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ORIGINAL CONTRIBUTION

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Down-regulation of α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase by polyunsaturated fatty acids in hepatocytes is not mediated by PPARα

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Abstract *Background* α-Amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD) is a key enzyme in NAD biosynthesis from tryptophan. Dietary polyunsaturated fatty acids (PUFA) have been shown to suppress hepatic ACMSD activity and its mRNA level in rat. However the mechanism of the suppressive action has not been clarified yet. Although the phenomena that fatty acids suppress the expression of ACMSD in rat liver have been established in vivo experiment, it is still obscure whether the effect of fatty acids on the expression of the enzyme is caused by its direct or indirect action, because there have been very few investigations performed in vitro. *Aim of the study* In this study, to examine whether down-regulation of ACMSD mRNA by PUFA involves peroxisome proliferator-activated receptor (PPAR) α mediated mechanism or not, we investigated the effect of PUFA on the ACMSD expression by using primary cultured rat hepatocytes. *Methods* For this purpose we investigated the effect of PUFA (linoleic acid and eicosapentanoic acid) on the ACMSD mRNA level in primary-cultured rat hepatocytes and compared its effect with that of WY-14,643 (a PPARα agonist). After the incubation of hepatocytes with fatty acids, WY-14,643 and/or MK886 (a PPARα antagonist), mRNA levels of ACMSD and a peroxisome marker enzyme acyl-CoA oxidase (ACO) were determined by competitive reverse transcription-polymerase chain reaction (RT-PCR) method. *Results* ACMSD mRNA level in primary hepatocytes were decreased by the incubation with high concentrations of linoleic acid, eicosapentaenoic acid (EPA) and WY-14,643. The appearance of ACO mRNA by WY-14,643 was remarkably increased, and those by linoleic acid and EPA were increased less than that by WY-14,643. Moreover, the suppression of ACMSD mRNA and the augmentation of ACO mRNA by WY-14,643 were inhibited by MK886, but the suppression by PUFA was not substantially affected by MK886. *Conclusions* The present study suggesting that the mechanism of decrease in ACMSD mRNA level by PUFA was different from that by WY-14,643, and that there would be any pathway other than PPARα mediated one for PUFA to regulate ACMSD expression.

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Key words α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase – polyunsaturated fatty acid – peroxisome proliferator-activated receptor α – primary cultured rat hepatocytes

Introduction

In the main catabolic pathway of L-Tryptophan, α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD) [EC4.1.1.45] catalyzes the decarboxylation of α-amino-β-carboxymuconate-ε-semialdehyde (ACMS) to α-aminomuconate-ε-semialdehyde (AMS) (Fig. 1), which enters into TCA cycle via glutalate pathway, leading to complete oxidation of tryptophan, or changes non-enzymatically to picolinic acid. In the absence of ACMSD, ACMS molecule can close non-enzymatically to make pyridine ring and is converted to quinolinic acid, which is metabolized further to NAD. Therefore, ACMSD plays a key role in NAD biosynthesis from L-tryptophan. It has been reported that human and murine ACMSD is present in kidney,

