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ORIGINAL ARTICLE

Arachidonic acid induces acetyl-CoA carboxylase 1 expression via activation of CREB1

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Abstract Acetyl-CoA carboxylase (ACC; EC 6.4.1.2) is the major enzyme of fatty acid synthesis and oxidation in response to dietary changes. In animals, there are two major isoforms of ACCs, ACC1 and ACC2, which are encoded by different genes and display distinct tissue and cellular distribution. We examined the effect of high concentration of arachidonic acid (AA) on the expression of ACC1 mRNA in HepG2 hepatoma cells cultured in the absence of insulin. After 12 h of treatment, AA was found to significantly up-regulate ACC1 mRNA level as well as that of cAMP regulatory element binding protein 1 (CREB1), implying the possible interactions between ACC1 and CREB1. In support of the hypothesis, several potential CREB1 binding sites were identified within the PII promoter of ACC1. Further experiments demonstrated that transient over-expression of CREB1 in HepG2 cells activates ACC1 PII promoter and induces the production of triacylglycerol in response to AA, indicating that the effect of AA on ACC1 is possibly regulated via CREB1.

Keywords Acetyl-CoA carboxylase 1 · Arachidonic acid · CREB1 · Promoter · HepG2

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Introduction

Increasing evidence has demonstrated that the right amount (10–100 μM) of polyunsaturated fatty acids (PUFAs) can reduce insulin resistance (IR) in vivo [1, 2] and ameliorate the illnesses such as cardiovascular diseases, diabetes, and cancer in humans. Regarded as a negative regulator of hepatic lipogenesis, PUFAs can suppress the expression and activity of adipogenic transcription factors peroxisome proliferators-activated receptor (PPAR), liver X receptor (LXR), carbohydrate response element-binding protein (ChREBP), and sterol regulatory element binding protein-1c (SREBP-1c) [3–6]. On the other hand, saturated fatty acids (SFAs) could cause IR and induce the expression of genes limiting fatty acid and glucose supply in muscle [7]. Actually, high-fat diet has been shown to be associated with abdominal fatness, which induces the increase of free fatty acids (FFAs) in blood and results in IR in vivo [8]. However, excess intake of PUFAs could bring about obesity, IR, and atherosclerosis [9].

As one of the genes controlled by ChREBP and SREBP-1c, ACC plays an important role in energy balance [10, 11]. ACC catalyzes the ATP-dependent carboxylation of acetyl-CoA to produce malonyl-CoA, a material of the fatty acid synthesis and a key regulator of the fatty acid oxidation. Two isoforms of ACC have been identified. ACC1 is mainly expressed in the cytoplasm of liver and adipose tissue, and is proposed to be involved in fatty acid synthesis, whereas ACC2 is predominantly expressed in skeletal muscle and heart, and is responsible for fatty acid β-oxidation [12, 13]. The distinct expression pattern, as well as the finding that malonyl-CoA produced by ACC1 is not involved in fatty acid β-oxidation, indicates the considerably different role of ACC1 and ACC2 in physiology.

Despite these differences, both ACC1 and ACC2 are responsive to FFAs level in vivo [14, 15]. Dietary intake of right amount of PUFAs may decrease the expression and activity of both ACC isoforms [16, 17].

In humans and ruminants, the transcription of ACC1 is initiated from three promoters, named as promoters I (PI), II (PII), and III (PIII) [18–20], whereas in rodents only two ACC1 promoters, PI and PII, have been identified [21]. Transcripts from PI and PII give rise to the same protein, while that from PIII produces an N-terminal variant of ACC1 expressed only in mammary gland during lactation [20, 22]. PI and PII promoters have different roles during the transcription. PI is the main promoter driving ACC1 transcription in liver and adipose tissue [19, 22], whereas PII is a housekeeping promoter and acting in all tissues [19, 22]. Besides, glucose and insulin can synergistically activate the transcription of ACC1 from promoter PI but not from PII [23, 24]. Fat-free diet, however, can activate the transcription from PII in liver [25].

