

## Docosahexaenoic acid regulates serum amyloid A protein to promote lipolysis through down regulation of perilipin<sup>☆</sup>

Ya C. Wang<sup>a,1</sup>, Wen H. Kuo<sup>b,1</sup>, Ching Y. Chen<sup>a</sup>, Hsin Y. Lin<sup>a</sup>, Hsin T. Wu<sup>a</sup>, Bing H. Liu<sup>a</sup>, Chia H. Chen<sup>a</sup>, Harry J. Mersmann<sup>a,2</sup>, King J. Chang<sup>b,\*</sup>, Shih T. Ding<sup>a,\*</sup>

<sup>a</sup> Department of Animal Science and Technology/Institute of Biotechnology, National Taiwan University, Taipei 106, Taiwan

<sup>b</sup> Department of Surgery, National Taiwan University Hospital and College of Medicine, Taipei 100, Taiwan

Received 20 September 2008; received in revised form 31 December 2008; accepted 6 January 2009

### Abstract

Docosahexaenoic acid (DHA) increases lipolysis and decreases lipogenesis through several pathways. DHA also enhances the expression of serum amyloid A protein (SAA), a possible lipid metabolism related gene. The question of whether DHA regulates the expression of SAA to affect lipid metabolism and increase lipolysis needs to be demonstrated in human adipocytes. We designed experiments to determine the role of SAA in regulating lipid metabolism in HepG2 cells using microarray technology. In human hepatocytes, recombinant human SAA1 (hSAA1) inhibited the expression of genes related to lipogenesis and promoted the expression of those involved in lipolysis. When human breast adipocytes were treated with hSAA1 or DHA in vitro, the expression of peroxisome proliferator-activated receptor  $\gamma$  and other lipogenic genes was decreased, whereas the expression of several lipolytic genes was increased. Glycerol release was increased by both SAA and DHA treatments, suggesting that they increased lipolytic activity in human adipocytes. The expression of perilipin, a lipid droplet-protective protein, was decreased, and hormone-sensitive lipase was increased by both of hSAA1 and DHA treatment. We speculate that the mechanism of lipolysis by DHA or SAA is at least partially the result of increased expression of hormone-sensitive lipase and decreased expression of perilipin. Whereas DHA treatment increased expression of hSAA1 in human adipocytes, the DHA-mediated reduction in expression of lipogenesis genes and enhancement of lipolysis may be through the activity of hSAA1. These results may be useful in developing new approaches to reduce body fat deposition.

© 2010 Elsevier Inc. All rights reserved.

**Keywords:** Docosahexaenoic acid; Lipolysis; Glycerol release; Perilipin; Serum amyloid A protein

### 1. Introduction

Adipose tissue not only stores energy but also plays a role in the pathogenesis of obesity [1]. Polyunsaturated fatty acids (PUFA) increase lipolysis and inhibit the expression of adipogenesis-related genes to affect physiology and disease conditions including cardiovascular disease, immunoresponses [2], insulin sensitivity [3], cancer growth [4], nerve development and visual function [5]. PUFA can also regulate signaling molecules to modify fatty acid transport and expression of metabolism-related genes to increase lipid oxidation and suppress adipogenesis [6]. Docosahexaenoic acid (DHA), an n-3 fatty acid, decreases the transcription factor, sterol regulatory element binding protein 1c (SREBP1c) mRNA and expression of the lipogenesis genes for fatty acid synthase (FAS [7,8]) and acetyl-CoA carboxylase

[7–9]. These findings suggest that DHA may reduce lipogenesis and subsequent lipid accumulation.

Serum amyloid A protein (SAA) is a secreted protein that is expressed primarily in human liver and adipose tissues [10]. Microarray data show that expression of SAA is four times greater in large versus small adipocytes; the expression of SAA is positively correlated with the diameter of the adipocytes [11]. The SAA expressed in human adipocytes is also positively correlated with body mass index (BMI) [12]. Although SAA is linked to obesity and lipid metabolism and may be contributing to obesity-associated cardiovascular disease, the regulatory mechanisms and effects of SAA on lipid metabolism are yet to be demonstrated. There are four subtypes of SAA: SAA1, SAA2, SAA3 and SAA4, with SAA3 a pseudogene in human [13]. DHA increases SAA expression, and recombinant human SAA1 inhibits the mRNA expression of a lipid droplet protective protein, perilipin [14]. The surface of lipid droplets is coated with perilipin protein to protect lipid droplets. When energy is needed, the enzyme adenylyl cyclase located in the adipocyte plasma membrane is activated to produce cAMP which then activates protein kinase A (PKA). Activated PKA can phosphorylate perilipin to allow the PKA-activated (phosphorylated) hormone sensitive lipase (HSL) to hydrolyze triacylglycerols to release free fatty acids and glycerol [15]. In the current study, we

<sup>☆</sup> The project was funded in part by the National Science Council in Taiwan.

\* Corresponding authors. Tel.: +886 2 33664175; fax: +886 2 27324070.  
E-mail address: [sdting@ntu.edu.tw](mailto:sdting@ntu.edu.tw) (S.T. Ding).

<sup>1</sup> These authors contributed equally to this work.

<sup>2</sup> Visiting Professor at National Taiwan University.

demonstrated that DHA increased the expression of SAA1, and recombinant human SAA1 inhibited the expression of perilipin and increased lipolysis in human adipocytes.

## 2. Materials and methods

### 2.1. HepG2 cell culture

HepG2 cells (hepatocellular carcinoma, ATCC no. CRL-11997) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, NY, USA) in six-well plates. When the cells had proliferated to approximately 80% confluence, they were treated with DMEM containing hSAA1 (Acris, Hiddenhausen, Germany) diluted in 5 mM Tris, pH=7.6. The hSAA1 final concentration in treatment medium was 2  $\mu$ M. The control medium was hSAA1-free DMEM with the same amount of Tris buffer added. Human adipocytes were cultured with hSAA1 and control medium for 72 h at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. Total RNA was extracted for gene expression profile analysis.

### 2.2. Human adipose tissue

Adipose tissues were obtained from the breast of five women undergoing mastectomy. The subjects were 33–51 years of age (mean=43.6, S.D.=6.18) with a BMI of 16–29 kg/m<sup>2</sup> (mean=21.5, S.D.=4.50). All participants gave permission in writing, and the study was approved by the Ethics Committee of National Taiwan University Hospital.

### 2.3. Isolation of human stromal/vascular cells and differentiation

The tissue was dissected under sterile conditions and immediately transferred to sterile transport buffer (0.9% NaCl, 56 mM glucose, 25 mM HEPES, penicillin 100 U/ml, streptomycin 100  $\mu$ g/ml, pH=7.4) for transportation to the laboratory. Transfer to the laboratory took approximately 30–35 min, and the tissue was maintained at 37°C. The tissue was minced into small pieces, and 13 g tissue was digested with 6000 U collagenase (C6885, collagenase from clostridium histolyticum, Type II, Sigma-Aldrich, St. Louis, MI, USA) in 15 ml Krebs-Ringer bicarbonate with glucose (KRBG) buffer (121 mM NaCl, 4.85 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 30 mM glucose, 1.25 mM CaCl<sub>2</sub>, penicillin 100 U/ml, streptomycin 100 $\mu$ g/ml) at 37°C for 50 min. The released stromal/vascular (S/V) cells or preadipocytes were centrifuged at 800 g for 10 minutes at room temperature. The S/V cells were washed 3 times with DMEM/F12 medium followed by centrifugation. The cells were plated in DMEM/F12 with 10% fetal bovine serum at a density of 8 $\times$ 10<sup>4</sup> cell/cm<sup>2</sup> [16].

