

# Docosahexaenoic Acid Enhances Hepatic Serum Amyloid A Expression via Protein Kinase A-dependent Mechanism\*

Received for publication, June 3, 2009, and in revised form, September 4, 2009. Published, JBC Papers in Press, September 15, 2009, DOI 10.1074/jbc.M109.024661

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Serum amyloid A (SAA) reduces fat deposition in adipocytes and hepatoma cells. Human *SAA1* mRNA is increased by docosahexaenoic acid (DHA) treatment in human cells. These studies asked whether DHA decreases fat deposition through *SAA1* and explored the mechanisms involved. We demonstrated that DHA increased human *SAA1* and *C/EBPβ* mRNA expression in human hepatoma cells, SK-HEP-1. Utilizing a promoter deletion assay, we found that a CCAAT/enhancer-binding protein β (*C/EBPβ*)-binding site in the *SAA1* promoter region between –242 and –102 bp was critical for DHA-mediated *SAA1* expression. Mutation of the putative *C/EBPβ*-binding site suppressed the DHA-induced *SAA1* promoter activity. The addition of the protein kinase A inhibitor H89 negated the DHA-induced increase in *C/EBPβ* protein expression. The up-regulation of *SAA1* mRNA and protein by DHA was also inhibited by H89. We also demonstrated that DHA increased protein kinase A (PKA) activities. These data suggest that *C/EBPβ* is involved in the DHA-regulated increase in *SAA1* expression via PKA-dependent mechanisms. Furthermore, the suppressive effect of DHA on triacylglycerol accumulation was abolished by H89 in SK-HEP-1 cells and adipocytes, indicating that DHA also reduces lipid accumulation via PKA. The observation of increased *SAA1* expression coupled with reduced fat accumulation mediated by DHA via PKA suggests that *SAA1* is involved in DHA-induced triacylglycerol breakdown. These findings provide new insights into the complicated regulatory network in DHA-mediated lipid metabolism and are useful in developing new approaches to reduce body fat deposition and fatty liver.

Serum amyloid A (SAA)<sup>3</sup> is a family of apolipoproteins mainly synthesized in mammalian liver. There are constitutive family members (SAA4) and acute-phase members (SAA1 and SAA2) that respond to tissue damage and inflammation. The acute-phase SAA is induced primarily by IL-1, tumor necrosis factor α, and IL-6 through the down-regulation of NF-κB, CCAAT/enhancer-binding protein (*C/EBP*)

family, and SAA activating factor (SAF) whose binding elements have been located and characterized in the acute-phase SAA promoter region (1). The *SAA1* is considered to be a marker for obesity and cardiovascular disease because the expression of *SAA1* is correlated well with the degree of obesity (2) and the risk of cardiovascular disease (3). However, the discoveries of the effect of *SAA1* in lipid metabolism suggest that *SAA1* is a mediator to reduce fat deposition. For example, several lipogenic enzymes, including acetyl-CoA carboxylase 1, lipoprotein lipase, and adipocyte fatty acid-binding protein, are reduced in adipocytes by SAA treatments (4). A glycerol-releasing effect of SAA can also be observed in porcine and human adipocytes (4, 5).

The *n*-3 polyunsaturated fatty acids (PUFA) are beneficial in many chronic diseases, including obesity, coronary heart disease, and fatty livers. A high docosahexaenoic acid/eicosapentaenoic acid diet increases weight loss and decreases fat deposition in mice and humans (6, 7). The intake of *n*-3 PUFA reduces coronary heart disease-related mortality (8, 9) and improves hepatic steatosis in nonalcoholic fatty liver patients (10, 11). The *n*-3 PUFA mainly exert their protective effect through their direct and extensive regulation of lipid metabolism.

The DHA is implicated in the regulation of lipid metabolism-related hepatic gene expression via its impact on several transcription factors and protein kinases (12). For example, DHA decreases fatty-acid synthase through the suppression of SREBP-1 (sterol regulatory element-binding protein-1) in hepatocytes (13, 14). Extracellular signal-related kinase (ERK) is found to be involved in DHA-mediated degradation of SREBP-1 (15). Besides the well known effect on lipogenesis and β-oxidation, *n*-3 PUFA also influence lipolysis. The *n*-3 PUFA promote glycerol release in adipocytes (16, 17). The mechanism of DHA-mediated lipolysis is not clearly defined. Our previous studies demonstrated DHA treatments increased SAA expression and lipolysis in porcine liver and hepatocytes (4, 18). Knowledge regarding DHA regulation of the expression of SAA will be helpful to unravel the underlying mechanism of DHA-mediated effects on lipid metabolism.

## EXPERIMENTAL PROCEDURES

**Cells and Culture Media**—The SK-HEP-1 cells (a human hepatoma cell line) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel). For the experiment to induce fat accumulation, SK-HEP-1 cells

\* This work was supported in part by the National Science Council in Taiwan and National Taiwan University (Grant 97R0066-43).

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<sup>3</sup> The abbreviations used are: SAA, serum amyloid A; DHA, docosahexaenoic acid; PKA, protein kinase A; *C/EBPβ*, CCAAT/enhancer-binding protein β; PUFA, polyunsaturated fatty acid; IL, interleukin; SAF, SAA activating factor; CREB, cAMP-response element-binding protein.

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were treated with serum-free medium containing 5 mM oleic acid (Sigma) for 2 days. All DHA treatment experiments were performed in serum-free medium with 1% bovine serum albumin for 24 or 48 h. Oleic acid, DHA (Nu-Chek, Elysian, MN), and H89 (Cayman Chemical, Ann Arbor, MI), a PKA inhibitor, were dissolved in DMSO (Sigma), and an equal amount of DMSO was used in the control group. Dibutyl cAMP (Sigma) was dissolved in deionized water and added in serum-free medium to treat cells for 12 h.

**Human Adipose Tissue Samples**—Adipose tissues were obtained from the breasts of six women undergoing mastectomy. The subjects were 37–54 years of age (mean = 47.1, S.D. = 5.93) with a body mass index of 22–34 kg/m<sup>2</sup> (mean = 26.6, S.D. = 3.92). All participants gave permission in writing, and the study was approved by the ethics committee of National Taiwan University Hospital.

**Adipocyte Isolation and Differentiation**—The adipocyte isolation and differentiation process was performed as described previously (19). The differentiated cells were treated with only Dulbecco's modified Eagle's medium/F-12 for 24 h, and the medium was then replaced with Dulbecco's modified Eagle's medium/F-12 containing 100  $\mu$ M DHA (Nu-Chek) or an equal amount of DMSO. After 24 h, the culture medium was collected to determine the glycerol concentration by the glycerol assay kit (Cayman Chemical).

**Quantitative RT-PCR**—The real time PCR procedure followed the one previously described (19). The following human primers were used: SAA1 sense 5'-CTGCAGAAGTGATCAGCG-3' and antisense 5'-ATTGTGTACCCTTCCC-3', annealing temperature 56 °C;  $\beta$ -actin sense 5'-CATCGAGCACGGCATCGTCA-3' and antisense 5'-TAGCAGCCTGGATAGCAAC-3', annealing temperature 55 °C; C/EBP $\beta$  sense 5'-AGCAAGGCCAAGAAGACCGT-3' and antisense 5'-TTGTGCTGCGTCTCCAGGTT-3', annealing temperature 56 °C; and SREBP-1c sense 5'-CTGTTGGTGCTCGTCTCCTTGG-3' and antisense 5'-CAGCAGGTGACGGATGAGGTTCC-3', annealing temperature 62 °C. Threshold cycle ( $C_t$ ) values were obtained, and relative gene expression ratio was calculated (20). The  $\beta$ -actin gene was used as a house-keeping gene.

