form expression plasmids were co-transfected with each Cide promoter plasmid into Cos-1 cells according to the same protocol as above (n=3-4, mean ± S.E.).