### 2.4. Incubation of human primary adipocytes with recombinant hSAA1 or DHA

When the S/V cells were approximately confluent, the medium was removed and replaced with serum-free, hormone-supplemented differentiation medium (DMEM/F12 containing 14.2 mM sodium bicarbonate, 2 mM L-glutamine, 10 mg/ml transferrin, 33  $\mu$ M biotin, 17  $\mu$ M pantothenate, 0.5  $\mu$ M insulin, 1  $\mu$ M dexamethasone, 1 nM triiodothyronine, 1  $\mu$ M rosiglitazone, 0.25 mM 3-isobutyl-methylxanthine, 100 U penicillin/ml, 100 mg streptomycin/ml and 1.5  $\mu$ g/ml amphotericin B) for 3 days to induce adipogenesis. The medium was replaced with maintenance differentiation medium, the same medium without rosiglitazone and 3-isobutyl-methylxanthine [16]. On the 13th day, approximately 70% of the cells were differentiated (visual appraisal of cells with lipid droplets). The cells were treated with only DMEM/F12 for 24 h. After that, the medium was replaced with DMEM/F12 containing 0, 0.2 or 1  $\mu$ M of hSAA1 (Acris, Hiddenhausen, Germany) for culturing 24 h. Due to the limitation of cell number harvested, cells from only four women were used in the SAA experiment. The treatment concentrations were chosen based on the physiological concentration of SAA which ranges from 0.59 to 0.74  $\mu$ M in human [1]. The cells were also treated with 0, 50 or 100  $\mu$ M DHA (Nu-Check, Elysian, MN). The fatty acids were bound to 1% bovine serum albumin before addition to the medium to reduce the toxicity of free fatty acids. The treatment concentrations were chosen because the physiological concentration of DHA ranges 82 to 91  $\mu$ M in human [17,18]. Under our treatment procedures, the cell viability estimated by lactate dehydrogenase activity in the medium was not affected by the DHA treatments (data not shown). After 24 h, the culture medium was collected to determine the glycerol concentration using a Free Glycerol Assay Kit (BioVision, Mountain View, CA, USA), and RNA was extracted from the cells.

### 2.5. Total RNA extraction and RNA amplification

Total RNA was extracted from cells using the TRI Reagent (Ambion, Foster City, CA, USA). The mRNA for microarray analysis was amplified with a MessageAmp II aRNA amplification reagent kit (Ambion). Amplified aRNA was quantified at 260 nm using the Nano Drop Spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA). The quality of RNA was also monitored by the 260-/280-nm absorbance.

### 2.6. Microarray analysis for gene expression

The aRNA was fluorescence-labelled using the ULS Labeling Kit (Kreatech, Amsterdam, Netherlands) following the manufacturer's procedure. The efficiency of fluorescence labeling achieved was >2.0%. The CustomArray metabolism 12K microarray (CombiMatrix, Mukilteo, WA, USA) for gene expression analysis was used. Fluorescence-labeled aRNA 4  $\mu$ g was used for hybridization for 16 h. The fluorescent images of the chips were scanned by GenePix 4000B microarray chip scanner (Molecular Devices, Sunnyvale, CA, USA). The data were analyzed by the online software (Microarray Imager, CombiMatrix). Several differentially expressed genes identified by microarray analysis were confirmed using quantitative real-time polymerase chain reaction (qPCR).

### 2.7. Quantitative real-time PCR analysis

For quantitative real-time PCR (qPCR), 5  $\mu$ g RNA was digested with DNase I (Epicentre, Madison, WI, USA) and 3  $\mu$ g RNA was reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) using a random primer procedure. The cDNA for various genes was amplified using the DyNAmo Flash SYBR Green Kit (Finnzymes, Espoo, Finland) with paired sense and antisense primers designed from human gene sequences. The primer pairs and optimized annealing temperature for individual genes are shown in Table 1. The conditions for qPCR were denatured at 95°C for 10 s (7 min in the first cycle), annealed at the optimized annealing temperature for 15 s and extended at 74°C for 5 s. The qPCR reaction was carried out for 40 cycles. Melting curve analysis was routinely performed by increasing the temperature from 55°C to 95°C and detecting signals every 0.5°C. The mRNA concentration of each gene was normalized to the  $\beta$ -actin mRNA concentration in the same sample. Amplification of specific transcripts was further confirmed by melting curve profile analysis and agarose gel electrophoresis. Primer efficiency was calculated using the formula  $[10^{(-1/\text{slope})}] - 1$  [19]. Threshold cycle (Ct) values were obtained, and relative gene expression ratio was calculated using the formula  $(1 + \text{efficiency of target gene})^{\Delta\text{Ct of target } (\beta\text{-actin} - \text{target gene})} / (1 + \text{efficiency of } \beta\text{-actin})^{\Delta\text{Ct of } \beta\text{-actin } (\beta\text{-actin} - \text{target gene})}$  [19].

### 2.8. Statistical analysis

For each replicate, the control value for a variable was set to one with other variables expressed relative to the control. Homogeneity of the variance was determined, and the treatment effects were analyzed using analysis of variance to determine the effects of DHA or hSAA1 at different concentrations. Tukey's test was used to evaluate differences among means (SAS Institute, Cary, NC, USA), except Fig. 1 which was analyzed by *t* test. A significant difference indicates that the *P* value was  $\leq 0.05$ .

## 3. Results

### 3.1. Effect of recombinant hSAA1 on the expression of genes related to lipid metabolism in HepG2 cells

The microarray served as a preliminary screening tool that allowed us to examine the effect of hSAA on overall gene expression. Only one pooled sample from each treatment was used in the analysis, so the results were only used to pick up target genes for further analysis and confirmation. The data showed that 2  $\mu$ M hSAA1 clearly affected the expression of genes related to lipid metabolism. Using qPCR, we confirmed that treatment for 72 h with 2  $\mu$ M hSAA1 significantly decreased the mRNA concentrations (Fig. 1) approximately 30% for the lipogenic gene, FAS; 50% for peroxisome proliferator-activated receptor (PPAR)  $\alpha$  and 30% for acyl-CoA oxidase (ACO), genes involved with fatty acid oxidation and 30% each for hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) and liver fatty acid binding protein (L-FABP), genes involved with cytoplasmic fatty acid transport. SREBP1c was not affected by hSAA1 (Fig. 1).

