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Docosahexaenoic acid inhibits proteolytic processing of sterol regulatory element-binding protein-1c (SREBP-1c) via activation of AMP-activated kinase

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ABSTRACT

In hyperinsulinemic states including obesity and T2DM, overproduction of fatty acid and triglyceride contributes to steatosis of the liver, hyperlipidemia and hepatic insulin resistance. This effect is mediated in part by the transcriptional regulator sterol responsive element binding protein-1c (SREBP-1c), which stimulates the expression of genes involved in hepatic fatty acid and triglyceride synthesis. SREBP-1c is up regulated by insulin both via increased transcription of nascent full-length SREBP-1c and by enhanced proteolytic processing of the endoplasmic reticulum (ER)-bound precursor to yield the transcriptionally active n-terminal form, nSREBP-1c. Polyunsaturated fatty acids of marine origin (n-3 PUFA) prevent induction of SREBP-1c by insulin thereby reducing plasma and hepatic triglycerides. Despite widespread use of n-3 PUFA supplements to reduce triglycerides in clinical practice, the exact mechanisms underlying their hypotriglyceridemic effect remain elusive. Here we demonstrate that the n-3 PUFA docosahexaenoic acid (DHA; 22:5 n-3) reduces nSREBP-1c by inhibiting regulated intramembrane proteolysis (RIP) of the nascent SREBP-1c. We further show that this effect of DHA is mediated both via activation of AMP-activated protein kinase (AMPK) and by inhibition of mechanistic target of rapamycin complex 1 (mTORC1). The inhibitory effect of AMPK on SREBP-1c processing is linked to phosphorylation of serine 365 of SREBP-1c in the rat. We have defined a novel regulatory mechanism by which n-3 PUFA inhibit induction of SREBP-1c by insulin. These findings identify AMPK as an important negative regulator of hepatic lipid synthesis and as a potential therapeutic target for hyperlipidemia in obesity and T2DM.

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1. Introduction

Sterol response element binding proteins (SREBPs) are helix-loop-helix transcription factors that regulate the expression of genes involved in cholesterol and fatty acid synthesis. The SREBP-1 gene (*Srebf1*) generates two transcripts, SREBP-1c and SREBP-1a that arise from alternate first exons. In the liver, SREBP-1c is the primary regulator of lipogenesis along with the carbohydrate response element binding factor, ChREBP [1]. SREBP-1c is induced at the transcriptional level by insulin [2] and in response to high fat/high carbohydrate diets [3]. The insulin effect is mediated through a number of factors bound to the *Srebf1* promoter including the liver X receptor (LXRα), Sp1 and SREBP-1c itself [4–6]. The abundance of SREBP-1c is regulated both by transcription of nascent full-length SREBP-1c and by the ER to Golgi transport of the precursor form and subsequent proteolytic processing to yield the transcriptionally active nuclear fragment [7]. SREBP-1c stimulates expression of many genes involved in lipogenesis including fatty acid synthase (*Fasn*) and acetyl-CoA carboxylase (*Acaca*) [8]. Elevated SREBP-1c contributes to the hepatic steatosis and hyperlipidemia associated with obesity and insulin resistance [9,10]. This effect is reversed by polyunsaturated fatty acid (n-3 PUFA) feeding [11–13]. Given the central role of SREBP-1c in the pathogenesis of the hyperlipidemia and hepatic steatosis that accompanies obesity and T2DM [14], understanding the mechanisms by which n-3 PUFA inhibit SREBP-1c is of great importance.

Abbreviations: SREBP, Sterol regulatory element binding protein; AMPK, AMPK-activated kinase; DHA, Docosahexaenoic Acid; PUFA, Polyunsaturated fatty acids; Akt, Akt/Protein Kinase B; mTORC1, Mammalian target of rapamycin complex 1; p70S6K, p70S6Kinase; T2DM, Type II Diabetes Mellitus; McA, McArdle H7777 rat hepatoma cell line; n-3, Omega three fatty acids.