It has proved that ACC1 is related closely to the IR control in liver [7]. In order to define the effect of high concentration PUFAs on ACC1 mRNA expression, we examined the variation of ACC1 mRNA expression in HepG2 cells cultured with arachidonic acid and oleic acid in the absence of insulin. Shortly after the treatment, arachidonic acid significantly up-regulated the mRNA level of ACC1, which is possibly mediated by the transcriptional activation of CREB1 on ACC1.

Results

AA up-regulates ACC1 expression in HepG2 cells

The effect of AA on ACC1 expression was investigated in HepG2 cells cultured in the absence of insulin. Shortly after the treatment (12 h), AA significantly increased the mRNA level of ACC1 in a dose-dependent manner at final concentrations ranging from 200 to 600 μM, with the peak level (fourfold versus control) at 300–500 μM. The expression of ACC1 was decreased when the final AA concentration is higher than 600 μM, probably due to the high concentration AA-induced cell toxicity [26] (Fig. 1a). In contrast, OA treatment had no significant effect on ACC1 expression at final concentrations ranging from 100 to 500 μM (Fig. 1a). At a final concentration of 300 μM, AA up-regulated the mRNA level of ACC1 during 3–12 h, the peak level (fivefold versus control) was observed at 3 h. After 24 h treatment, the expression of ACC1 started to decrease (Fig. 1b). No significant effect was observed using OA treatment (Fig. 1b).

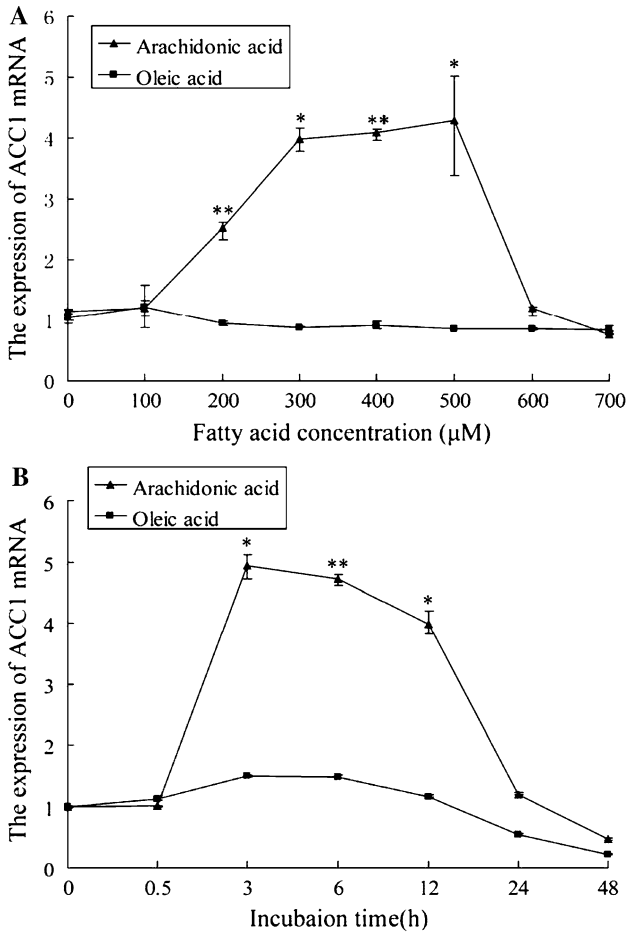

Fig. 1 ACC1 expression is upregulated by arachidonic acid in a concentration- and time-dependent manner. **a** HepG2 cells were incubated with OA or AA at indicated final concentrations for 12 h, and subjected to real-time RT-PCR analysis for ACC1 expression. The level of ACC1 mRNA was significantly increased in cells treated with AA at final concentrations higher than 200 μM. **b** HepG2 cells were incubated with 300 μM OA or AA for various time periods, and subjected to real-time RT-PCR analysis for ACC1 expression. ACC1 mRNA was upregulated in cells incubated with AA, and reached peak level 3 h after treatment. Data represent mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$

The regulation of AA on ACC1 transcription

Bioinformatic analysis revealed that ACC1 promoter contains potential binding sites for various transcript factors including ChREBP, SREBP-1c, PPARγ, LXR, and CREB1. In order to explore the transcriptional control of ACC1, we examined the expression patterns of these transcription factors using qRT-PCR in HepG2 cells treated with 300 μM OA or AA for 3 h. The results showed that the mRNA levels of ChREBP and SREBP-1 were markedly reduced by both OA and AA. OA, rather than AA, significantly reduced the expression of PPARγ. Compared to the control, AA induced a twofold increase in CREB1 mRNA level, whereas OA slightly reduced CREB1