### 3.2. Effect of hSAA1 on human adipocytes

The treatment of human preadipocytes that were differentiated into adipocytes with 1  $\mu$ M hSAA1 protein treatment significantly increased the release of glycerol into the culture medium (Fig. 2A). This indicates that recombinant hSAA1 increased lipolysis in these cells. The 1  $\mu$ M hSAA1 treatment also promoted HSL mRNA expression (Fig. 2B). The expression of perilipin mRNA was reduced about 60% by incubation with either 0.2 or 1  $\mu$ M hSAA1 (Fig. 2C), suggesting that the lipolytic

Table 1  
Primer sequences for real-time PCR

Gene name	Primers 5'→3'	Length (bp)	Annealing temperature(°C)	Reference sequence
SAA1-s	CTGCAGAAGTGATCAGCG	237	54	DQ367410
SAA1-a	ATTGTGTACCCCTCCTCC			
LPL-s	CCGCCGACCAAGAAGAGAT	159	57	NM_000237
LPL-a	TTCTGTACCGTCCAGCCAT			
TNF $\alpha$ -s	GGACCTCTCTAATCAGCCCTC	49	64	NM_000594.2
TNF $\alpha$ -a	TCGAGAAGATGATCTGACTGCC			
IL-6-s	TGGCTGAAAAGATTGGATGCT	50	55	NM_000600.2
IL-6-a	AACTCCAAAAGACCAGTGATGATTT			
PPAR $\gamma$ -s	TCCATGCTGTTATGGGTGAAACT	72	60	NM_015869.4
PPAR $\gamma$ -a	CTTTACCTTGTGATATGTTGCAGACA			
HSL-s	TCAGTGTCTAGGTGACTGG	135	62	NM_005357.2
HSL-a	AGGCTTCTGTGGGTATTGGA			
FAS-s	ACAGGGACAACCTGGAGTTC	151	60	NM_004104.4
FAS-a	CTGTGGTCCCACTGATGAGT			
PPAR $\alpha$ -s	GCAGAAACCCAGAATCAGC	141	56	NM_001001928
PPAR $\alpha$ -a	ATGGCCCACTGTAAGAAACG			
Perilipin-s	GACAACGTGGTGACACAGT	78	57	NM_002666.3
Perilipin-a	CTGGTGGGTTGTGATGTC			
L-FABP-s	TTTCTCCGGCAAGTACCAAC	227	60	NM_001443
L-FABP-a	TCTCCCTGTCATTGTCTCC			
HNF4 $\alpha$ -s	AGAGCAGGAATGGGAAGGAT	151		NM_000457
HNF4 $\alpha$ -a	GCACTGGCTTCAACATGAGA			
SREBP1c-s	GGTGAGTGGCGAACCATC	73	63	NM_001005291
SREBP1c-a	GAGCCGGTTGATAGGCAGC			
ACO-s	CTCGACACCAGATGAAAT	61	150	NM_007292
ACO-a	AGGTCATAAGTGGCTGTGG			

effect produced by incubation with hSAA1 may result from increased expression of HSL coupled with decreased expression of perilipin.

Treatment with hSAA1 resulted in the expression of mRNA for the transcription factors PPAR $\gamma$  and PPAR $\alpha$  mRNA at only 30% and 50%, respectively, of the controls (Fig. 2D). The expression of lipogenesis-related genes was decreased by either 0.2 or 1  $\mu$ M hSAA1 treatment, approximately 50% for both lipoprotein lipase (LPL) and FAS (Fig. 2E).

In human adipocytes, the expression of interleukin (IL)-6 and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) were increased by both 0.2- and 1- $\mu$ M hSAA1 treatments for 24 h. The IL-6 mRNA was increased fivefold (Fig. 2F), and the TNF $\alpha$  mRNA was increased 8–12-fold (Fig. 2G) compared to the control group.

### 3.3. Regulation of SAA mRNA by DHA in human adipocytes

Both 50- and 100- $\mu$ M DHA treatments increased the expression of SAA1 mRNA compared with the control group (Fig. 3). The increments ranged from 2.5- to 2.7-fold.

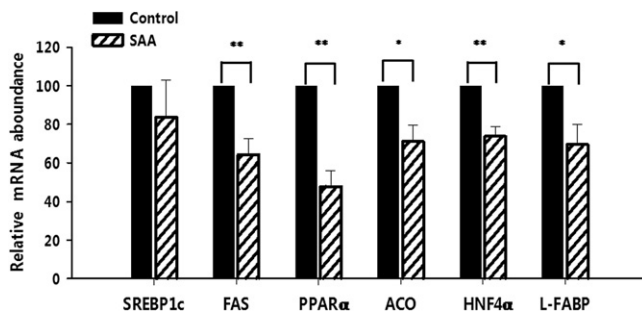


Fig. 1. Modulation of lipid metabolism genes by hSAA1 in HepG2 cells. Quantitative real-time PCR confirmed microarray data that mRNA expression of some lipid metabolism related genes were decreased by hSAA1. FAS, PPAR $\alpha$ , ACO, HNF4 $\alpha$  and L-FABP mRNA were significantly reduced by incubation with 2  $\mu$ M hSAA1 for 72 h in vitro. The mRNA concentrations of individual genes were determined and normalized to the mRNA concentration for  $\beta$ -actin in the same sample using real-time PCR. The bars indicate the means  $\pm$  S.E. for cells from three independent replicates ( $n=3$ ). \* $P \leq .05$ ; \*\* $P \leq .01$ .

### 3.4. Effects of DHA on human adipocytes

The expression of the IL-6 mRNA was increased 6 and 10 times compared with the control group by 50  $\mu$ M and 100  $\mu$ M DHA, respectively (Fig. 4A). The expression of TNF $\alpha$  mRNA tended to be increased by DHA treatments (Fig. 4B). The treatment of human adipocytes with DHA increased the release of glycerol (Fig. 5A). The 100 $\mu$ M DHA treatment doubled the expression of HSL mRNA and decreased perilipin mRNA to 60% of the control group (Fig. 5B). The PPAR $\gamma$  mRNA was reduced by 50- or 100- $\mu$ M DHA treatment compared to the control group (Fig. 5C). The expression of PPAR $\alpha$  mRNA was increased by DHA treatments compared to the control group (Fig. 5C). The FAS and LPL mRNAs were significantly decreased by DHA treatments (Fig. 5D).

## 4. Discussion

Because hepatic SAA mRNA is induced by dietary DHA in pigs [20], we chose the human hepatocarcinoma cell line, HepG2, to study the effect of human SAA on human hepatic gene expression. Hepatic SREBP1c and FAS are involved in lipogenesis [14,21], and SREBP1c up-regulates FAS expression [22]. Expression of FAS is also regulated by PPAR $\alpha$  because the addition of PPAR $\alpha$  ligands increase FAS expression in wild type mice, but not in PPAR $\alpha^{-/-}$  mice [23]. In our experiments, SAA down-regulated the expression of PPAR $\alpha$ , which may then reduce FAS mRNA expression. The SAA treatment had no effect on SREBP1c, suggesting that there is no role of this transcription factor in the SAA regulation of FAS. PPAR $\alpha$  can regulate fatty acid oxidation by binding to the PPAR response element of the ACO promoter to increase the expression of ACO [24], a key enzyme in the  $\beta$ -oxidation of fatty acids in peroxisomes. We found that ACO expression was down-regulated by hSAA1 treatment speculatively from the SAA-decreased PPAR $\alpha$ . HNF4 $\alpha$  and L-FABP, two proteins that participate in lipid transport within the cytoplasm, were decreased by hSAA1 treatment. Thus, SAA1 may decrease the transportation of fatty acids in cytoplasm and consequently decrease the utilization of fatty acids in adipocytes. L-FABP is a target gene of PPAR $\alpha$  which suggests that the fatty acid transport ability is affected by regulation of PPAR $\alpha$  [25].