**Western Blot Analysis**—For immunoblotting analysis of C/EBP $\beta$ , aliquots of 50  $\mu$ g of protein were subjected to SDS-12% PAGE, and the proteins were blotted onto a polyvinylidene difluoride membrane (PerkinElmer Life Sciences). Membranes were blocked with 0.25% gelatin and 0.05% Tween 20 in Tris-buffered saline. C/EBP $\beta$  antibody (sc-7962, Santa Cruz Biotechnology, Santa Cruz, CA) and  $\beta$ -actin antibody (sc-4778, Santa Cruz Biotechnology) were used as primary antibodies. Secondary goat anti-mouse antibody coupled to horseradish peroxidase was used with the chemiluminescence reagent (Immobilon Western, Millipore, Billerica, MA). Specific bands were quantified using the ImageQuant software (GE Healthcare) and corrected for the intensity of the  $\beta$ -actin band in the same sample.

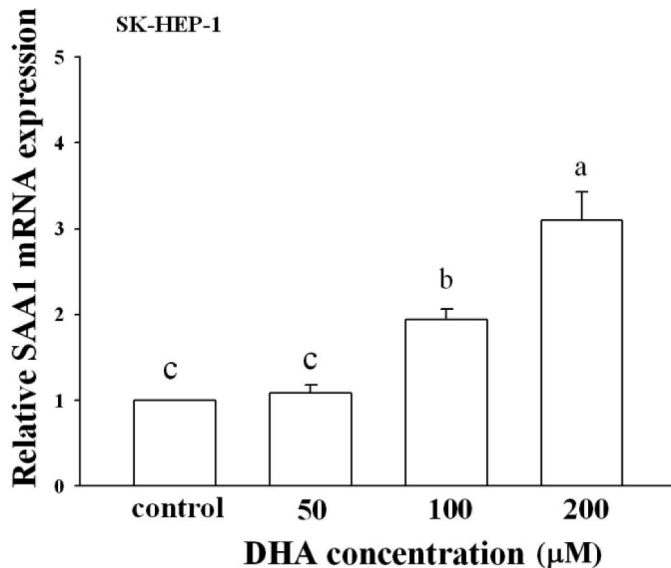
**Plasmids**—The human SAA1 promoter region was cloned and the sequence listed in Fig. 1. Three different lengths of the promoter region, SP1 (–910  $\rightarrow$  +32), SP2 (–242  $\rightarrow$  +32), and SP3 (–101  $\rightarrow$  +32) plus a normal and a mutated C/EBP $\beta$  site

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CTGTGCTGAGGAGGTAAATCTCGGGATATCTAGACTGAGGGGTGGAAGG -810
AGCCTTCCAGGGCACACATGAGACATGGCAGGGCTAGGCTGCTAGTTTAA -760
TTTTGTTTTCTTTAGACACAGGGTCTTGCTCTGTTAACCAGGCTGGAGTGC -708
AGTGCGGTGATTATAGCTCACTGCAGCCTTGACCTCTGGGTCTCCACAA -657
TCCTCCGCTTCAGCCTCTTGAGTAGCTGGGACTGCAGGTGCACACTACCA -606
CACCCGGTCCATTATTTTATATTTTCGTAGAGACAAGATCTTACAGTTTTG -554
CACA GAGTGATCTTAAACTCTTGACCCCAAGTGATCTCTGCTTGGCCT -503
CCAA AAGCATTGGGATTATAGGAGTGAGCCACTGTGCTGGACCTAGTCTG -453
TCAGCTTTGAAGCTTTAGATATGAAGTCAAGAGGACTTCATTTAGAGGCA -402
TCTG CCATGTGGCCAGCAGAGCCCATCTGAGGAAATGACTGGTAGAGT -352
CAGGAGCTGGCTTCAAAGCTGCCTCACTTCACACCTTCCAGCAGCCAGG -302
TGCCGCCATCACGGGGCTCCCACTCTCAACTCCGAGCCTCAGCCCCCTCA -251
ATGCTGAGGAGCAGAGCTGGTCTCTGCTGCTGACAGCTGCCAGGCACATCT -200
TGTTCCCTCAGGTTGACAACTGGGATAAATGACCCGGATGAAGAAACCA -149
C/EBP $\beta$ 
CTGGCATCCAGGAAGTGTCTTAGACCGTTTGTAGGGGAAATGACCTGCA -98
GGGACTTCCAGGAGCACATCCAGCTTTTCTGCTCCCAAGAAACCA -47
NF- $\kappa$ B
GCAGGGAAGGCTCAGTATAAATAGCAGCCACCGCTCCCTGGCAGGCAGG +4
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FIGURE 1. Nucleotide sequence of the promoter region of human SAA1 gene plus 32 bases of 5'-untranslated region. There is a TATA box at –30 to –25. C/EBP $\beta$  consensus binding site is at –190 to –177. NF- $\kappa$ B-binding site is at –97 to –86.

containing the SAA1 promoter regions (SP-normal and SP-mutated), were obtained by PCR with the introduction of MluI and XhoI restriction sites at the 5' and 3' ends, respectively. The sense primers for SP1, SP2, and SP3 were 5'-ACGCGTTTCC-TTCCTTCCTAATTTCC-3', 5'-ACGCGTGCACAACTGGGATAAATGACC-3', and 5'-ACGCGTTGCAGGGACTT-TCC-3', respectively. The SP-normal sense primer was 5'-ACGCGTAGGTTGCACAACTGGGATA-3', and the SP-mutated sense primer was ACGCGTAGGCTACAAACATGGATAAATGA-3'. All constructs, SP1, SP2, SP3, SP-normal, and SP-mutated shared the same antisense primer, 5'-CTCG-AGTGATCTGTGCTGTAGCTGAG-3'. The PCR products were cloned into pGL3-Basic vector (Promega, Madison, WI) with MluI and XhoI restriction sites for promoter activity analysis.

**Transient Transfection and Dual Luciferase Assay**—The SK-HEP-1 cells were seeded in 24-well plates, and 3  $\mu$ g of pGL3-SP1, pGL3-SP2, pGL3-SP3, pGL3-SP normal, or pGL3-SP mutated vectors were transfected into SK-HEP-1 cells using the PolyFect transfect reagent (Qiagen, Valencia, CA). The Renilla luciferase control vector, pRL-TK (0.3  $\mu$ g; Promega), was cotransfected with the constructed vectors. The cells were transfected for 24 h before replacement of culture medium with or without 200  $\mu$ M DHA. Promoter activities were measured using the Dual-Glo luciferase assay system (Promega) with a luminometer (Hidex, Turku, Finland). The firefly luciferase activities were normalized by Renilla luciferase activities. Each treatment was carried out in triplicate, and the mean ratio of luciferase to Renilla activity and standard deviations was calculated.