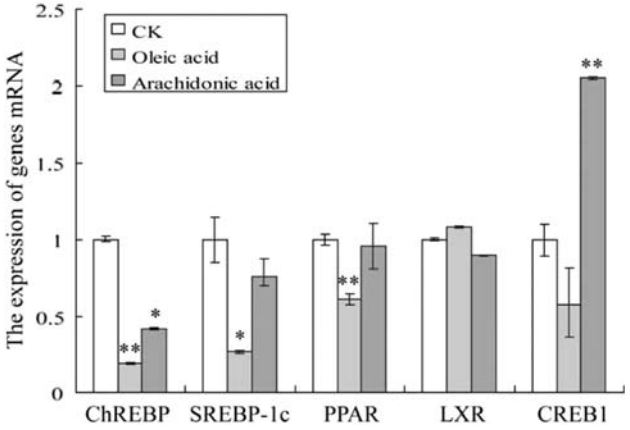

Fig. 2 Effect of arachidonic acid on expression of adipogenic transcription factors. HepG2 cells were incubated with 300 μM OA or AA for 12 h, and subjected to real-time RT-PCR analysis for ChREBP, SREBP-1c, PPARγ, LXR, and CREB1. Only CREB1 mRNA was upregulated in response to AA treatment. Data represent mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$

expression. No significant effect was observed for LXR mRNA expression (Fig. 2).

Transient overexpression of CREB1 increased the expression of ACC1

The previous results implied that CREB1 is a potential mediator of AA-regulated ACC1 expression. Real-time PCR was performed to determine the ACC1 mRNA expression in HepG2 cells transiently transfected with pcDNA3.1-CREB1. ACC1 was obviously activated 24 h after transfection whereas the expression of ChREBP and SREBP-1c remained unchanged. The expression level of ACC1 in the cells overexpressed CREB1 is over 140-fold higher than that in the control cells (transfected with empty vector) (Fig. 3).

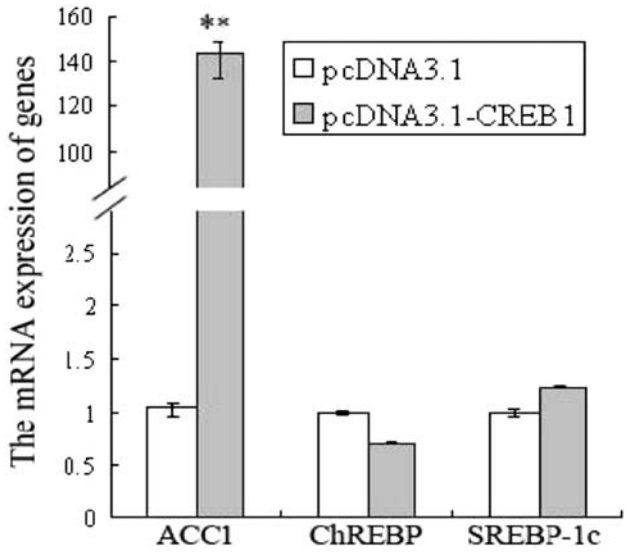

Fig. 3 ACC1 expression is upregulated by overexpression of CREB1. HepG2 cells were transfected with pcDNA3.1 (control) or pcDNA3.1-CREB1, and subjected to real-time RT-PCR for ACC1, ChREBP, and SREBP-1c. Overexpression of CREB1 induced ACC1 mRNA level, but has no effect on ChREBP and SREBP-1c. Data represent mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$

AA-regulated ACC1 expression, lipometabolism, and glycometabolism in liver

We tested the expression of the marker genes FAS, GLUT2, and G6Pase in HepG2 cells treated with OA or AA. FAS mRNA expression was highly activated by AA and increased by fourfold compared to control. AA treatment had no obvious effect on GLUT2 and G6Pase expression (Fig. 4a). To further clarify the role of AA-induced effect, we tested the glucose expenditure and TG