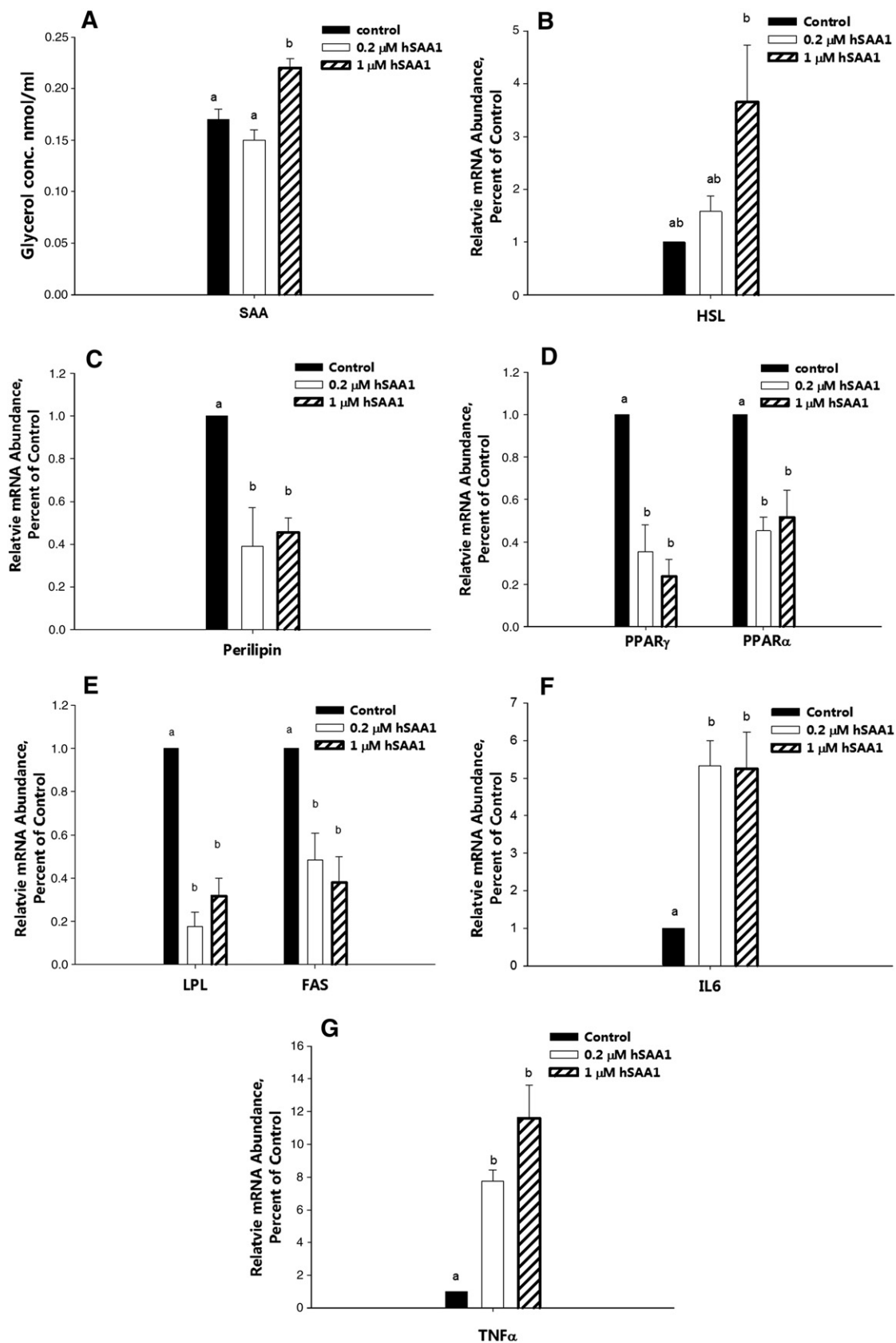


Fig. 2. The effect of hSAA1 on the expression of genes related to lipid metabolism and the release of glycerol in human adipocytes. Human breast preadipocytes were isolated, differentiated and then treated with 0.2 and 1  $\mu$ M hSAA1 for 24 h. Glycerol was analyzed in the medium and RNA was extracted from the cells. The mRNA concentration was quantified by real-time PCR analysis and related to the  $\beta$ -actin mRNA concentration in the same sample. Glycerol release to the culture medium (A), HSL mRNA (B), Perilipin mRNA (C), PPAR $\gamma$  and PPAR $\alpha$  mRNA (D), LPL, FAS mRNA (E), IL-6 mRNA (F) and TNF $\alpha$  mRNA (G). Data are expressed as mean  $\pm$  S.E.M.,  $n=4$  independent experiments. Different superscripts indicate a statistical significance ( $P<0.05$ ).

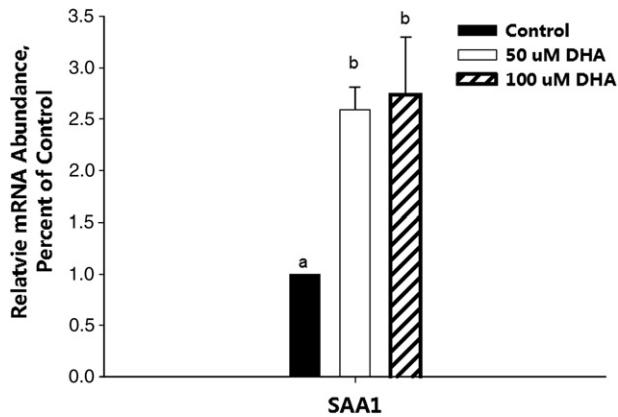


Fig. 3. The effect of DHA on the expression of SAA1 in adipocytes. Human breast preadipocytes were isolated, differentiated and then treated with 0, 50 or 100 μM DHA for 24 h. The cellular mRNA concentration of SAA was analyzed by real time PCR and related to the β-actin mRNA concentration in the same sample. The data were expressed as means ± S.E.M. from five independent replicates ( $n=5$ ). Different superscripts indicate a statistical significance ( $P<.05$ ).