**FIGURE 2. DHA increased human SAA1 mRNA expression in SK-HEP-1 cells.** SK-HEP-1 cells were treated with different concentrations of DHA for 24 h before SAA1 mRNA expression was measured and normalized to  $\beta$ -actin mRNA. Data are expressed as means  $\pm$  S.E. ( $n = 5$ ). Significant differences are indicated by letters  $a-c$  ( $p < 0.05$ ). Control value was set as 1.

**PKA Activity**—The PKA activity of SK-HEP-1 cells was measured using the PepTag assay for nonradioactive detection of cAMP-dependent PKA following the manufacturer's instructions (Promega).

**cAMP Analysis**—After 12- or 24-h DHA of treatment, the SK-HEP-1 cells were collected, and the cAMP concentration was determined by the cAMP direct immunoassay kit (BioVision, Mountain View, CA). The protein concentrations were determined by the BCA protein assay (Pierce) and were used to normalize cAMP concentration in each sample.

**Staining**—Oil red-O staining was used to detect intracellular neutral lipids. The cells were counterstained by the Harris modified hematoxylin solution (Sigma) for 10 min, and the solution was washed out by deionized water.

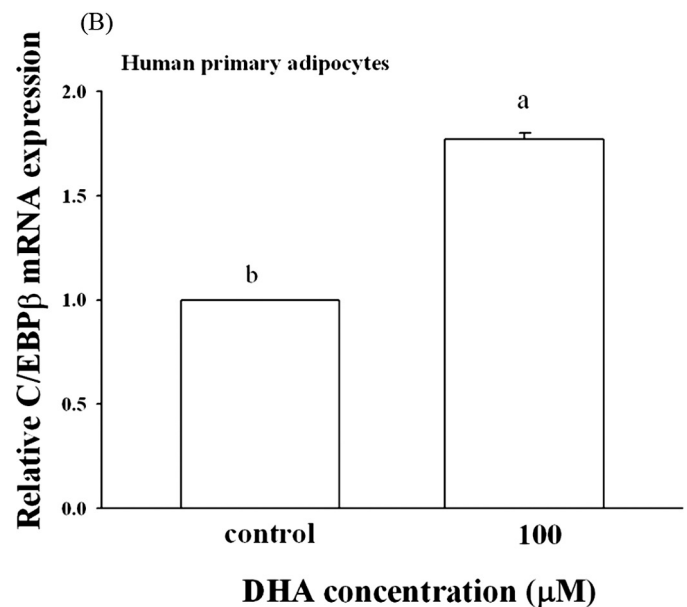
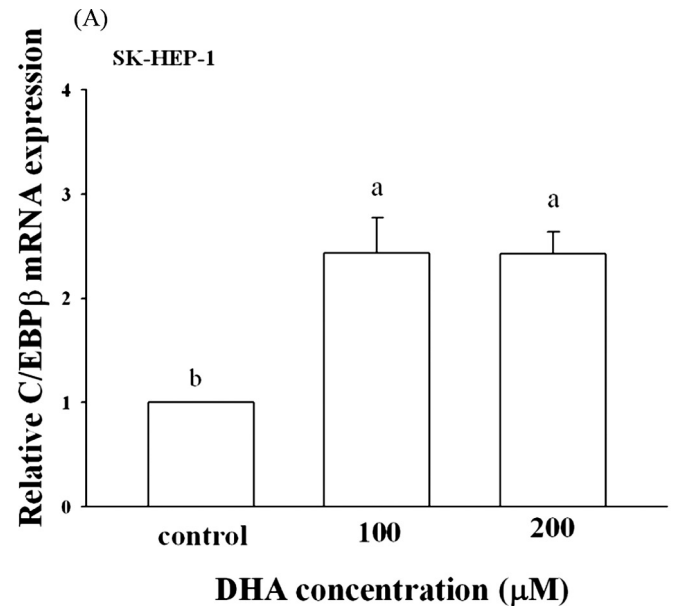
**Triacylglycerol Measurements**—Triacylglycerols were extracted from SK-HEP-1 cells and quantified using a triglyceride assay kit (BioVision). The triacylglycerol contents were normalized by the protein concentration in each sample.

**Enzyme-linked Immunosorbent Assay**—The SAA1 production in SK-HEP-1 cells was quantified by human SAA Cytoset kit (BIOSOURCE). The detection limit of the assay was 9.4 ng/ml.

**Statistical Analysis**—Data were expressed as means  $\pm$  S.E. Results of two groups were compared by the Student's  $t$  test. Statistical significance among different experimental treatments was determined by one-way analysis of variance. Tukey's test was used to evaluate differences among means (SAS Institute, Cary, NC).  $p$  values  $\leq 0.05$  were considered statistically significant.

## RESULTS

**DHA Increased Human SAA1 mRNA Expression in SK-HEP-1 Cells**—We treated several human hepatoma cell lines, including PLC, HEP G2, HEP-3B, and SK-HEP-1 cells, with 200  $\mu$ M DHA and found that only SK-HEP-1 showed increased



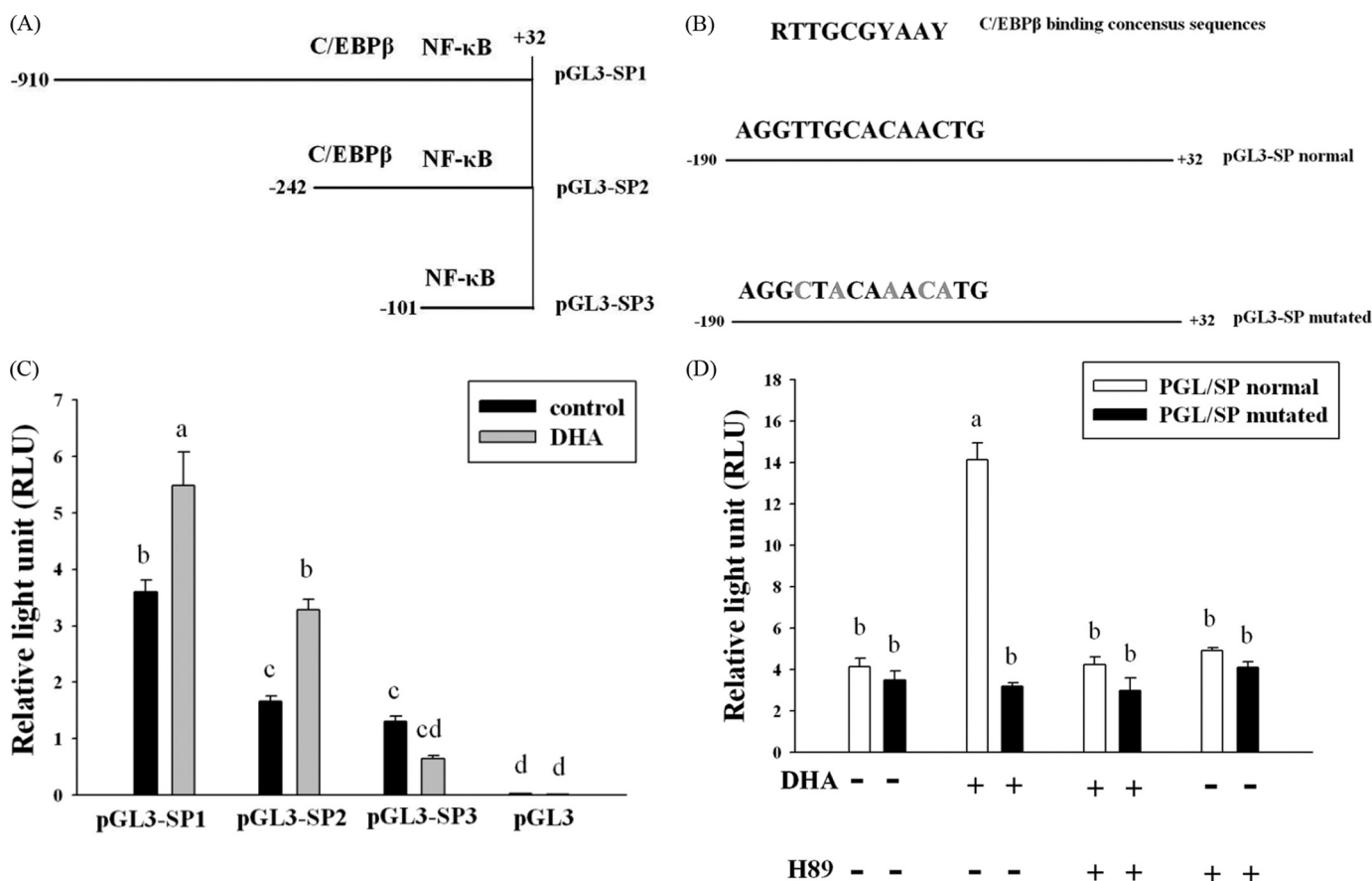
**FIGURE 3. DHA increased human C/EBPβ mRNA expression in SK-HEP-1 cells.** The SK-HEP-1 cells were treated with DHA for 24 h before C/EBPβ mRNA expressions were measured and normalized to  $\beta$ -actin mRNA (A);  $n = 4$  independent measures. Human primary adipocytes were treated in the same way, and C/EBPβ mRNA expression was measured (B);  $n = 6$  independent measures. Data are expressed as means  $\pm$  S.E. Significant differences are indicated by letters  $a$  and  $b$  ( $p < 0.05$ ). Control value was set as 1.