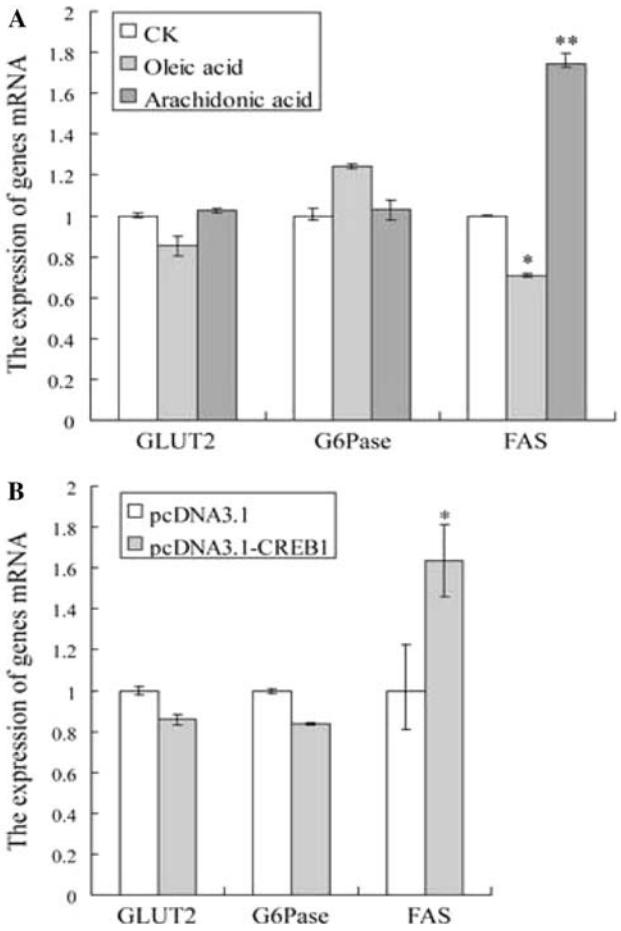

Fig. 4 Effect of arachidonic acid and CREB1 on expression of genes for glucose transport and fatty acids synthesis. HepG2 cells were incubated with 300 μM OA or AA for 12 h (**a**), or transfected with pcDNA3.1-CREB1 (**b**), and subjected to real-time RT-PCR analysis for FAS and GLUT4. The mRNA level of FAS was significantly increased in response to AA treatment (**a**). Overexpression of CREB1 also induced the transcription of FAS (**b**). Data represent mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$

