Serum Amyloid A Protein Regulates the Expression of Porcine Genes Related to Lipid Metabolism

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Introduction

Adipose tissue is an important organ for fatty acid (FA) synthesis and energy metabolism in pigs. Porcine adipocytes are frequently used to study the effect of PUFA on gene expression (1,2). Both eicosapentaenoic acid and docosahexaenoic acid (DHA) downregulate sterol regulatory element binding protein 1c (SREBP1c) mRNA and protein expression (3); this major transcription factor regulates lipogenesis by increasing expression of FA synthase and acetyl-CoA carboxylase 1 (ACC1) (2,4,5). Although there are differences in the effects of individual (n-3) PUFA on lipolysis, with considerable disparity between eicosapentaenoic acid and DHA (6), we have concentrated on studying the bioactivity of the latter.

In the past, one emphasis of research on the effect of dietary PUFA to reduce lipogenesis and increase lipolysis concerned the molecular mechanisms involved in the function of the SREBP1c and PPARα transcription factors in mammals (7–11). Many other genes mediating PUFA effects have also been reported, and the gene list is still growing (reviewed by (12,13)). Pigs fed DHA supplements have decreased hepatic SREBP1c mRNA and protein, whereas there is no change in adipose tissue SREBP1c (4,14). Dietary DHA also significantly reduced plasma triacylglycerol concentration in pigs (14). Because hepatic SREBP1c is not highly expressed in porcine liver (1), reduction in this protein may only partially explain the reduction in triacylglycerol. These findings suggest that there may be other mechanisms by which DHA reduces lipid deposition in pigs.

We previously demonstrated that dietary DHA treatments increased the expression of porcine hepatic serum amyloid A protein (SAA), a secretory protein that circulates in the blood and may affect overall body metabolism (15). This protein was expressed in the liver but not in the adipose tissue (15). Recent data show that SAA is associated with obesity and lipid metabolism and is highly correlated with BMI (16,17). However, the ability of SAA to regulate the expression of genes involved in lipid metabolism has not been demonstrated. Therefore, we studied

Abstract

Serum amyloid A protein (SAA) is an apolipoprotein that can replace apolipoprotein A1 (apoA1) as the major apolipoprotein of HDL. Porcine hepatic SAA mRNA is increased by dietary docosahexaenoic acid (DHA) treatment. The purpose of this study was to investigate the role of SAA protein in regulating gene expression related to lipid metabolism in pigs. First, we demonstrated that the 100-μmol/L DHA treatment increased SAA and apoA1 mRNA expression in porcine hepatic cell cultures (P < 0.05). Secondly, we produced porcine SAA recombinant protein and found that the addition of SAA to porcine preadipocytes in culture stimulated interleukin-6 (IL-6) mRNA expression (P < 0.05), indicating a similar biological function of porcine SAA and human SAA. We also found PPARα and PPARγ mRNA were decreased (40 and 60%, respectively) in differentiated adipocytes after treatment with 2 μmol/L SAA. SAA treatment also increased inflammatory cytokine gene expression (IL-6 and tumor necrosis factor α) and glycerol release (P < 0.05), indicating increased lipolysis. Because the expression of perilipin, a lipid droplet–protective protein, was reduced by the SAA treatment, we hypothesized that SAA increased lipolysis by decreasing the expression of perilipin, which would then allow an increase in hormone sensitive lipase activity. In conclusion, we demonstrated that the DHA-induced SAA gene expression decreased PPAR expression and consequently downregulated the expression of several genes involved in lipid metabolism. Accordingly, SAA may play a critical role in mediating the function of dietary DHA on lipid metabolism and could be a factor in regulating obesity. J. Nutr. 138: 674–679, 2008.
the effect of DHA on the expression of SAA in hepatocytes and generated the porcine SAA recombinant protein (pSAA) to study its function in adipocytes.