expression of SAA1 (data not shown). The positive response of SAA1 expression to DHA treatment was similar to results with porcine primary hepatocyte cell culture (4); therefore, SK-HEP-1 was chosen for the following studies. The SAA1 mRNA was increased by treatments of 50, 100, or 200  $\mu$ M DHA in SK-HEP-1 cells (Fig. 2).

**DHA Increased Human C/EBPβ mRNA Expression in SK-HEP-1 Cells**—We measured the responses of the putative SAA1-regulatory transcription factor, C/EBPβ, to DHA treatments in SK-HEP-1 cells. The DHA treatments enhanced the expression of C/EBPβ mRNA in SK-HEP-1 cells and primary adipocytes (Fig. 3, A and B), suggesting that C/EBPβ may be



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**FIGURE 4. C/EBPβ was involved in DHA-mediated increment of SAA1 promoter activities.** A, diagram of variable length constructs of the human SAA1 promoter luciferase vectors. B, diagram of normal and mutated C/EBPβ site-containing human SAA1 promoter luciferase vectors. C, effect of DHA on the induction of SAA1 promoter activities. The SK-HEP-1 cells were transfected with constructed pGL3-SP1, pGL3-SP2, and pGL3-SP3 plasmid vectors, and their promoter activities were then assayed after 24 h of DHA treatment. The pGL3 is a luciferase vector without the SAA1 promoter insertion and was used as a negative control. D, effect of DHA and the PKA inhibitor H89 on the induction of normal and mutated C/EBPβ site-containing SAA1 promoter activities. The promoter activities of each well were normalized for transfection efficiency using the *Renilla* luciferase activity. Data are expressed as means  $\pm$  S.E. ( $n = 3$ ). Significant differences are indicated by letters *a–d* ( $p < 0.05$ ).

involved in regulating the expression of SAA1 after DHA treatment.

**C/EBPβ Was Involved in DHA-mediated Increments of SAA1 Promoter Activities**—Various lengths of SAA promoter sequences were ligated to a luciferase vector (Fig. 4A). Promoter sequences containing a normal or a mutated C/EBPβ-binding site were also ligated to a luciferase vector (Fig. 4B). Treatment with DHA increased promoter activities in the pGL3-SP1 and pGL3-SP2 plasmid vectors containing the C/EBPβ-binding site (Fig. 4C). When the putative C/EBPβ-binding site was removed (pGL3-SP3 plasmid vector), the enhancing ability of DHA was not observed (Fig. 4C). The putative C/EBPβ-binding site-containing SAA1 promoter region between -242 and -102 bp was required for DHA-regulated SAA1 expression. The pGL3-SP normal and pGL3-SP mutated plasmid vectors were also used to verify the role of C/EBPβ in DHA-mediated SAA1 expression. Mutation of the putative C/EBPβ-binding site sequences eliminated the DHA-induced increase in SAA1 promoter activity (Fig. 4D), further indicating that C/EBPβ is involved in DHA-regulated SAA1 expression.

**H89 Inhibited DHA-induced C/EBPβ Production in SK-HEP-1 Cells**—The PKA inhibitor H89 suppressed the DHA-induced increase in SAA1 promoter activity in pGL3-SP normal

plasmid vectors but not in pGL3-SP mutated plasmid vectors (Fig. 4D). The data suggest that PKA is involved in mediating the increase of C/EBPβ to enhance DHA-regulated SAA1 expressions. To explore whether inhibition of PKA reduces DHA-mediated C/EBPβ expression, total protein was isolated from SK-HEP-1 cells treated with 200  $\mu$ M DHA with or without 1  $\mu$ M H89. The DHA treatment alone enhanced the C/EBPβ protein concentration, but the PKA inhibitor counteracted the DHA-mediated increase of C/EBPβ (Fig. 5), suggesting strongly that PKA is involved in DHA-induced C/EBPβ expression.

**H89 Inhibited DHA-induced SAA mRNA Production in SK-HEP-1 Cells**—When SK-HEP-1 cells were treated with 200  $\mu$ M DHA alone, SAA1 expression was increased, whereas the PKA inhibitor abolished the DHA-mediated increase in SAA1 (Fig. 6A). Similar results were obtained for DHA and H89 effects on SAA1 promoter activity (Fig. 6B). To demonstrate the role of PKA in the regulation of SAA1 expression, the analogue of cyclic AMP, dibutyryl cAMP, was used. The SAA1 expression was increased about 2-fold by 500 and 700  $\mu$ M dibutyryl cAMP (Fig. 6C).

**DHA Increased PKA Activities and Intracellular cAMP Levels in SK-HEP-1 Cells**—Treatments of SK-HEP-1 cells with 100 or 200  $\mu$ M DHA increased PKA activity, whereas co-treatment of

10  $\mu\text{M}$  H89 negated the increase in PKA activity caused by 200  $\mu\text{M}$  DHA (Fig. 7, A and C). A similar effect of DHA was found in adipocytes (Fig. 7, B and D). The 100  $\mu\text{M}$  DHA treatment enhanced the intracellular cAMP levels in SK-HEP-1 cells (Fig. 7E). The data show that DHA treatment enhanced cAMP concentration and PKA activity.