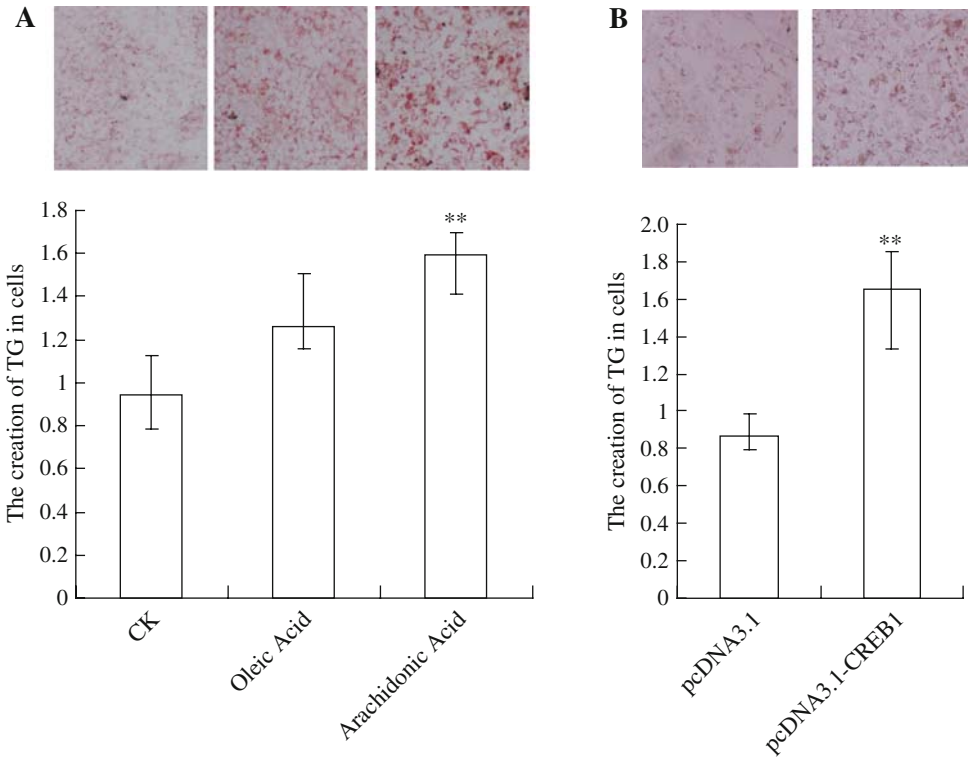

Fig. 5 Effect of arachidonic acid and CREB1 on TG production. HepG2 cells were treated with 300 μM AA (**a**) or transfected with pcDNA3.1-CREB1 (**b**) for 24 h. Cellular TG content was determined by oil red O staining. Both AA treatment and CREB1 overexpression significantly increased the production of TG. Data represent mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$

synthesis of the cells. The TG synthesis increased obviously after 24 h (Fig. 5a) of AA or OA treatment, while the glucose expenditure had no significant changes. The expression of FAS, GLUT2, and G6Pase, as well as the glucose expenditure and TG synthesis were also examined in cells transfected with pcDNA3.1-CREB1. CREB1 overexpression increased TG synthesis, as well as the mRNA level of FAS (1.5-fold vs. control), and had no significant effect on that of GLUT2 and G6Pase, suggesting that CREB1 may be a mediator of AA-induced regulation (Figs. 4b, 5b).

AA-induced ACC1 expression via CREB1

To further investigate the mechanism of AA-induced up-regulation of ACC1, HepG2 cells were transfected with ACC1 promoters and stimulated with AA or OA, and subjected to firefly luciferase activity assay. After transfection, pGL3-P II induced a significant increase in ACC1 expression in HepG2 cells cultured with AA, but not in cells cultured with OA (Fig. 6a). In the cells co-transfected with pcDNA3.1-CREB1, the activity of PII promoter was markedly increased in the absence of AA (Fig. 6b).

Discussion

ACC1 is generally considered as a rate-limiting enzyme in fatty acid synthesis. Consequently, the enzymatic activity of ACC1 is tightly controlled by a variety of mechanisms including phosphorylation, covalent modification, and allosteric control. In addition, high-carbohydrate diet and insulin can stimulate ACC1 production through the activation of ChREBP and SREBP-1c, the two transcription factors capable of binding to ACC1 promoter [27]. Right amount of PUFAs can restrain the effect of insulin on ACC1 expression [3–6], whereas high concentration of FFAs in blood induce IR [8, 28, 29] and increase the mRNA level of ACC1 [7], suggesting that FFAs at different concentrations may lead to various physiological conditions, as well as the involved genes, such as ACC1.

In order to check the effect of high concentration of PUFAs (>100 μM) on ACC1 expression in a short term, we did the experiments in HepG2 cells cultured in the absence of insulin. Our results showed that AA promotes a marked increase in ACC1 expression independent of serum constituents, implying AA might be a physiological regulator of ACC1 expression in hepatocytes. In contrast to the previous experimental results on long-term effects of AA on ACC1 expression, the mRNA level of ChREBP and SREBP-1c were not up-regulated by AA during 12 h treatment. Further experiments also confirmed that the short-term effect of AA on ACC1 expression is not mediated via ChREBP and SREBP-1c.

AA is one of the essential fatty acids which distributes in neutral adipose of mammals. As a precursor of many useful substances, such as prostaglandin (PG) [30], AA and its derivatives have various biological functions. For example,