DHA has been shown to increase SAA expression in porcine liver [20] and hepatic cells [14] so we treated 50 and 100 μM DHA in human preadipocytes to study the gene expression pattern compared to the SAA treatment. DHA increased the expression of SAA, TNFα and IL-6, three genes previously considered as proinflammatory elements. However, the DHA-induced SAA expression was about twofold in human adipocytes and fourfold in porcine hepatocytes [14]. These effects are much lower than what is observed in inflammatory responses (15–250 times) in animal studies [1,26,27]. When human monocytes are infected with bacteria, the concentrations of IL-6 and TNFα are increased to 30 and 80 times, respectively [28]. During murine cytomegalovirus infection, TNFα and IL-6 are increased to 150 and 1500 times, respectively [29]. These are acute-phase inflammatory responses. The lower SAA1, IL-6 and TNFα responses induced by DHA compared to true inflammatory responses may represent divergent physiological responses. The IL-6 treatment inhibits lipopolysaccharide-induced TNFα production in both human monocytes and human monocytic line U937 [30,31]. Therefore, IL-6 is a likely anti-inflammatory cytokine that can stimulate the expression of other anti-inflammatory cytokines and factors, for example, IL-10, IL-1 receptor antagonist, soluble TNFα receptor and cortisol during acute inflammation in normal subjects [32–34]. It has also been reported that the administration of recombinant IL-6 to IL-6<sup>-/-</sup> mice suppressed the expression of TNFα [35], suggesting an anti-inflammatory effect of IL-6. Other reports also indicate that DHA treatment decreases IL-2 and interferon gamma production (both are regarded as inflammation cytokines), whereas the production of IL-10 was increased in Jurkat T cell [36,37]. Furthermore, the definition of inflammation should include several clinical signs: the inflammation-related cytokines expression; the elevation of macrophage or polymorphonuclear leukocyte infiltration; the increase of white blood cell accumulation; the increased expression of cyclooxygenase, eicosanoids and leukotrienes and the production of reactive oxygen species. According to these signs, we cannot define whether DHA induces inflammation in our system, based only on the increased expression of IL-6 and TNFα.

The expression of PPARγ occurs primarily in adipocytes, and it participates in the transcriptional activation of numerous adipogenic genes, including FAS, SREBP1c and LPL [38,39]. We found that the mRNA for PPARγ, FAS and LPL was decreased by hSAA1 treatment in human adipocytes, indicating that SAA1 can reduce adipogenic and lipogenic genes and potentially decrease the accumulation of triacylglycerol in adipocytes. Moreover, DHA

increased the expression of SAA and decreased PPARγ, LPL and FAS mRNA. Therefore, DHA and SAA treatments had similar effects on adipogenic and lipogenic genes in human adipocytes. The data suggest that the mechanism for the DHA effects may be through an increase in SAA. The direct evidence for this hypothesis has not yet been shown.

The function of perilipin protein is to prevent untimely lipid mobilization and maintain the structure of lipid droplets. When energy is needed, the PKA-phosphorylated perilipin allows PKA-activated (phosphorylated) HSL to hydrolyze triacylglycerols and release free fatty acids and glycerol [15,40]. There is no glycerol kinase to metabolize glycerol in adipocytes, so glycerol is released into the circulation in vivo or into the culture medium in vitro. Hence, glycerol release is an indication of lipolysis. In the current study, both hSAA1 and DHA treatments increased the glycerol concentration in culture medium, indicating elevated lipolysis.

Because IL-6 increases the release of glycerol [41] and stimulates fatty acid oxidation [41,42] in human adipocytes, our finding that hSAA1 and DHA treatments increased the expression of IL-6 suggest that at least part of the effect was mediated by IL-6. In 3T3L1 adipocytes, TNFα down-regulates the expression of LPL and increases glycerol release [43,44]. TNFα also can inhibit the activity of perilipin by phosphorylation with mitogen activated protein kinases [44] and can activate PKA to phosphorylate perilipin [45]. The current study found that hSAA1 and DHA treatments increased the expression of

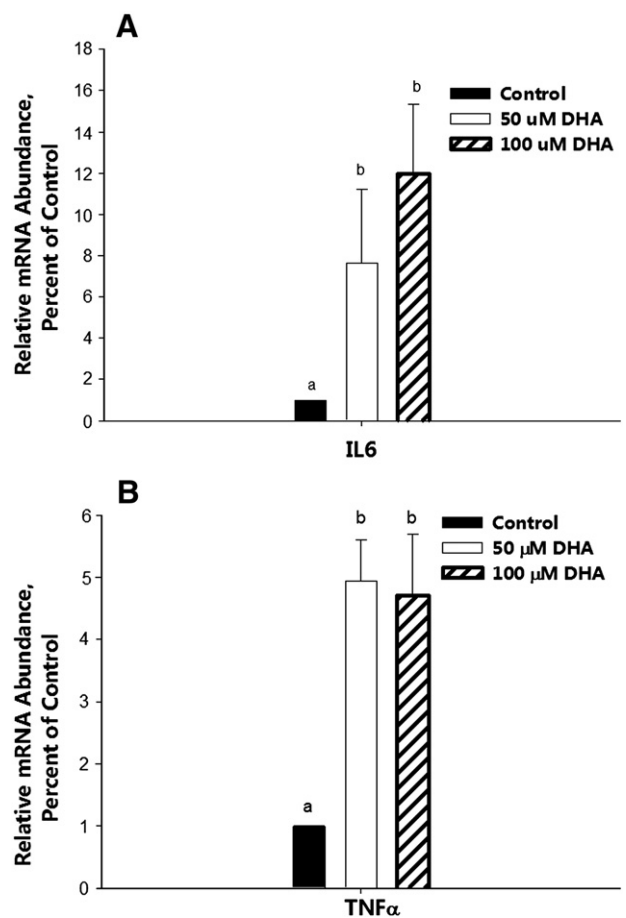


Fig. 4. The effect of DHA on the expression of inflammation-related genes in adipocytes. Human breast preadipocytes were isolated, differentiated and then treated with 0, 50 or 100 μM DHA for 24 h. The cellular mRNA concentrations for IL-6 (A) and TNFα were analyzed by qPCR and related to the β-actin mRNA concentration in the same sample (B). The data were expressed as means ± S.E.M. from five independent replicates ( $n=5$ ). Different superscripts indicate a statistical significance ( $P<.05$ ).