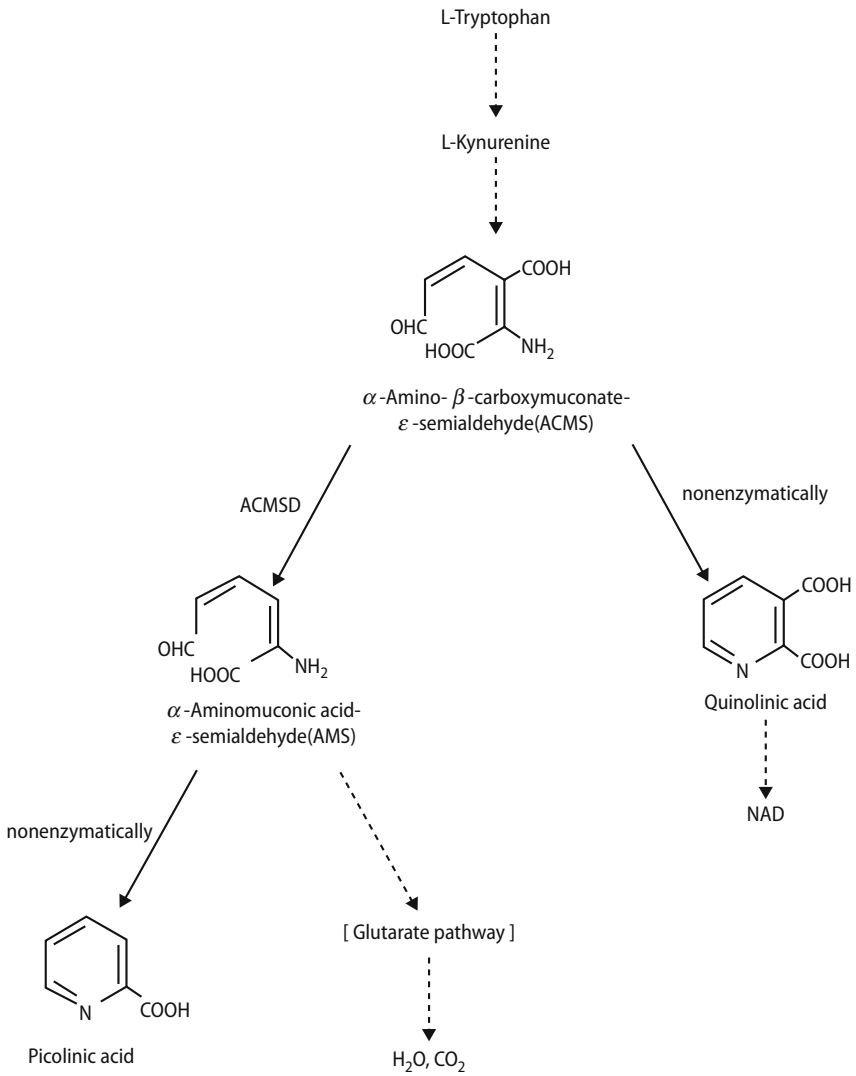

Fig. 1 Schematic diagram of tryptophan catabolism. *ACMSD* α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase

liver and brain, whereas rat ACMSD activity in normal rat has been detected only in kidney and liver at the moment. Since ACMSD activity is extremely lower in liver and brain than kidney and tryptophan-2,3-dioxygenase activity exists only in liver of animals [12], it is considered that NAD biosynthesis from tryptophan occurs exclusively in liver and that this biosynthetic flow is affected by hepatic ACMSD activity. It has been reported that hepatic ACMSD activity is influenced by some nutrients such as fats and amino acids [7, 21], in addition to hormones [26], diseases [5, 6, 9, 26] and some drugs [10, 24]. As for the nutrient-induced alteration of hepatic ACMSD activity, several studies have been conducted in vivo on down-regulation of hepatic ACMSD activity by dietary fats in rats, and its suppressive intensity was shown to be correlated with number of double bonds in the fatty acids, thereby the suppressive effect is stronger by linoleic acid than by oleic acid [7, 8].

In recent years, dietary fats have been interested in not only as sources of energy and membrane structural components but also as biological functional factors. Indeed, a number of studies have been conducted on the effects of fatty acids as a regulator of gene expression and indicate that the regulation of gene expression by fatty acids is multiple mechanisms including activation of peroxisome proliferator-activated receptors (PPARα, β, γ1 and γ2), liver X receptors (LXRs) and hepatic nuclear factor-4 (HNF-4)α and others [4, 18, 20]. Hepatic ACMSD activity and its mRNA expression in rat liver have been reported to be decreased not only by fatty acids [7] but also by peroxisome proliferators (PPs) [24]. Since this effect of PPs has been considered to be induced through a PPARα-dependent mechanism [16] and fatty acids are known as ligands of PPARα too, we can hypothesize that down-regulation of ACMSD activity and its mRNA expression by polyunsaturated fatty acid (PUFA) would be also caused by the PPARα involving mechanism. Although the phenomena that fatty acids suppress the expression of ACMSD in rat liver have been established in vivo experiment, it is still obscure whether the effect of fatty acids on the expression of the enzyme is caused by its direct or indirect action, because there have been very few investigations performed in vitro. In this study, we focused on eicosapentaenoic acid (EPA) and linoleic acid which were shown to down-regulate ACMSD in vivo experiments, and investigated the effect of PUFA on the ACMSD expression by using primary-cultured rat hepatocytes to examine whether down-regulation of ACMSD mRNA by PUFA would involve PPARα-mediated mechanism or not. Since acyl-CoA oxidase (ACO) has been shown to be induced by PPARα in hepatocytes [16], ACO was used as a metabolic marker of PPARα action in this study.