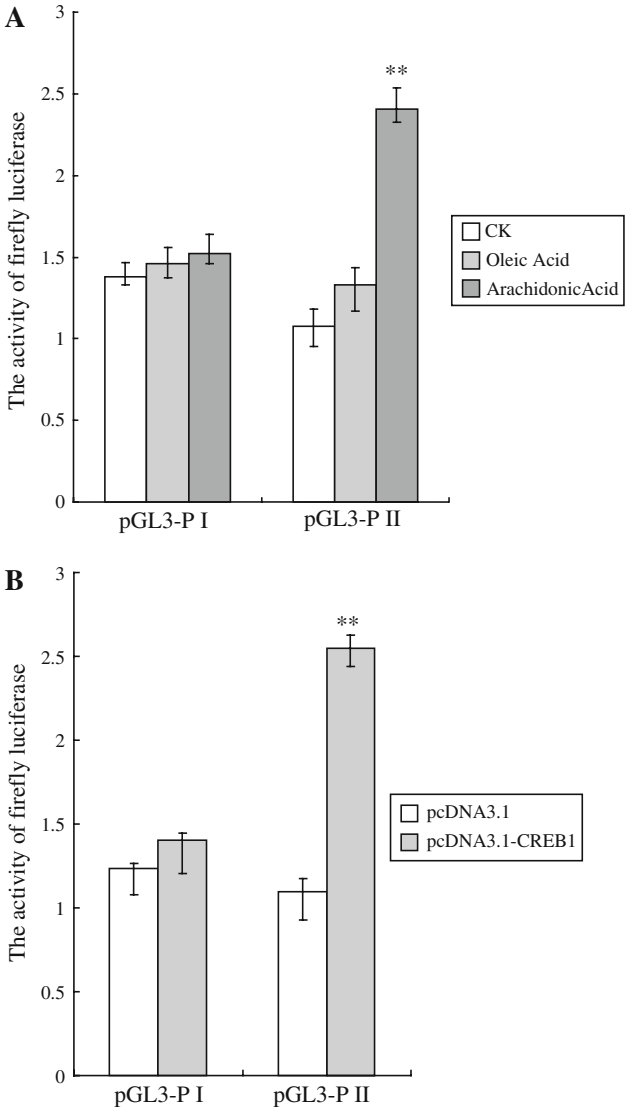

Fig. 6 Effect of arachidonic acid and CREB1 on ACC1 promoter activity. HepG2 cells were transfected with pGL3-PI or pGL3-PII for 6 h, followed by treatment with 300 μM AA (**a**) or transfection with pcDNA3.1-CREB1 (**b**) for 24 h, and subjected to luciferase activity assay. Compared with controls, the activity of PII was increased over twofold within 24 h after AA treatment (**a**) or transfection with pcDNA3.1-CREB1 (**b**). Data represent mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$

PG (E1, E2, I2, D2) can activate adenyl cyclase and increase cAMP in cytoplasm [31]. Bioinformatic analysis predicted several potential transcript factors binding sites, as well as ChoRE and SRE within ACC1 promoter [32]. Among these factors, our results identified CREB1 as a possible mediator of AA-induced ACC1 expression.

As ACC1 is associated with energy metabolism in liver, we examined the relationships between the AA-induced ACC1 expression and lipometabolism or glycometabolism in HepG2 cells. The results showed that right amount of AA can promote fatty acid synthesis but not glycometabolism. The effect of high concentration of AA on lipometabolism and glycometabolism is similar to that of the high-fat diet [33].

In rodents, the transcription of ACC1 gene is under control of two promoters (PI and PII), resulting in transcripts with or without the exons near 5′UTR. Transcripts from the PII promoter are expressed in all tissues and responded to fat-free diet in liver. In contrast, PI-generated transcripts are expressed predominantly in liver and adipose, two major tissues of lipometabolism and glycometabolism in mammals. Contradicting studies in rodents, we have found AA treatment dramatically activates PII promoter in HepG2 cells, with no significant effect on PI promoter activity. Such observation is further confirmed by the co-transfection experiments that demonstrate CREB1 may bind to PII, but not to PI promoter, and activate ACC1 transcription in response to AA. Furthermore, transient overexpression of CREB1 did not influence the expression of ChREBP and SREBP-1c, indicating that the regulation of CREB1 on ACC1 is independent of ChREBP and SREBP-1c.

In conclusion, we observed that high concentration of AA increases ACC1 mRNA level in HepG2 cells in a short-term incubation via CREB1. Since AA is the precursor of PG, which is involved in the regulation of lipometabolism and glycometabolism, we proposed that the effect of AA on ACC1 expression might be related to the PG pathway.