Materials and Methods

Isolation of porcine hepatocytes and cell culture. The animal protocol was approved by the Animal Usage Committee at National Taiwan University. A total of 3-7-d-old small-eared pigs were killed by electrocution and exsanguination for hepatocyte isolation. The liver was perfused with PBS to wash out RBC before tissue was removed, minced, and passed through a 100-μm mesh. This hepatic mince contains hepatocytes but also other hepatic cells, including Kupffer cells, bile duct cells, vascular cells, etc. These hepatic cells were used for hepatocyte culture experiments. Hepatic cells were treated with different concentrations of DHA (0, 25, 50, and 100 μmol/L) in Williams’ Medium E for 24 h (37°C, 5% CO2). The TRI reagent (Sigma) was used to extract total RNA. A total of 2 μg RNA was then reverse transcribed to cDNA. The mRNA concentrations of apolipoprotein A1 (apoA1), SAA, and β-actin were determined by real-time PCR. Liver tissue from these pigs was used to clone the full-length cDNA for SAA (15) by RT-PCR using AccuPrime pfx DNA polymerase (Invitrogen). The primer pair for the cloning of porcine SAA was 5’-5′-GCA GCT CAG CTT CAC CAG GA-3′ and 3′-3′-CTG CTC ACA GGA GCC TCA CA-3′. Primers were designed from the human sequence and included sequences before the start codon for the sense primer and after the stop codon for the antisense primer (15). The cDNA was used for recombinant protein production.

Gene construction and recombinant protein production. The full-length cDNA fragment without a signal peptide sequence was subcloned into the QIAexpressionist protein expression vector, pQE30 (Qiagen). An Opticon 2 Real-Time PCR Detection System (Bio-Rad Laborato-
ries). The PCR was performed under conditions typically consisting of 40 cycles with paired-sense and antisense primers designed from porcine gene sequences. The primer pairs and optimized annealing temperature for the genes are listed in Supplemental Table 1. The conditions for PCR were denaturation at 94°C for 30 s (10 min in cycle 1), annealing at optimized annealing temperature for 30 s, and extension at 72°C for 30 s. The mRNA concentration of each gene was normalized to its β-actin mRNA concentration. Amplification of specific transcripts was further confirmed by melting curve profile analysis and agarose gel electrophoresis. Threshold cycle (Ct) values were obtained and relative gene expression was calculated using the formula (1/2)^ΔΔCt target genes−(1/2) β-actin (20). The PCR amplification efficiency was high for all treatments.

Statistical analyses. For each replicate, the control value for a variable was set to 100, with other variables expressed relative to the control. Routinely, there were 3 replicates, each using preadipocytes isolated from a different pig. Homogeneity of the variance was determined and data were analyzed using ANOVA to determine the effects of DHA or pSAA at different concentrations. The data for tumor necrosis factor (TNF)-α and IL-6 mRNA (Fig. 5) were log transformed before analysis to establish homogeneity of the variance. Tukey’s test was used to evaluate differences among means (SAS Inst.). Differences were considered significant at P ≤ 0.05.

Results

Effects of DHA on the expression of genes in porcine hepatic cells. Treatment with 100 μmol/L DHA tended to increase the expression of apoA1 mRNA ~100% (P = 0.09, Fig. 1A) and significantly increased SAA mRNA expression ~3-fold concentration, following the method of Kreutz (19) for estimation of li-

cer for S/V cells was removed and replaced by serum-free, hormone-

Cell culture and differentiation of porcine adipocytes. The me-
dium for SV cells was removed and replaced by serum-free, hormone-supplemented differentiation medium (DMEM/F12 containing sodium bicarbonate, 0.5 μmol/L insulin, 10 mg/L transferrin, 2 mmol/L L-glutamine, 33 μmol/L biotin, 17 μmol/L pantothenate, 1 μmol/L dexamethasone, 1 mmol/L triiodothyronine, 0.25 mmol/L 3-isobutyl-methylxanthine, 100 μL/L penicillin, 100 μg/L streptomycin, 1.5 mg/L amphotericin B, and 1 μmol/ L rosiglitazone) for 3 d to induce adipogenesis. The medium was then changed to a differentiation medium without rosiglitazone. The medium was replaced every 3 d. After 6 d, the well-differentiated adipocytes (~80% differentiation) were treated with a medium containing 0, 0.2, or 2 μmol/L of pSAA for 24 h to test the effect of pSAA on adipocyte gene expression. The medium from all treatments was collected to determine its glycerol concentration, following the method of Kreutz (19) for estimation of lipolysis activity. Total glycerol was determined (mg/L) and relative concentra-
tions were calculated and presented. The results were the means of 3 independent experiments with SV cells isolated from 3 different pigs.