Materials and methods

Animals

Sprague-Dawley rats (5-week-old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). The rats were housed in an air-conditioned room at 22 ± 1°C with 12 h light and dark cycles. They were fed on a fat-free high protein diet for 1 week [21]. The composition of the diet was as follows (% in diet): casein 40, cornstarch 40.25, sucrose 10, vitamin mixture (AIN-93) 1, mineral mixture (AIN-93) 3.5, choline bitartrate 0.25, cellulose 5. The care and treatment of the rats were in accordance with the guidelines prescribed by the Faculty of Horticulture, Chiba University, and National Institutes of Health Guide for the Care and Use of Laboratory Animals [17].

Primary culture of rat hepatocytes

The hepatocytes were obtained from the rats fed with fat-free high protein diet for 1 week by collagenase perfusion method [27]. The cells were plated in 60 mm Primaria dishes (Falcon) with alpha-MEM (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 15 mM HEPES, 100 units/ml penicillin, and 100 units/ml streptomycin, 10 mg/l amphotericin B, 100 nM insulin, 1 nM glucagon, and 10 μM dexamethasone. After the first 4 h of cell attachment periods, the medium was replaced by the medium of which composition was the same as that described above except FBS was free and the concentration of insulin was 10 nM. After 16 h of preincubation, hepatocytes were then incubated for 48 h in the same medium with or without fatty acids, WY-14,643 (a PPARα agonist) and MK886 (a PPARα antagonist). The fatty acids used in the present study were linoleic acid ($C_{18:2}$, $n-3$) and EPA ($C_{20:5}$, $n-3$). These fatty acids were dissolved in acetone and then were bond to small amount of fatty acid-free albumin (Sigma) with antioxidants (α-tocopherol and BHT) before its addition to the medium as described by Armstrong and Towle [1]. The final concentrations of acetone in the media were 0.3% (v/v). The media in the control dishes were added with identical concentrations of fatty acid-free albumin, acetone and the antioxidants.

Isolation of total RNA from primary cultured rat hepatocytes

Total RNA was isolated using SV total RNA Isolation System (Promega, Madison, WI, USA). The quality of RNA preparations was evaluated by measuring optical

density at absorbance of 260/280 nm and by agarose-gel electrophoretic analysis, and its concentration was calculated based on the value of absorbance at 260 nm. The obtained RNA samples were stored at −80°C until their analysis by PCR methods described below.