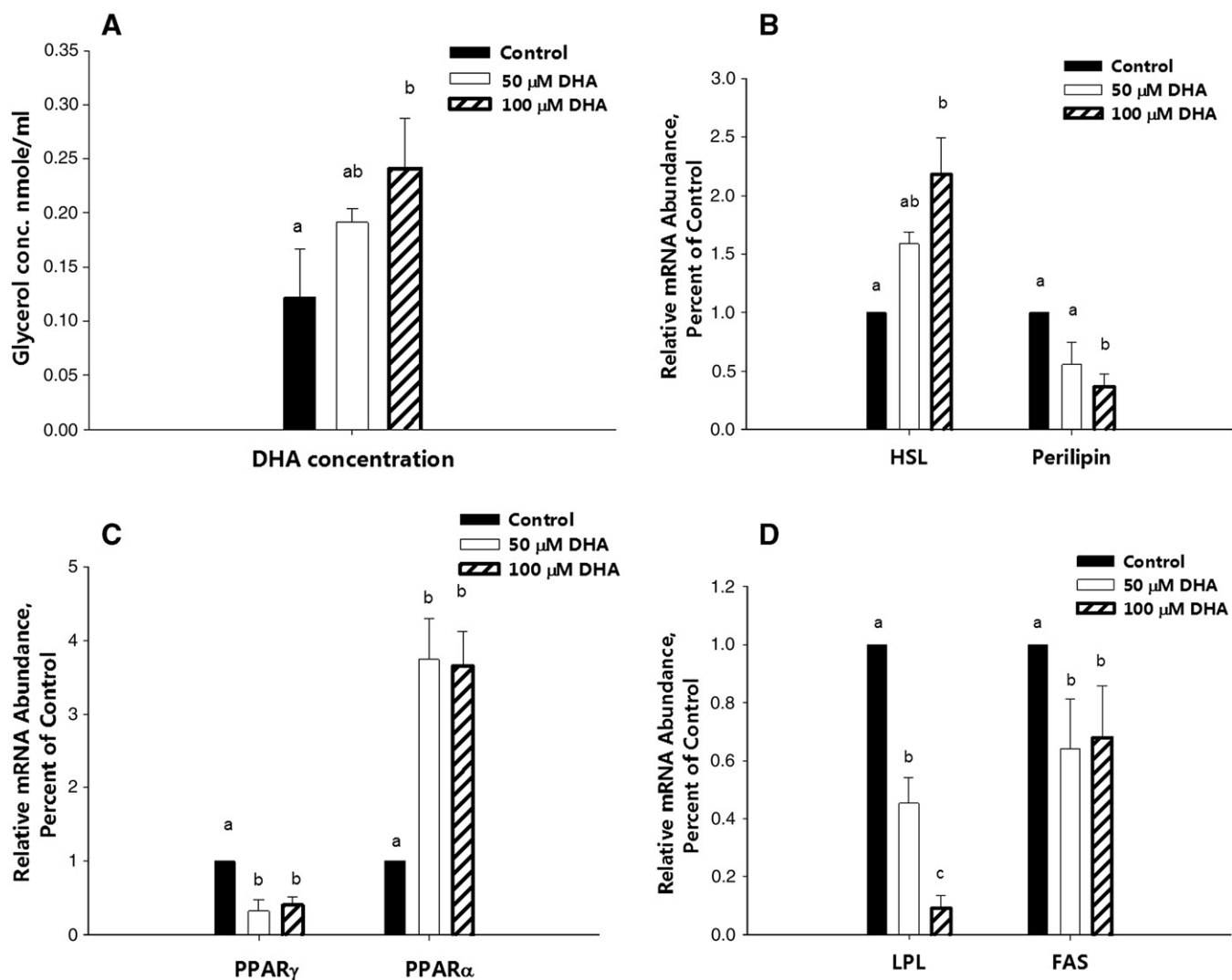


Fig. 5. The effect of DHA on the expression of genes related to lipid metabolism and the release of glycerol in human adipocytes. The human breast preadipocytes were isolated and differentiated and then treated with 0, 50 or 100  $\mu$ M DHA for 24 h. The RNA was extracted from the cells and the mRNA concentration was quantified by qPCR analysis and related to the  $\beta$ -actin mRNA concentration in the same sample. Glycerol release in the culture medium (A), HSL and perilipin mRNA (B), PPAR $\gamma$  and PPAR $\alpha$  mRNA (C), Lipoprotein lipase (LPL) and FAS mRNA (D). Data are expressed as mean  $\pm$  S.E.M.,  $n=5$  independent experiments. Different superscripts indicate a statistical significance ( $P<0.05$ ).

TNF $\alpha$ , so we speculate that hSAA1- and DHA-induced TNF $\alpha$  reduced the expression of perilipin. Moreover, perilipin expression can be stimulated by PPAR $\gamma$  to accumulate lipid [46]. Our current study showed that both DHA and hSAA1 treatments reduced the expression of PPAR $\gamma$  mRNA to potentially reduce expression of perilipin. The combined mechanisms to reduce perilipin and increase HSL expression coupled with accentuated phosphorylation potential may all work in concert to stimulate lipolysis.

DHA may inhibit or increase TNF $\alpha$  and IL-6 expression or concentration depending on the cell type or culture medium conditions. Dietary DHA-rich fish oil supplementation up-regulates serum IL-6 and TNF $\alpha$  concentrations in human leukocytes [47]. Treatment with DHA increases the production of TNF $\alpha$  and IL-6 in macrophages [48]. In contrast, 50 or 500  $\mu$ M of DHA had no effect on TNF $\alpha$  in murine 3T3L1 adipocytes [49]. Furthermore, TNF $\alpha$  and IL-6 secretion by human mononuclear cells is inhibited by dietary fish-oil supplementation [50]. In the current study, we demonstrated that DHA increased the expression of TNF $\alpha$  and IL-6. These increments may then modify the cellular function of human adipocytes.

The DHA treatment promotes PPAR $\alpha$  mRNA expression [51,52]. The PUFA are natural PPAR $\alpha$  ligands, with simultaneous stimulation

of fatty acid oxidation and inhibition of fatty acid synthesis [53]. Incubation of human adipocytes with DHA increased the expression of PPAR $\alpha$ , whereas incubation with hSAA1 decreased the expression of PPAR $\alpha$ , so that the effects of DHA and SAA were opposite. Which regulatory mechanisms are associated with these divergent effects of DHA and SAA are not clear now. In liver cells (HepG2), SAA reduced both PPAR $\alpha$  and ACO mRNAs suggesting inhibition of fatty acid oxidation.

Although conjugated linoleic acid (CLA) and DHA are different in the position and number of double bonds, they have similar effects on alleviating obesity and the expression of lipid metabolism related genes [54]. Microarray profiling of white adipose tissue of mice fed trans-10, cis-12 CLA indicated that CLA reduced the expression of genes involved in lipogenesis and adipogenesis such as LPL, FAS, fatty acid binding protein 4 and PPAR $\gamma$  [55]. In the current study, DHA treatment also reduced LPL, FAS and PPAR $\gamma$  in human adipocytes and increased transcript levels for IL-6 and TNF $\alpha$ . Similar to our findings, CLA reduces the expression of lipid synthesis genes, such as FAS, HSL, LPL [56] and perilipin [57]. Moreover, CLA increases the gene expression of IL-6 in human adipose tissue [57]. DHA treatment reduces body weight and energy intake in human subjects [58,59] and

promotes gene expression of TNF $\alpha$  and reduces PPAR $\gamma$ . However, CLA has no effect on these genes and body weight in humans [57]. Although there are similar effects between CLA and DHA on prevention or improvement of obesity, the role and function of different fatty acids can be very different.

In conclusion, we have demonstrated that DHA increases the expression of SAA1 in human adipocytes and that the effects of SAA1 and DHA on expression of the lipolytic genes, TNF $\alpha$ , IL-6, HSL and perilipin in human adipocytes are parallel. In contrast, PPAR $\gamma$ , LPL and FAS mRNAs were decreased by the SAA1 and DHA treatments, suggesting that DHA and SAA1 also inhibit adipogenesis in human adipocytes. These results suggest that DHA may enhance lipolytic activity and decrease lipogenic and adipogenic activity by regulating the expression of SAA1. Such DHA function will be useful for developing new approaches to reduce body fat deposition.