Materials and methods

Cell culture

HepG2 hepatoma cells were cultured in DMEM supplemented with 20% FBS (Sanli) at 37°C in 5% CO_2 and seeded in 24-well plates after trypsinization. Fatty acids (Sigma), including oleic acid (C18:1) and arachidonic acid (C20:4) were dissolved in ethanol as 3 mM stock solutions and stored at −20°C [34]. For treatment, cells were washed and pre-incubated in serum-free DMEM for 6 h, and further cultured with a serial dilution of each fatty acid for 12 h.

RNA preparation and real-time RT-PCR

Total RNA was prepared using the TRIzol method and reverse transcribed by M-MLV reverse transcriptase (Promega). For real-time PCR, primer pairs specific for ACC1, ChREBP, SREBP-1c, CREB1, PPARγ, LXR, FAS, GLUT2, GLUT4, and G6Pase (Table 1) were designed with Primer premier 5. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in parallel as an internal control. Real-time PCR was performed using the

Table 1 Primers used for real-time RT-PCR

Gene	FP sequence	RP sequence
ACC1	5′-TGGTAATGCGGTATGGAAGTCG-3′	5′-TGTATGTTGTCCCTAAGGATTGTGC-3′
SREBP-1c	5′-CACCGTTTCTTCGTGGATGG-3′	5′-GTCACACAGTTCAGTGCTCGCTC-3′
ChREBP	5′-CGTTTTGACCAGATGCGAGAC-3′	5′-GGCGTAGGGAGTTCAGGACAG-3′
PPAR	5′-TCTCCAGTGATATCGACCAGC-3′	5′-TTTTATCTTCTCCCATCATTAAGG-3′
LXR	5′-TTTGCCTTGCTCATTGCTATCAG-3′	5′-GGGACAGAACAGTCATTCGTGC-3′
CREB1	5′-ATACCTGGGCTAATGTGGCAATC-3′	5′-GTGAACGAAAGCAGTGACGGAG-3′
GLUT2	5′-TTTTCAGACGGCTGGTATCAGC-3′	5′-CACAGAAGTCCGCAATGTACTGG-3′
G6Pase	5′-CGACCTACAGATTTCGGTGCTTG-3′	5′-AGATAAAATCCGATGGCGAAGC-3′
FAS	5′-CAAATTCGACCTTTCTCAGAACCAC-3′	5′-CCCCCTTCAACACTGCCTCC-3′
GAPCH	5′-TGGACCTGACCTGCCGTCTAG-3′	5′-AGTGGGTGTCGCTGTTGAAGTC-3′

PCR mixture with SYBR Green I as described previously [35].

Plasmid construction

ACC1 mRNA (GenBank accession no. NM_133360) was used as a bait to search and identify the mouse ACC1 promoter using Genomatix. The region of −1060 to +61 of ACC1 PI promoter and −1677 to +28 of PII promoter were amplified by PCR, and subcloned into pGL3-basic vector, respectively.

Transfection and luciferase assay

HepG2 cells were transiently transfected with expression vector containing mouse CREB1 (pcDNA3.1-CREB1), using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions. For luciferase assay, cells were transfected with 8 μg of ACC1 promoter vectors (pGL3-PI and pGL3-PII) for 6 h, and further cultured in media containing supplements of AA or OA for 24 h. Cell extracts were then prepared and assayed for firefly luciferase activity with the Dual-Luciferase® Reporter Assay System (Promage). For co-transfection, ACC1 promoter vector (pGL3-PI or pGL3-PII) and pcDNA3.1-CREB1 were simultaneously introduced into HepG2 cells. After 36 h, the activity of firefly luciferase in cell extracts was checked as above-mentioned.

Glucose expenditure and triacylglycerol (TG) synthesis assay

After 24 and 36 h, the culture media with AA or without AA were collected and analyzed using an automatic biochemical analyzer at Wuhan Asia Heart Hospital.

Triacylglycerol (TG) content in cells was determined by oil red O staining as previously described [36], and quantitatively analyzed at 510 nm using a spectrophotometer.

Statistical analysis

Data are presented as mean ± SEM of at least three independent experiments. The comparison was carried out using Student's *t* test. A *P* value of less than 0.05 was considered to be statistically significant.

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