Synthesis of competitors for competitive RT-PCR

Competitive reverse transcription-polymerase chain reaction (RT-PCR) was performed as described by A. Tanabe et al. [26]. As a positive marker of PPARα activation, ACO mRNA level was measured together with ACMSD mRNA. ACMSD, β-actin and ACO competitors were synthesized by PCR using pGEM-T 4Z plasmid DNA (Promega Corp.) as the template. The PCR mixture contained 2.5 unit/100 μl TaKaRa Ex Taq (Takara Shuzo, Kyoto, Japan), 0.2 μM corresponding sense and antisense primers, 2 mM Mg^{2+}, 0.2 mM deoxyribonucreotides and template DNA. The following primers were used: ACMSD competitor: forward-5′-CTACCAAAGGAATGGCCTGATGTTTTCCCAGTCACGAC-3′; reverse-5′-TGGTCTCCGATGGCATTCCTACAGGAAACAGCTATGAC-3′, β-actin competitor: forward-5′-GTGGGCCGCCCTAGGCACCAGGTTTTCCCAGTCACGAC-3′; reverse-5′-CTCTTTAATGTCACGCACGATTTCCAGG AAACAGCTATGAC-3′, ACO competitor: forward-5′-ATTCGGTG TTGTAAGTGCGTTTTCCCAGTCACGAC-3′; reverse-5′-TTGGTGG GTGGG TGTTGACAGGAAACAGCTATGAC-3′. The sequences underlined were the complementary sequences to pGEM-T 4Z plasmid DNA. The PCR was performed using a iCycler (BioRad) as follows: ACMSD: one cycle of 96°C, 3 min, 40 cycles of 96°C, 45 s; 58°C, 30 s; 72°C, 20 s, one cycle of 72°C, 1 min, β-actin: one cycle of 94°C, 2 min, 35 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 45 s, one cycle of 72°C, 1 min, ACO: one cycle of 94°C, 3 min, 28 cycles of 94°C, 15 s; 54°C, 30 s; 72°C, 1 min, one cycle of 72°C, 1 min. These PCR products were separated and purified by agarose gel electrophoresis using QIAquick gel Extraction Kit (QIAGEN).

Competitive RT-PCR

In the RT-PCR methods, 1 μg of total RNA was reverse transcripted by using first-strand cDNA Synthesis Kit for RT-PCR (AMV; Roch Diagnostics Corp., IN, USA). The PCR reaction mixtures were added to make the final volume of 50 μl containing 1 μl of RT product solution, 1 μl of various concentrations of competitor cDNA, 0.2 mM deoxiribonucleotide mix, 0.2 μM each primers and 0.25 units Taq DNA polymerase. The primers used for ACMSD were: forward-5′-CTACCAAAGGAATGGCCTGAT-3′; reverse-5′-TG GTCTCCGATGGCATTCCTA-3′, β-actin: forward-5′-GTGGGCCGCCCTAGGCACCAG-3′; reverse-5′-CTCT TTAATGTCACGCACGATTTC-3′, ACO [25]: forward-5′-ATTCGGTGTTGTAAGTGC-3′; reverse-5′-TTGGT GGGTGGGTGTTGA-3′. The competitive-PCR products were electrophoresed on 1% (w/v) agarose gel, and then analyzed by densitometry using Scion Image (Scion Corp.). These results were normalized by respective β-actin mRNA levels, because in our preliminary experiments we confirmed that fatty acids and other reagents used in the present experiments had no appreciable effect on the level of β-actin mRNA.

Statistical analysis

The values of mRNA levels normalized by respective β-actin mRNA expression were expressed as mean ± SE. Statistical analysis was performed by two-way ANOVA and Scheffe test. In the experiments which used agonist and antagonist, the differences within a group were analyzed by Student's t test. Difference among treatments with $P < 0.05$ were considered significant.

Results

Dose dependence of EPA on ACMSD mRNA level

In order to examine the effect of PUFA concentration on ACMSD mRNA level in primary-cultured rat hepatocytes, we used EPA as a representative of PUFAs in final concentration of 3–300 μM. In the results of competitive-RT-PCR, ACMSD mRNA level was markedly low in the hepatocytes treated with 300 μM EPA (approximately 40% of control). It is also shown that EPA treatment in primary culture of rat hepatocytes decreased mRNA level of ACMSD (Fig. 2). It was found from the result that EPA acted on liver parenchymal cells directly, hence decreasing ACMSD mRNA. Although ACO mRNA level was not examined in this experiments, it has been reported that PUFAs regulate ACO mRNA level in dose-dependent manner [2].