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Fish oil concentrates containing the n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) effectively reduce plasma triglycerides [15,16]. n-3 PUFA exert their hypotriglyceridemic effect largely via inhibition of SREBP-1c leading to reduced hepatic fatty acid and triglyceride synthesis [11,12]. Initial studies focused on inhibition of transcription of nascent precursor SREBP-1c and accelerated transcript decay as mechanisms underlying inhibition of SREBP-1c by n-3 PUFA [17–19]. Others attributed the suppression of hepatic lipid synthesis by PUFA to reduced levels of the mature cleaved form of SREBP-1c [12,20]. Based upon n-3 PUFA feeding studies using an in vivo reporter system for SREBP-1c, Takeuchi et al. proposed that the predominant mechanism by which n-3 PUFA (EPA) reduced levels of mature nSREBP-1c was via inhibition of proteolytic processing and reduced feed-forward activation of the *Srebf1* gene [21]. The mechanisms underlying this effect of n-3 PUFA remain undefined.

N-3 PUFA may inhibit proteolytic activation of SREBP-1c via attenuation of insulin signaling. Insulin promotes the ER to Golgi transport and proteolytic processing of SREBP-1c via an Akt dependent mechanism [22]. mTORC1, which is activated by Akt, and p70S6 kinase (p70S6K) the downstream target of mTORC1 have both been implicated in mediating enhanced proteolytic processing of SREBP-1c in response to insulin [23,24]. Given that n-3 PUFA specifically antagonizes the ability of insulin to induce SREBP-1c [4,25], the kinase pathways that oppose insulin effects are prime candidates for mediating the inhibitory effect of n-3 PUFA. Of these, AMP-activated kinase (AMPK) is a likely candidate kinase for promoting the inhibitory actions of n-3 PUFA. AMPK is activated by nutrient deprivation and is a physiologic antagonist to the mTORC1 pathway [26–28]. Significantly, PUFA have been reported to activate AMPK in vivo [29] and in vitro in adipocytes [30] and endothelial cells [31]. AMPK activators including metformin and polyphenols, on the other hand, have been shown to suppress SREBP-1c cleavage and nuclear translocation potentially via phosphorylation of serine 372 on the human SREBP-1c [32].

We undertook an investigation of the mechanisms underlying inhibition of SREBP-1c processing by the n-3 PUFA, docosahexaenoic acid. We established a mechanistic link between activation of AMPK by DHA and phosphorylation of SREBP-1c leading to the inhibition of its proteolytic processing. We also identified an inhibitory loop between AMPK and mTORC1 as a complementary mechanism by which DHA inhibits proteolytic activation of SREBP-1c. Our findings implicate activation of AMPK as a mechanism by which n-3 PUFA exert their inhibitory effects on SREBP-1c.

2. Materials and methods

2.1. Cell cultures and reagents

McArdle 7777 (McA) rat hepatoma cells were purchased from ATCC (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified chamber supplied with 5% CO_2. N-(beta-D-Ribofuranosyl)-5-aminoimidazole-4carboxamide (AICAR), Compound C, and MG132 were obtained from R & D Systems (Minneapolis, MN). DAPI, protease inhibitor, and phosphatase inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN). The transfection reagent GenJet Plus was purchased from SignaGen Laboratories (Gaithersburg, MD). Antibodies to SREBP-1 were purchased from BD Biosciences (cat. 557,036, San Jose, CA). The following antibodies were purchased from Sigma-Aldrich (St. Louis, MO): β-Actin (Cat. A1978), mTOR (Cat. T2949), p70S6K (Cat. SAB4502691), Phospho-p70S6K-T389 (Cat. SAB4503957) and Phospho-FoxO1 (Cat. SAB4300094). All other antibodies were purchased from Cell Signaling Technology (Danvers, MA) including HA-Tag (Cat. 3724), Akt (Cat. 4685), P-Akt T308 (Cat. 4056), AMPK-alpha (Cat. 5832), P-AMPKalpha-T172 (Cat. 2535), P-mTOR-Ser2448 (Cat. 5536), Acetyl-CoA Carboxylase (Cat. 3676), P-Acetyl-CoA Carboxylase (Cat. 3661), FoxO1 (Cat. 2880), FAS (Cat. 3180), GFP (Cat. 2555) and Lamin A/C (Cat. 4777).