**Induction of Steatosis in SK-HEP-1 Cells**—To further understand the role of PKA in DHA-mediated triacylglycerol breakdown, a model of fatty hepatocytes was established *in vitro*. Steatosis in SK-HEP-1 cells was induced by the addition of a high concentration of oleic acid. Greater lipid droplets were

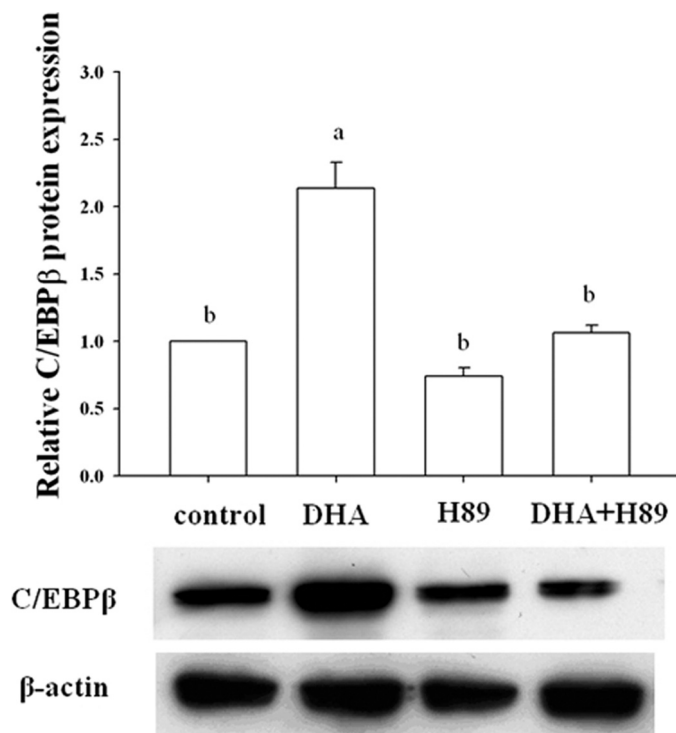
observed in oleic acid-treated cells (Fig. 8A). There was a 13-fold increase in the amount of intracellular triacylglycerols in steatotic SK-HEP-1 cells compared with normal cells (Fig. 8B). We examined the *SAA1* expression level in these cells and found there was no difference in steatotic and normal cells (Fig. 8C).

**H89 Inhibited DHA-induced Triacylglycerol Breakdown in SK-HEP-1 Cells and Adipocytes**—The role of PKA in DHA-mediated triacylglycerol breakdown was examined in the fatty hepatocyte model. Treatment of fatty SK-HEP-1 cells with 100  $\mu\text{M}$  DHA decreased triacylglycerol accumulation (Fig. 9A) and the mRNA level of *SREBP-1c* (Fig. 9B). DHA also decreased fatty-acid synthase and *ACC1* mRNA, suggesting that DHA decreased lipogenesis (data not shown). The inhibitory effects of DHA on triacylglycerol accumulation and *SREBP-1c* expression were counteracted by H89 (Fig. 9, A and B), as was the DHA-enhanced increase in *SAA1* mRNA and protein expression (Fig. 9C). The PKA inhibitor H89 also inhibited the DHA-mediated increase in glycerol release (Fig. 9D). Together, the data strongly suggest that PKA participates in the DHA-mediated mechanism to increase triacylglycerol breakdown.

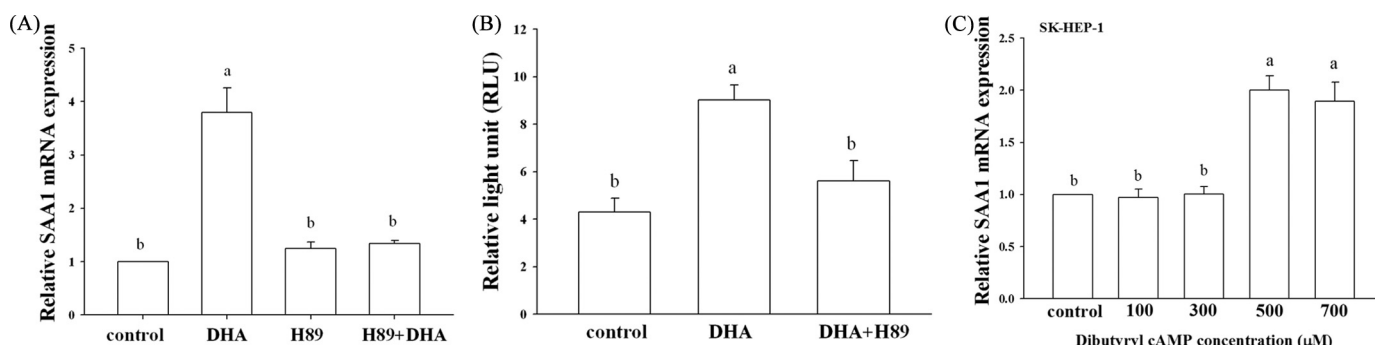
## DISCUSSION

The *n*-3 PUFA suppressed expression of lipogenic genes, *e.g.* fatty-acid synthase, acetyl-CoA carboxylase 1, and stearoyl-CoA desaturase, through down-regulation of *SREBP1* in the liver (13, 21) and adipocytes (22, 23) to reduce fat deposition. The *n*-3 PUFA also can promote lipolysis and  $\beta$ -oxidation to increase lipid utilization (4, 24). Treatment with DHA increases the expression of hepatic *SAA1* (18), and *SAA1* treatment increases lipolytic gene expression and glycerol release in adipocytes (4, 19). These observations link DHA treatments and *SAA1* expression to the increased lipolysis and therefore reduced fat deposition. In this study, we demonstrated the mechanisms by which DHA enhances the expression of *SAA1* in a human hepatoma cell line.