Effect of WY-14,643 on ACMSD mRNA level with or without PPARα antagonist

Since PPs and are known to regulate various gene expressions including ACO, we measured the increase of ACO mRNA level as a positive response to WY-14,643 to compare with its effect on ACMSD mRNA level. Figure 3 shows effects of WY-14,643 on ACMSD and ACO mRNA level in hepatocytes with or without

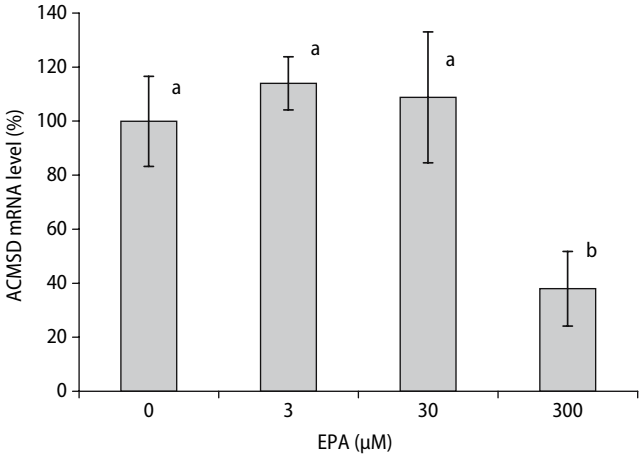

Fig. 2 Effects of EPA concentrations on ACMSD mRNA level in rat hepatocytes. Hepatocytes were incubated for 48 h in αMEM including 3–0.3 μM EPA. Total RNA is isolated from hepatocytes and analyzed for the expression of ACMSD mRNA by competitive-RT-PCR. Values are mean ± SD ($n = 4$). Values with *different letters* are significantly different ($P < 0.05$)

MK886 one of the PPARα antagonist. As a result, WY-14,643 caused a large increase in ACO mRNA levels when antagonist was not present in the medium, but that increase was not observed in the cells treated with WY-14,643 together with MK886 (Fig. 3B). Similarly, the ACMSD mRNA down-regulation by WY-14,643 was moderated by adding MK886 (Fig. 3 A). These results clearly suggest that the effect of WY-14,643 on ACMSD mRNA level was mediated by PPARα.

Effect of PUFA on ACMSD mRNA level with/without PPARα antagonist

The effects of linoleic acid and EPA in the presence or absence of MK886 on ACMSD and ACO mRNA levels in hepatocytes are shown in Fig. 4. Cells treated with 300 μM linoleic acid or EPA resulted in significant augmentation of ACO mRNA levels. Furthermore, addition of MK886 moderated these effects (Fig. 4B). On the contrary, treatment with linoleic acid or EPA resulted in the reduction of ACMSD mRNA levels when PPARα antagonist was absent, and addition of MK886 to the media did not influence the suppressive effects of these fatty acids (Fig. 4A). These results indicate that the mechanism of the suppression of the ACMSD mRNA level by PUFA was different from that by PPs such as WY-14,643.

Discussion

ACMSD plays a key role in tryptophan metabolism in mammals. The enzyme catalyzes the decarboxylation