The plasmid for GFP-SREBP-1c fusion protein expression was constructed by inserting the cDNA sequence for the full-length rat SREBP-1c into the pEGFP-C2 vector (Clontech Laboratories, CA). Plasmids encoding the amino-terminal HA-tagged wild-type SREBP-1c, or the serine-to-alanine mutation at Ser365 (KSK**S**LKDLV) SREBP-1c, or the serine-to-aspartic acid mutation SREBP-1c, were created by inserting the wild-type SREBP-1 cDNA sequence, or the S365A, S365D mutated sequences in to the pcDNA3.1 vector (Life Technologies, NY). The primers for creating the site-directed mutagenesis of rat SREBP-1c S365A and S365D were as follows.

SREBP1c-S365A-F: 5′-GCTCACAAAAGCAAAGCACTGAAAGACCTG GTG-3′,

SREBP1c-S365A-R: 5′-CACCAGGTCTTTCAGTGCTTTGCTTTTGTGAGC-3′,

SREBP1c-S365D-F: 5′-GCTCACAAAAGCAAAGACCTGAAAGACCTG GTG-3′,

SREBP1c-S365D-R: 5′-CACCAGGTCTTTCAGGTCTTTGCTTTTGTGAGC-3′.

Docosahexaenoic acid (DHA) was purchased from Cayman Chemicals (Ann Arbor, MI) and was conjugated with bovine serum albumin (BSA) to be used for the treatment of cell cultures. One hundred milligram of DHA was diluted in 1 ml of absolute ethanol. Then the dissolved DHA was diluted in 2 mM lipid-free BSA dissolved in DMEM medium by adding DHA drop-wise beneath the liquid surface of BSA-DMEM with stirring by a magnetic bar. The process was continued for 30 min under N_2 flow. Stock solutions of the BSA-DHA complexes were stored at −80 °C until use. A BSA vehicle media was prepared in the same manner as that for DHA except that only the ethanol solvent was added to the BSA-DMEM.

2.2. Quantitative real-time PCR (qRT-PCR)

RNA was prepared from McA cells using RNeasy kits (Qiagen, CA), and cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche, IN) and analyzed by real time PCR (RT-PCR). The amplification of target cDNA was detected by SYBR Green and analyzed by the ΔΔCt method. Cyclophilin D mRNA was used as a reference. The primers used for amplifying the genes studied are as follows: rat *Srebf1* forward primer 5′-catggattgcacatttgaagac-3′ and reverse primer 5′-gcaggagaagagaagctctcag-3′; rat fatty acid synthase (*Fasn*) forward primer 5′-ggccacctcagtcctgttat-3′ and reverse primer 5′-agggtccagctagagggtaca-3′.

2.3. Western blot analysis

Whole-cell lysates from McA cells were prepared as described previously [5,6]. To prepare the microsomal fractions and nuclear extracts, McA cells were incubated for 10 min in Buffer A composed of 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 250 mM sucrose. Then the cells were passed through 23 gauge needles 30 times followed by centrifugation at 1000 ×*g* for 10 min. The supernatant was further centrifuged at 16,000 ×*g* for 2 h to obtain the pellet, which contained the microsomal membranes. The pellet was incubated for 30 min in Buffer B containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Igepal CA-630, and 1% SDS. After the incubation, the lysed content was centrifuged at 15,000 ×*g* for 30 min. The supernatant was collected as microsomal fraction. To obtain the nuclear extracts, the pellet of the aforementioned 1000 ×*g* centrifugation underwent two cycles of wash and spin (1000 ×*g*, 10 min) in buffer A, followed by incubation for 30 min on an shaking platform with buffer C composed of 20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 10% glycerol. The nuclear extracts were collected from the supernatant after 20 min of centrifugation at 15,000 ×*g*.