## References

- [1] Yang RZ, Lee MJ, Hu H, Pollin TI, Ryan AS, Nicklas BJ, et al. Acute-phase serum amyloid A: an inflammatory adipokine and potential link between obesity and its metabolic complications. *PLoS Med* 2006;3:e287.
- [2] Stulnig TM. Immunomodulation by polyunsaturated fatty acids: mechanisms and effects. *Int Arch Allergy Immunol* 2003;132:310–21.
- [3] Suresh Y, Das UN. Long-chain polyunsaturated fatty acids and chemically induced diabetes mellitus. Effect of omega-3 fatty acids. *Nutrition* 2003;19:213–28.
- [4] Narayanan BA, Narayanan NK, Reddy BS. Docosahexaenoic acid regulated genes and transcription factors inducing apoptosis in human colon cancer cells. *Int J Oncol* 2001;19:1255–62.
- [5] Rojas CV, Martinez JI, Flores I, Hoffman DR, Uauy R. Gene expression analysis in human fetal retinal explants treated with docosahexaenoic acid. *Invest Ophthalmol Vis Sci* 2003;44:3170–7.
- [6] Duplus E, Glorian M, Forest C. Fatty acid regulation of gene transcription. *J Biol Chem* 2000;275:30749–52.
- [7] Hsu JM, Ding ST. Effect of polyunsaturated fatty acids on the expression of transcription factor adipocyte determination and differentiation-dependent factor 1 and of lipogenic and fatty acid oxidation enzymes in porcine differentiating adipocytes. *Br J Nutr* 2003;90:507–13.
- [8] Hsu JM, Wang PH, Liu BH, Ding ST. The effect of dietary docosahexaenoic acid on the expression of porcine lipid metabolism-related genes. *J Anim Sci* 2004;82:683–9.
- [9] Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 1997;89:331–40.
- [10] Gomez-Ambrosi J, Salvador J, Rotellar F, Silva C, Catalan V, Rodriguez A, et al. Increased serum amyloid A concentrations in morbid obesity decrease after gastric bypass. *Obes Surg* 2006;16:262–9.
- [11] Jernas M, Palmring J, Sjöholm K, Jennische E, Svensson PA, Gabrielson BG, et al. Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *Faseb J* 2006;20:1540–2.
- [12] Poutou C, Viguier N, Cancellor R, De Matteis R, Cinti S, Stich V, et al. Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. *Diabetologia* 2005;48:519–28.
- [13] Urieli-Shoval S, Cohen P, Eisenberg S, Matzner Y. Widespread expression of serum amyloid A in histologically normal human tissues. Predominant localization to the epithelium. *J Histochem Cytochem* 1998;46:1377–84.
- [14] Chen CH, Wang PH, Liu BH, Mersmann HJ, Ding ST. Serum amyloid A protein regulates the expression of porcine genes related to lipid metabolism. *J Nutr* 2008;138:674–9.
- [15] Festuccia WT, Laplante M, Berthiaume M, Gelinat Y, Deshaies Y. PPARgamma agonism increases rat adipose tissue lipolysis, expression of glyceride lipases, and the response of lipolysis to hormonal control. *Diabetologia* 2006;49:2427–36.
- [16] Liu BH, Kuo CF, Wang YC, Ding ST. Effect of docosahexaenoic acid and arachidonic acid on the expression of adipocyte determination and differentiation-dependent factor 1 in differentiating porcine adipocytes. *J Anim Sci* 2005;83:1516–25.
- [17] Pawlosky RJ, Hibbeln JR, Herion D, Kleiner DE, Salem N. Compartmental analysis of plasma and liver n-3 essential fatty acids in alcohol-dependent men during withdrawal. *J Lipid Res* 2008.
- [18] Attar-Bashi NM, Weisinger RS, Begg DP, Li D, Sinclair AJ. Failure of conjugated linoleic acid supplementation to enhance biosynthesis of docosahexaenoic acid from alpha-linolenic acid in healthy human volunteers. *Prostaglandins Leukot Essent Fatty Acids* 2007;76:121–30.
- [19] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45.
- [20] Chang WJ, Chen CH, Cheng WTK, Ding ST. The effects of dietary docosahexaenoic acid enrichment on the expression of porcine genes. *Asian-Aust J Anim Sci* 2007;20:768–74.
- [21] Seo JB, Moon HM, Kim WS, Lee YS, Jeong HW, Yoo EJ, et al. Activated liver X receptors stimulate adipocyte differentiation through induction of peroxisome proliferator-activated receptor gamma expression. *Mol Cell Biol* 2004;24:3430–44.
- [22] Diraison F, Parton L, Ferre P, Foufelle F, Briscoe CP, Leclerc I, et al. Over-expression of sterol-regulatory-element-binding protein-1c (SREBP1c) in rat pancreatic islets induces lipogenesis and decreases glucose-stimulated insulin release: modulation by 5-aminimidazole-4-carboxamide ribonucleoside (AICAR). *Biochem J* 2004;378:769–78.
- [23] Knight BL, Hebbachi A, Hauton D, Brown AM, Wiggins D, Patel DD, et al. A role for PPARalpha in the control of SREBP activity and lipid synthesis in the liver. *Biochem J* 2005;389:413–21.
- [24] Marcus SL, Miyata KS, Zhang B, Subramani S, Rachubinski RA, Capone JP. Diverse peroxisome proliferator-activated receptors bind to the peroxisome proliferator-responsive elements of the rat hydratase/dehydrogenase and fatty acyl-CoA oxidase genes but differentially induce expression. *Proc Natl Acad Sci U S A* 1993;90:5723–7.
- [25] Poirier H, Niot I, Monnot MC, Braissant O, Meunier-Durmort C, Costet P, et al. Differential involvement of peroxisome-proliferator-activated receptors alpha and delta in fibrate and fatty-acid-mediated inductions of the gene encoding liver fatty-acid-binding protein in the liver and the small intestine. *Biochem J* 2001;355:481–8.
- [26] Tecles F, Fuentes P, Martinez Subiela S, Parra MD, Munoz A, Ceron JJ. Analytical validation of commercially available methods for acute phase proteins quantification in pigs. *Res Vet Sci* 2007;83:133–9.
- [27] Parra MD, Fuentes P, Tecles F, Martinez-Subiela S, Martinez JS, Munoz A, et al. Porcine acute phase protein concentrations in different diseases in field conditions. *J Vet Med B Infect Dis Vet Public Health* 2006;53:488–93.
- [28] Stauffer F, Petrow EP, Burgmann H, Graninger W, Georgopoulos A. Release of TNF alpha and IL6 from human monocytes infected with *Mycobacterium kansasii*: a comparison to *Mycobacterium avium*. *Infection* 1994;22:326–9.
- [29] Ruzek MC, Miller AH, Opal SM, Pearce BD, Biron CA. Characterization of early cytokine responses and an interleukin (IL)-6-dependent pathway of endogenous glucocorticoid induction during murine cytomegalovirus infection. *J Exp Med* 1997;185:1185–92.
- [30] Petersen AM, Pedersen BK. The anti-inflammatory effect of exercise. *J Appl Physiol* 2005;98:1154–62.
- [31] Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. *Blood* 1990;75:40–7.
- [32] Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* 1994;83:113–8.
- [33] Bethin KE, Vogt SK, Muglia LJ. Interleukin-6 is an essential, corticotropin-releasing hormone-independent stimulator of the adrenal axis during immune system activation. *Proc Natl Acad Sci U S A* 2000;97:9317–22.
- [34] Steensberg A, Fischer CP, Keller C, Moller K, Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am J Physiol Endocrinol Metab* 2003;285:E433–7.
- [35] Xing Z, Gaudie J, Cox G, Baumann H, Jordana M, Lei XF, et al. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 1998;101:311–20.
- [36] Verlengia R, Gorjao R, Kanunfre CC, Bordin S, Martins De Lima T, Martins EF, et al. Comparative effects of eicosapentaenoic acid and docosahexaenoic acid on proliferation, cytokine production, and pleiotropic gene expression in Jurkat cells. *J Nutr Biochem* 2004;15:657–65.
- [37] Fritsche KL, Anderson M, Feng C. Consumption of eicosapentaenoic acid and docosahexaenoic acid impair murine interleukin-12 and interferon-gamma production in vivo. *J Infect Dis* 2000;182(Suppl 1):S54–61.
- [38] Schadinger SE, Bucher NL, Schreiber BM, Farmer SR. PPARgamma2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes. *Am J Physiol Endocrinol Metab* 2005;288:E1195–205.
- [39] Ding ST, McNeel RL, Mersmann HJ. Expression of porcine adipocyte transcripts: tissue distribution and differentiation in vitro and in vivo. *Comp Biochem Physiol B Biochem Mol Biol* 1999;123:307–18.
- [40] Holm C. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem Soc Trans* 2003;31:1120–4.
- [41] Path G, Bornstein SR, Gurniak M, Chrousos GP, Scherbaum WA, Hauner H. Human breast adipocytes express interleukin-6 (IL-6) and its receptor system: increased IL-6 production by beta-adrenergic activation and effects of IL-6 on adipocyte function. *J Clin Endocrinol Metab* 2001;86:2281–8.
- [42] van Hall G, Steensberg A, Sacchetti M, Fischer C, Keller C, Schjerling P, et al. Interleukin-6 stimulates lipolysis and fat oxidation in humans. *J Clin Endocrinol Metab* 2003;88:3005–10.
- [43] Rahn Landstrom T, Mei J, Karlsson M, Manganiello V, Degerman E. Down-regulation of cyclic-nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes induced by tumour necrosis factor alpha and cAMP. *Biochem J* 2000;346(Pt 2):337–43.
- [44] Ryden M, Arvidsson E, Blomqvist L, Perbeck L, Dicker A, Arner P. Targets for TNF-alpha-induced lipolysis in human adipocytes. *Biochem Biophys Res Commun* 2004;318:168–75.
- [45] Fricke K, Heitland A, Maronde E. Cooperative activation of lipolysis by protein kinase A and protein kinase C pathways in 3T3-L1 adipocytes. *Endocrinology* 2004;145:4940–7.
- [46] Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H, et al. Adipose tissue expression of the lipid droplet-associated proteins S3-12