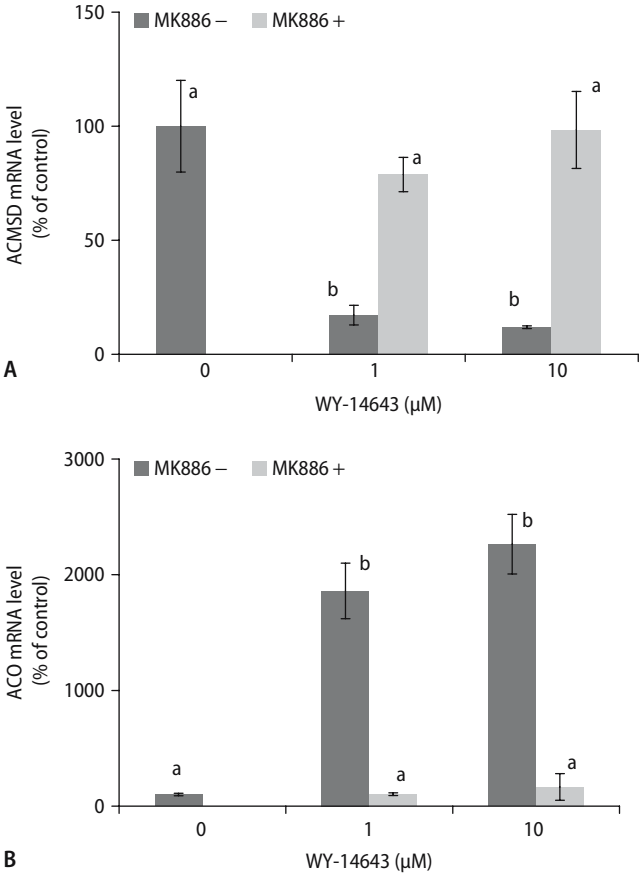

Fig. 3 Effects of WY-14,643 on ACMSD and ACO mRNA levels with or without PPARα antagonist. Hepatocytes were incubated for 48 h in αMEM including 1–10 μM WY-14,643 with or without 10 μM MK886. Total RNA was isolated from hepatocytes and analyzed for the expression of ACMSD (**A**) and ACO (**B**) mRNA by competitive-RT-PCR as described in the text. Values are mean ± SD ($n = 3$). Values without a *common letter* are significantly different ($P < 0.05$)

of ACMS to AMS, which enters into TCA cycle via glutarate pathway or changes non-enzymatically to picolinic acid. In the absence of ACMSD, however, ACMS can be non-enzymatically converted into quinolinic acid, which is metabolized further to NAD. Quionolinic acid acts as agonist of *N*-methyl-D-aspartate (NMDA)-sensitive glutamate receptors and causes the induction of neurotoxicity [19, 22] It is considered that quinolinate associated with the pathogenesis of neurodegenerative diseases and convulsive disorders, e.g. Huntington's desease [3, 11]. Furthermore, change of ACMSD activity is correlated with hepatic NAD levels [24]. Therefore, alteration in ACMSD activity is important in the regulation of the concentration of these compounds in vivo. Several studies have been conducted on the effects of various factors including nutrients, hormones, diseases and drugs on the rat liver ACMSD activity and its mRNA level [5–7, 9, 10, 21, 24, 26]. From these studies, it is shown that feeding a high PUFA diet or administra-

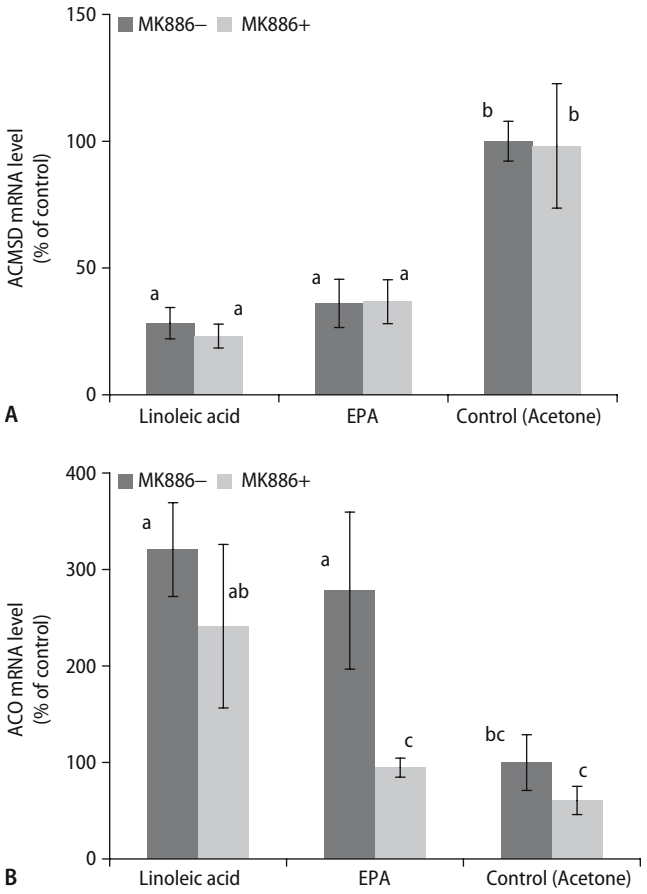

Fig. 4 Effects of PUFA on ACMSD and ACO mRNA level with or without PPARα antagonist. Hepatocytes were incubated for 48 h in αMEM including 0.3 mM Linoleic acid or EPA with or without 10 μM MK886. Total RNA was isolated from hepatocytes and analyzed for the expression of ACMSD (**A**) and ACO (**B**) mRNA by competitive-RT-PCR as described in the text. Values are mean ± SD ($n = 4$). Values without a *common letter* are significantly different ($P < 0.05$)