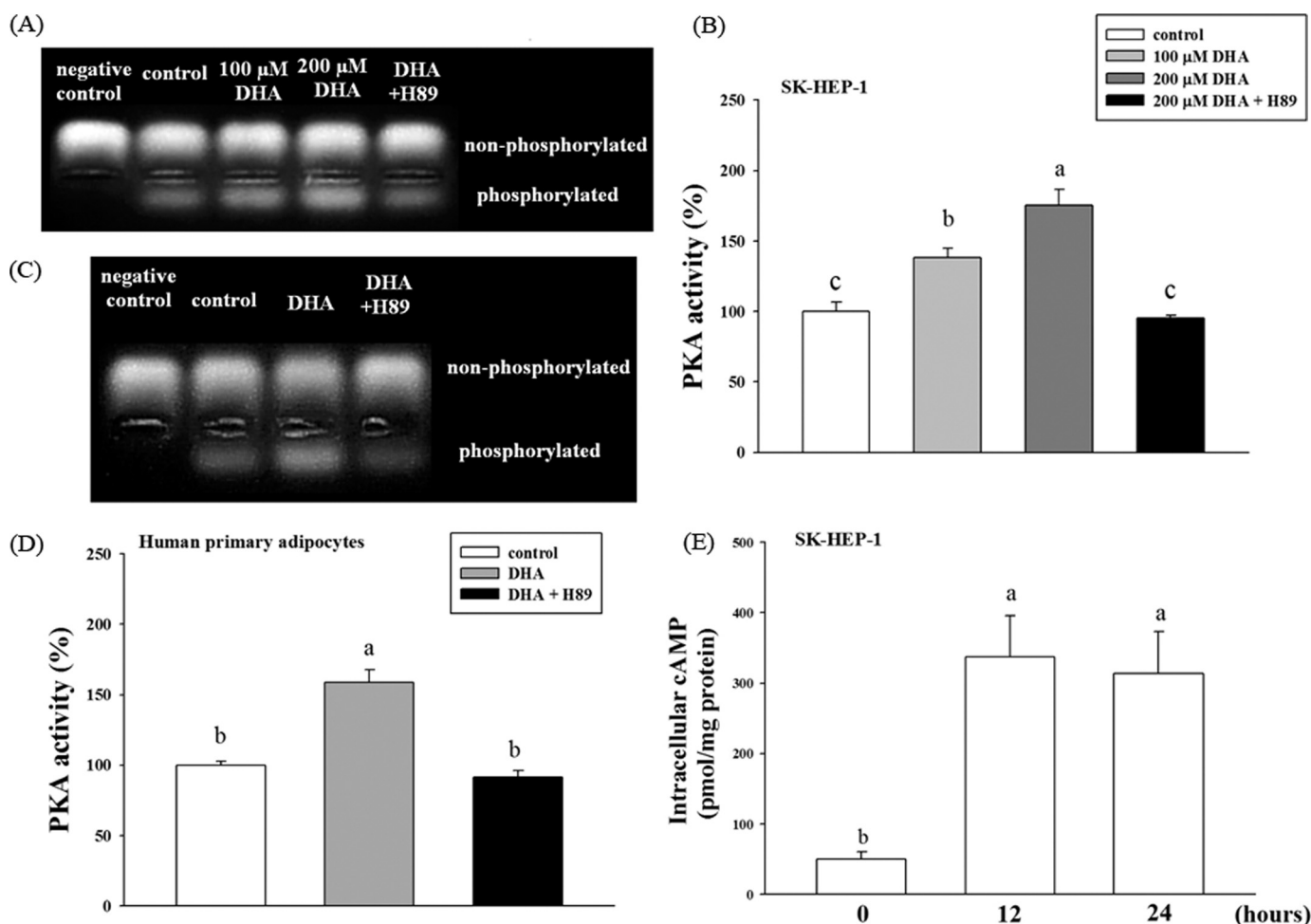
Protein kinase A, a cAMP-dependent protein kinase, plays an important role in regulating lipolysis. The PKA-mediated phosphorylation of perilipin and hormone-sensitive lipase increases the release of glycerol and free fatty acids through the breakdown of stored triacylglycerols in adipocytes (25,



**FIGURE 5. H89 inhibited DHA-induced C/EBP $\beta$  production in SK-HEP-1 cells.** The SK-HEP-1 cells were treated with 200  $\mu\text{M}$  DHA and 1  $\mu\text{M}$  H89, as indicated for 24 h, and total protein lysates were then obtained and analyzed by Western blotting. Relative protein levels were quantified by ImageQuant software, and data are shown in comparison with untreated cells. Data are expressed as means  $\pm$  S.E. ( $n = 3$ ). Significant differences are indicated by letters *a* and *b* ( $p < 0.05$ ). Control value was set as 1.



**FIGURE 6. H89 inhibited DHA-induced SAA expression in SK-HEP-1 cells.** A, SK-HEP-1 cells were treated with 200  $\mu\text{M}$  DHA with or without 10  $\mu\text{M}$  H89, and relative *SAA1* mRNA expression was assessed by real time PCR. Expression of *SAA1* mRNA was measured and normalized to  $\beta$ -actin mRNA. Data are expressed as means  $\pm$  S.E. ( $n = 4$ ). B, SK-HEP-1 cells were transfected with constructed pGL3-SP1 plasmid vectors, and their promoter activities were then assayed after 24 h of DHA  $\pm$  H89 treatment. The promoter activities of each well were normalized for transfection efficiency using the *Renilla* luciferase activity;  $n = 3$  independent measures. C, SK-HEP-1 cells were treated with various concentrations of dibutyl cAMP for 12 h, and relative *SAA1* mRNA expression was assessed by real time PCR. Expression of *SAA1* mRNA was measured and normalized to  $\beta$ -actin mRNA;  $n = 4$  independent measures. Data are expressed as means  $\pm$  S.E. Significant differences are indicated by letters *a* and *b* ( $p < 0.05$ ). Control value was set as 1.



**FIGURE 7. DHA increased PKA activities and intracellular cAMP levels in SK-HEP-1 cells.** The SK-HEP-1 cells were treated with 100 or 200  $\mu$ M DHA with or without 10  $\mu$ M H89 for 24 h. Human adipocytes were treated with 100  $\mu$ M DHA with or without 10  $\mu$ M H89 for 24 h. The cell lysates were assayed for PKA activities using PepTag assay. Data represent a typical gel pattern from three independent experiments in SK-HEP-1 cells (A) with average densitometric value (B) and six independent experiments in adipocytes (C) with average densitometric values (D). Densitometric analysis of the bands was quantified by ImageQuant software, and data are shown in comparison with untreated cells.  $n = 3$  independent measures in SK-HEP-1 cells, and  $n = 6$  independent experiments in adipocytes. The SK-HEP-1 cells were treated with 100  $\mu$ M DHA for 12 and 24 h before intracellular cAMP level was measured and normalized to protein concentration in each sample. E,  $n = 4$  independent measures. Data are expressed as means  $\pm$  S.E. Significant differences are indicated by letters a–c ( $p < 0.05$ ).

26). The  $n$ -3 PUFA activate PKA in rat heart and in the epithelial renal cell line A6 (27, 28). In this study, we demonstrated by either the use of a PKA inhibitor or by the direct measurement of cAMP levels and a PKA phosphorylation product that DHA stimulated the activity of PKA in both SK-HEP1 cells and human adipocytes. Others have reported (29–31) that  $n$ -3 PUFA influence PKA by increasing adenylyl cyclase activity and decreasing phosphodiesterase activity. Therefore, these findings illustrate that there is a lipolysis-promoting mechanism for  $n$ -3 PUFA through induction of PKA activity.

Similar to observations in smooth muscle cells (32), we found that DHA increased *C/EBP $\beta$*  expression in the SK-HEP-1 cell line. We also demonstrated that the up-regulation of *C/EBP $\beta$*  by DHA treatment was through a PKA-dependent pathway. The *C/EBP $\beta$*  expression is up-regulated by the cAMP-response element-binding protein (CREB) via the PKA-dependent pathway (33), and the phosphorylation of CREB by PKA is critical for CREB-induced gene expression (34). Recent data indicate that DHA treatment increases phosphorylated CREB (35).

These data suggest that CREB participates in the DHA-mediated induction of *C/EBP $\beta$*  in our studies.