Real-time PCR analysis. The RNA samples were digested with DNase I to remove genomic DNA contamination and were reverse transcribed at 42°C with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The mRNA for various genes was quantified with the FastStart SYBR Green real-time PCR kit (Roche). They were quantified by an Opticon 2 Real-Time PCR Detection System (Bio-Rad Laborato-

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(Fig. 1B). These results suggest that this hepatic cell culture system functions as expected and the hepatic cells are responsive to DHA treatment. The lower concentrations of DHA did not affect the expression of these 2 genes.

**Effect of pSAA on the expression of IL-6 in porcine preadipocytes.** Treatment of porcine preadipocytes (S/V cells) with 40 and 200 nmol/L pSAA for 24 h increased the expression of IL-6 mRNA by 1.3 and 2.2 times compared with the control group, respectively (P < 0.05, data not shown), indicating that the recombinant protein generated in the current study is biologically active.

**Effects of pSAA on porcine adipocytes.** Treatment with 2 μmol/L pSAA significantly increased the release of glycerol to adipocyte culture medium 4-fold (Fig. 2A), indicating that pSAA increased lipolysis in porcine adipocytes. We hypothesized that the increase was via an increase in the expression of hormone sensitive lipase (HSL) or a decrease in the expression of a lipid droplet–protective protein, perilipin. The expression of perilipin mRNA was reduced by 85% when cells were treated with 2 μmol/L pSAA (P < 0.05, Fig. 2B).

The expression of the transcription factors, CCAAT/enhancer binding protein α and retinoid X receptor α, was not affected by the pSAA treatments, whereas 2 μmol/L reduced the expression of PPARα mRNA ~40% and that of PPARγ mRNA ~60% (Fig. 3). The lower concentration of pSAA (0.2 μmol/L) had no effect. Expression of mRNA for HSL and acyl-CoA oxidase were decreased by 2 μmol/L SAA, suggesting that lipolysis and FA oxidation may be reduced, although carnitine palmitoylCoA transferase I (CPTI) mRNA was increased by 0.2 μmol/L SAA (Fig. 4A). The expression of several lipogenic genes, including ACC1, adipocyte fatty acid binding protein (aP2), and lipoprotein lipase (LPL), were significantly reduced to ~50% of the control group by the treatment with pSAA, indicating that pSAA may downregulate lipogenesis through effects on these genes (Fig. 4B). Concomitantly, the expression of TNFα and IL-6, 2 lipolytic adipocytokines, were greatly increased by pSAA treatment (Fig. 5).

**Discussion**

Because apoA1 is one of the PPARα-regulated genes (21) and DHA can enhance the activity of PPARα (22), we measured the response of porcine hepatic cell apoA1 mRNA to DHA treatment. The DHA treatment tended to increase the expression of apoA1 mRNA. In humans, dietary (n-3) PUFA supplementation increases serum apoA1, indicating that (n-3) PUFA increases the expression of apoA1 in the liver (23). We also found that the mRNA concentration for SAA was increased by the 100-μmol/L DHA treatment, confirming our experiment in the response of porcine hepatic cell apoA1 mRNA to DHA treatment. The DHA treatment tended to increase the expression of apoA1 mRNA. In humans, dietary (n-3) PUFA supplementation increases serum apoA1, indicating that (n-3) PUFA increases the expression of apoA1 in the liver (23). We also found that the mRNA concentration for SAA was increased by the 100-μmol/L DHA treatment, confirming our experiment in

**FIGURE 2** The effect of pSAA on glycerol release (A) and the expression of perilipin mRNA (B) in porcine preadipocytes cultured in serum-free, hormone-supplemented differentiation medium for 3 d to induce adipogenesis. After 6 d, the well-differentiated adipocytes were treated with medium containing 0, 0.2, or 2 μmol/L of pSAA for 24 h. Bars are means ± SE, n = 3 independent measures. Means without a common letter differ, P = 0.05.