- and perilipin is controlled by peroxisome proliferator-activated receptor-gamma. *Diabetes* 2004;53:1243–52.
- [47] Gorjao R, Verlengia R, Lima TM, Soriano FG, Boaventura MF, Kanunfre CC, et al. Effect of docosahexaenoic acid-rich fish oil supplementation on human leukocyte function. *Clin Nutr* 2006;25:923–38.
- [48] Tappia PS, Man WJ, Grimble RF. Influence of unsaturated fatty acids on the production of tumour necrosis factor and interleukin-6 by rat peritoneal macrophages. *Mol Cell Biochem* 1995;143:89–98.
- [49] Bradley RL, Fisher FM, Maratos-Flier E. Dietary fatty acids differentially regulate production of TNF-alpha and IL-10 by murine 3T3-L1 adipocytes. *Obesity (Silver Spring)* 2008.
- [50] Trebble T, Arden NK, Stroud MA, Wootton SA, Burdge GC, Miles EA, et al. Inhibition of tumour necrosis factor-alpha and interleukin 6 production by mononuclear cells following dietary fish-oil supplementation in healthy men and response to antioxidant co-supplementation. *Br J Nutr* 2003;90:405–12.
- [51] Delarue J, LeFoll C, Corporeau C, Lucas D. N-3 long chain polyunsaturated fatty acids: a nutritional tool to prevent insulin resistance associated to type 2 diabetes and obesity? *Reprod Nutr Dev* 2004;44:289–99.
- [52] Diep QN, Touyz RM, Schiffrin EL. Docosahexaenoic acid, a peroxisome proliferator-activated receptor-alpha ligand, induces apoptosis in vascular smooth muscle cells by stimulation of p38 mitogen-activated protein kinase. *Hypertension* 2000;36:851–5.
- [53] Larter CZ, Yeh MM, Cheng J, Williams J, Brown S, dela Pena A, et al. Activation of peroxisome proliferator-activated receptor alpha by dietary fish oil attenuates steatosis, but does not prevent experimental steatohepatitis because of hepatic lipoperoxide accumulation. *J Gastroenterol Hepatol* 2008;23:267–75.
- [54] Li JJ, Huang CJ, Xie D. Anti-obesity effects of conjugated linoleic acid, docosahexaenoic acid, and eicosapentaenoic acid. *Mol Nutr Food Res* 2008;52:631–45.
- [55] LaRosa PC, Miner J, Xia Y, Zhou Y, Kachman S, Fromm ME. Trans-10, cis-12 conjugated linoleic acid causes inflammation and delipidation of white adipose tissue in mice: a microarray and histological analysis. *Physiol Genomics* 2006;27:282–94.
- [56] Park Y, Storkson JM, Albright KJ, Liu W, Pariza MW. Evidence that the trans-10, cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids* 1999;34:235–41.
- [57] House RL, Cassady JP, Eisen EJ, McIntosh MK, Odle J. Conjugated linoleic acid evokes de-lipidation through the regulation of genes controlling lipid metabolism in adipose and liver tissue. *Obes Rev* 2005;6:247–58.
- [58] Kunesova M, Braunerova R, Hlavaty P, Tvrzicka E, Stankova B, Skrha J, et al. The influence of n-3 polyunsaturated fatty acids and very low calorie diet during a short-term weight reducing regimen on weight loss and serum fatty acid composition in severely obese women. *Physiol Res* 2006;55:63–72.
- [59] Thorsdottir I, Tomasson H, Gunnarsdottir I, Gisladdottir E, Kiely M, Parra MD, et al. Randomized trial of weight-loss-diets for young adults varying in fish and fish oil content. *Int J Obes (Lond)* 2007;31:1560–6.