There is a *C/EBP $\beta$* -response element on the human *SAA2* promoter (36). Because the sequence homology in the proximal promoter regions is high between human *SAA1* and *SAA2* and their promoter activities in response to cytokine treatments are also similar (37), the sequence AGGTTACACAAGT (–190/–177) is regarded as the *C/EBP $\beta$* -binding site in the human *SAA1* promoter. The *C/EBP $\beta$*  consensus sequences are identified as RTTGCGYAAY, where R is A or G and Y is C or T (38). The importance of T (at –4-position) and A (at +4-position) in the *C/EBP $\beta$*  consensus-binding site suggests that the mutated *C/EBP $\beta$* -binding sequence GCTACAAACA in the pGL3-SP mutated vector should eliminate the binding of *C/EBP $\beta$*  to the *SAA1* promoter. Indeed, our current data support this speculation and demonstrate that this binding site is very important in mediating DHA function in regulating *SAA1* expression.

It has been reported that cAMP potentiates the inflammation response (39, 40). The cAMP is able to potentiate IL-6

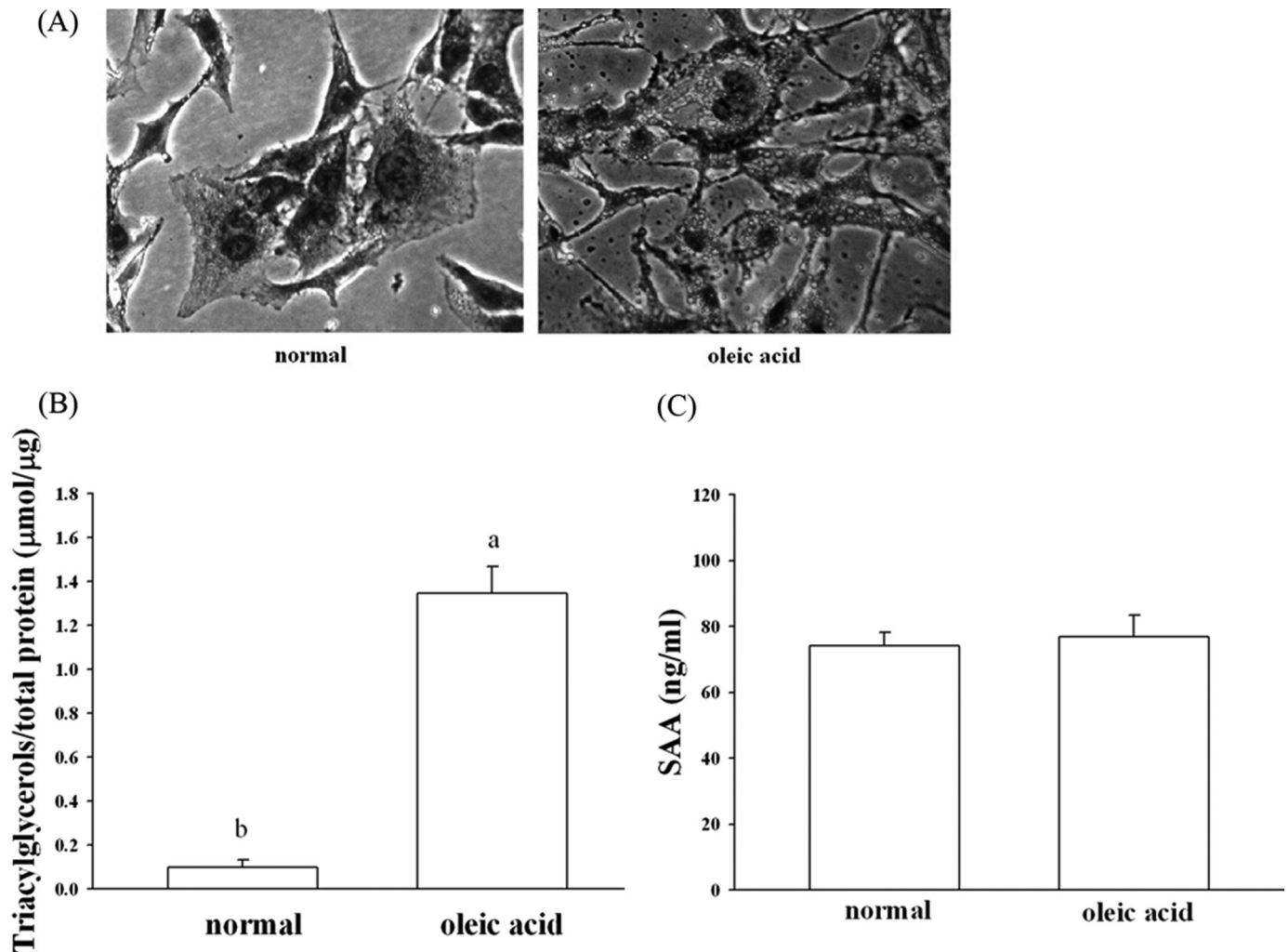


FIGURE 8. **Induction of steatosis in SK-HEP-1 cells.** A, SK-HEP-1 cells were stained with Oil red-O and counterstained with hematoxylin after 5 mM oleic acid treatment for 2 days. B, quantification of the triacylglycerol content in normal and steatotic SK-HEP-1 cells. Triacylglycerols were collected from SK-HEP-1 cells treated with or without 5 mM oleic acid for 2 days. Data are expressed as means  $\pm$  S.E. ( $n = 5$ ). Significant differences are indicated by letters *a* and *b* ( $p < 0.05$ ). C, total protein lysates were collected from normal and steatotic SK-HEP-1 cells and used in SAA enzyme-linked immunosorbent assay analysis;  $n = 4$  independent measures.

production after IL-1 $\beta$  treatment; this increased IL-6 production is related to increased DNA binding activities for several IL-6-regulating transcription factors induced by cAMP (39). The alterations in cytokine levels induced by cAMP can be quite small. For example, dibutyryl cAMP treatments induced a 2-fold *SAA1* expression in our studies. Our findings that connect the induction of *SAA1* expression to PKA illustrate the relationship of PKA to a proinflammatory factor. Regarding the mechanism for PKA regulation of the expression of *SAA1*, we speculate that PKA influences *C/EBP* $\beta$  function not only by increasing *C/EBP* $\beta$  expression but also by direct phosphorylation of *C/EBP* $\beta$  to elevate transcription of *C/EBP* $\beta$ -regulated genes (41, 42). In addition to *C/EBP* $\beta$ , another *SAA*-regulating transcription factor, SAF, may also participate in PKA-mediated *SAA* expression. The SAF is an *SAA*-regulating transcription factor binding to the rabbit *SAA* promoter, and MAZ is considered the homolog of SAF for the human *SAA* promoter (43). Treatment with cAMP increases binding of SAF to the SAF DNA-binding element on the *SAA* promoter in mouse and