tion of clofibrate, one of the PPs, causes to decrease the liver ACMSD activity and mRNA level in rat. Egashira et al. [7] reported that ACMSD activity decreased gradually after feeding a high PUFA diet, on the contrary ACMSD mRNA level decreased quickly. Hence, it is quite likely that PUFA regulate ACMSD gene transcription. So far, however, investigations have been made on the effect of ACMSD down-regulation by PUFA, very few attempts have been performed in in vitro. Therefore, the detailed mechanisms of the phenomena have not been elucidated yet. In this study, the fact that the effect is reproducible in primary-cultured rat hepatocytes offers evidence that PUFA directly regulate the expression of the ACMSD genes.

Recently, many reviews covering the regulation of various kinds of gene expression by fatty acids have been described with regard to several estimated mechanisms [14]. Fatty acids have been found to regulate the activity and/or the abundance of several transcription factors, including PPAR, LXR, HNF4, NFκB and SREBP. Highly unsaturated $n-3$ fatty acids and PPs, such as fibrates, up-regulate the expression of genes involved in fatty acid oxidation, including ACO and CYP4A2, by activation of PPARα. On the other hand, $n-3$ and $n-6$ PUFA down-regulate the transcription of hepatic fatty acid synthase (FAS), the S14 protein, and L-pyruvate kinase (L-PK). These effects are shown to be caused by the alteration of the nuclear content of transcription factor SREBP-1c, without direct binding of the fatty acids [13]. Only recently, it has been reported that PPs including fibrates, phtarate esters and WY-14,643 down-regulated expression of hepatic ACMSD protein and mRNA, and furthermore it was also demonstrated that the constitutive expression of ACMSD mRNA is positively controlled by HNF4α and down-regulated by the activation of PPARα by WY-14,643, using hepatic PPARα-null mice and HNF4α-null mice [23, 24].

The experimental results obtained from competitive RT-PCR methods show that suppression of ACMSD mRNA level and augmentation of ACO mRNA level in primary hepatocytes occurred by the incubation with high concentrations (0.3 mM) of FAs or 1–10 μM WY-14,643. Moreover, down-regulation of ACMSD mRNA and up-regulation of ACO mRNA by WY-14,643 were inhibited by addition of MK886. MK886 is an inhibitor of PPARα, and prevent activation of PPARα by WY-14,643 [15]. Therefore it is clear that the WY-14,643-induced down-regulation of ACMSD mRNA is caused by PPARα activation. These observations are consistent with previous findings [23]. On the other hand, the suppression of ACMSD mRNA by PUFAs was not substantially affected by the addition of MK886, although the augmentation of ACO mRNA by PUFAs was inhibited by addition of MK886 as observed in the case using WY-14,643 as one of PPs. These results suggest that the mechanism of the down-regulation of ACMSD mRNA by PUFAs was different from that of PPs. Since, MK886 is also a potent inhibitor of 5-lipoxygenase (LOX) activating protein (FLAP) and suppresses the leukotriene biosynthesis [15], the fact that the addition of MK886 did not affect the down-regulation of ACMSD mRNA caused by PUFAs indicate that lipoxin family derived from PUFAs should not be responsible for this regulation. However, the involvement of prostanoids derived from PUFAs in this regulation cannot be ruled out at present.

In conclusion, down-regulation of ACMSD mRNA caused by PUFAs is suggested not to be mediated by PPARα-dependent pathway, although that by PPs are PPARα dependent.

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