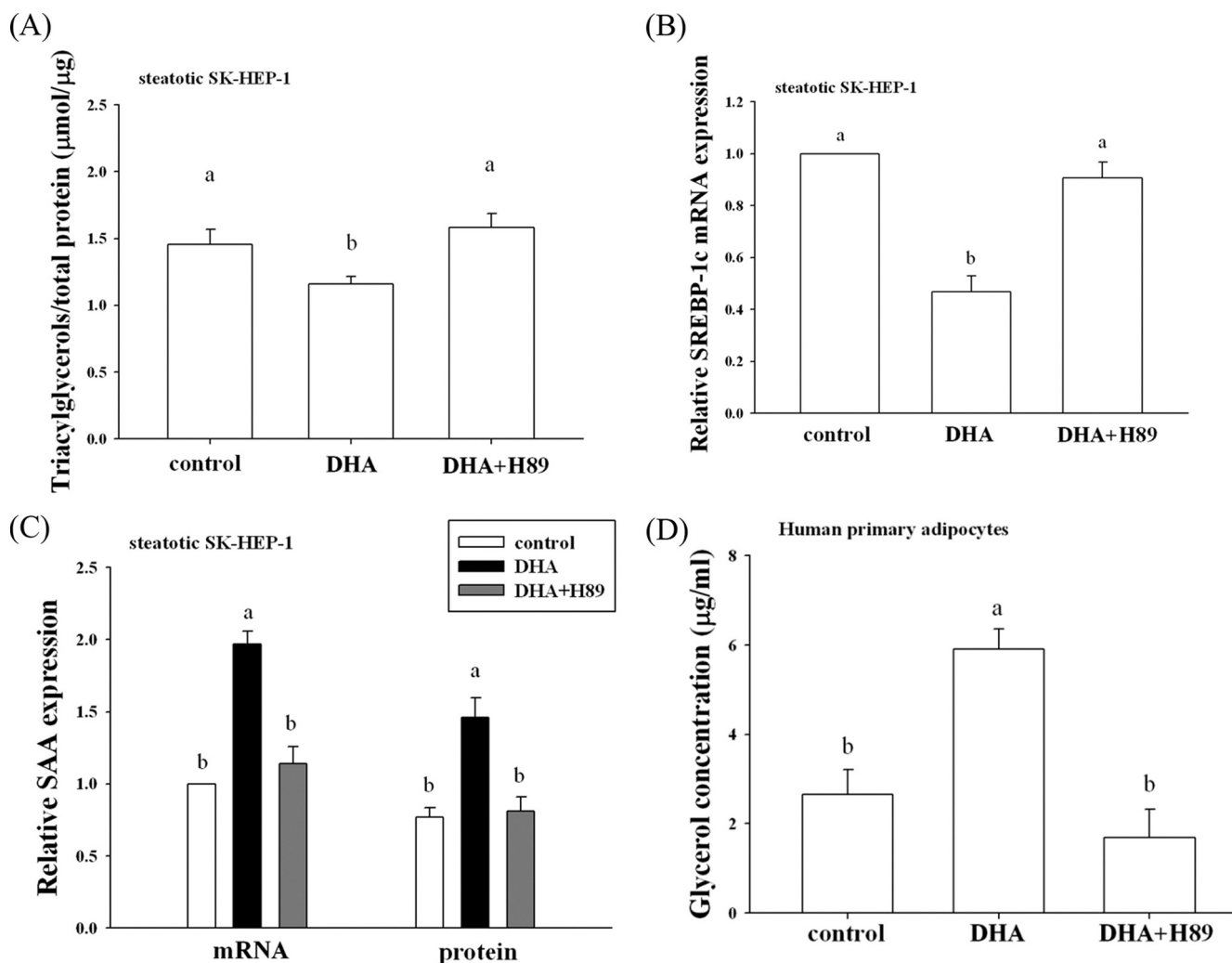
human cells, and consistently PKA increases the SAF-regulated promoter activities (43, 44). Therefore, PKA may also up-regulate *SAA* expression through SAF.

Besides *SAA1* and *C/EBP* $\beta$ , DHA affects *SREBP-1c* expressions via PKA. It has been reported that activation of PKA suppresses *SREBP-1* expression through phosphorylation of liver X receptor, thus inhibiting the liver X receptor stimulation of transcription of *SREBP-1c* (45). Our data supported that DHA can activate PKA to reduce the expression of *SREBP-1c*. Regardless of the mechanism involved, the inhibitory effect of DHA on the expression of *SREBP-1c* reduces the lipogenesis and therefore reduces lipid accumulation.

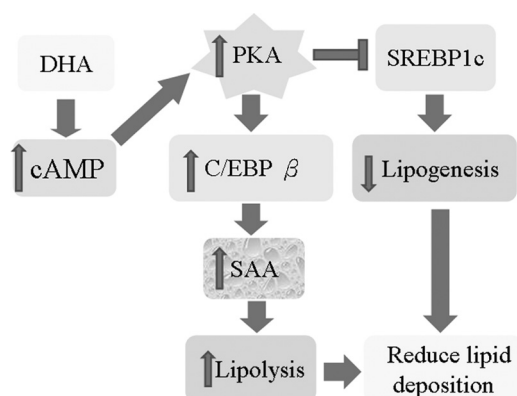
This study used the hepatoma cell line SK-HEP-1 as the primary study model to demonstrate the lipolytic promoting mechanism by DHA. Human adipocyte culture was also used to confirm the major mechanism by which DHA increases *SAA1* expression through PKA. A schematic diagram of a DHA-mediated signaling pathway is shown in Fig. 10. These findings indicate mechanisms involved in the DHA-mediated decrease in triacylglycerol deposition via



## DHA Enhances Expression of Human Serum Amyloid A Protein



**FIGURE 9. H89 inhibited DHA-induced triacylglycerol breakdown in SK-HEP-1 cells and adipocytes.** A, SK-HEP-1 cells were treated with 5 mM oleic acid for 2 days as the control group. The addition of 5 mM oleic acid and 100  $\mu\text{M}$  DHA with or without 10  $\mu\text{M}$  H89 was used in the DHA+H89 group and the DHA group. Triacylglycerol content in these cells was assayed;  $n = 5$  independent measures. B, SREBP-1c mRNA expression of steatotic SK-HEP-1 cells was measured and normalized to  $\beta$ -actin mRNA in the control, DHA, and the DHA+H89 group;  $n = 5$  independent measures. Control value was set as 1. C, SAA1 mRNA expression of steatotic SK-HEP-1 cells was measured and normalized to  $\beta$ -actin mRNA in the control, DHA, and the DHA+H89 groups;  $n = 5$  independent measures. As for SAA protein expression, the total protein lysates were collected from the control, DHA, and DHA+H89 groups and used in SAA enzyme-linked immunosorbent assay analysis;  $n = 4$  independent measures. D, human breast preadipocytes were isolated, differentiated, and then treated with 100  $\mu\text{M}$  DHA with or without 10  $\mu\text{M}$  H89 for 24 h. The culture mediums were collected for the glycerol assay;  $n = 6$  independent measures. Data are expressed as means  $\pm$  S.E. Significant differences are indicated by letters *a* and *b* ( $p < 0.05$ ).



**FIGURE 10. Schematic diagram of the proposed DHA-mediated signaling pathway.** DHA elicits PKA activation by the elevation of intracellular cAMP levels. DHA up-regulates human SAA1 via increasing PKA activity to increase expression of C/EBP $\beta$ . DHA also inhibits SREBP-1c expression by PKA activation. The increased SAA1 and the decreased SREBP-1c expressions can lead to increased lipolysis and decreased lipogenesis, respectively, resulting in reduced lipid deposition.

decreased lipogenesis and increased lipolysis and suggest use of *n*-3 PUFA to reduce lipid deposition in the liver and other tissues.

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