**FIGURE 3** The effect of pSAA on the expression of transcription factors related to lipid metabolism in porcine adipocytes. The well-differentiated adipocytes, as described in Figure 2, were treated with medium containing 0, 0.2, or 2 μmol/L of pSAA for 24 h. The total RNA was extracted to determine the effect of SAA on the expression of transcription factors, CCAAT/enhancer binding protein α (C/EBPα), retinoid X receptor α (RXRα), PPARα, and PPARγ in porcine adipocytes. Bars are means ± SE, n = 3 independent measures. Labeled means without a common letter differ, P ≤ 0.05.

**FIGURE 4** The effect of pSAA on the expression of genes related to lipid metabolism in porcine adipocytes. The well-differentiated adipocytes, as described in Figure 3, were treated with medium containing 0, 0.2, or 2 μmol/L of pSAA for 24 h. The total RNA was extracted to determine the effect of SAA on the expression of genes related to lipid metabolism, HSL, ACO, CPTI (A), ACC1, aP2, and LPL (B) in porcine adipocytes. Bars are means ± SE, n = 3 independent measures. Means without a common letter differ, P ≤ 0.05.
The expression of perilipin is regulated by PPARα (41). The major transcription factor to induce the expression of perilipin in adipocytes, as described in Figure 3, were treated with medium containing 0, 0.2, or 2 μmol/L of pSAA for 24 h. The total RNA was extracted to determine the effect of pSAA on the expression of cytokines related to lipid metabolism, i.e., TNFα (4) and IL-6 (8) by porcine adipocytes. The data were log transformed before statistical analysis. Bars are means ± SE, n = 3 independent measures. Means without a common letter differ, P ≤ 0.05.

The observation that porcine hepatic SAA mRNA is increased by dietary DHA suggests that the SAA protein was increased, secreted into the circulation, and affected physiological functions throughout the body. The expression of SAA mRNA is specific to the liver and not found in the adipose tissues in pigs (15). However, in humans, SAA is expressed in liver and adipose tissue and the SAA expression is associated with body composition and nutritional condition (16,26). In mice, there are 4 types of SAA expressed, with SAA3 mainly expressed in adipocytes and considered an adipocytokine (27). Therefore, there is species specificity for expression of the SAA genes in various tissues.

In human preadipocytes, treatment with SAA increases the expression of IL-6 (17). In this study, we found that the pSAA increased the IL-6 in porcine preadipocytes. Therefore, we demonstrated that this recombinant protein is biologically active.

In adipose tissue, energy is stored primarily as triacylglycerol in lipid droplets. The cellular lipid droplet is covered with perilipin protein to protect it from lipolytic enzyme action. When energy is needed, triacylglycerol is cleaved by HSL, monoacylglycerol lipase, or adipose triglyceride lipase to FFA and glycerol (28). The FFA is released to the blood circulation, transported into the mitochondria for β-oxidation to provide energy, or reesterified to triacylglycerol. Because there is no glycerol kinase in adipocytes, the glycerol is released into the circulation in vivo or into the culture medium in vitro. Therefore, the measurement of culture medium glycerol concentration monitors the lipolytic activity. In this experiment, we found that pSAA induced the release of glycerol into the culture medium (Fig. 2), indicating that pSAA can increase the lipolytic activity in the adipocytes. A similar observation for human SAA treatment in human adipocytes was reported (17). Because we have found that the perilipin mRNA was concomitantly reduced with the increase of medium glycerol, i.e., lipolysis, we speculate that pSAA increased lipolysis through reduction of the perilipin protein. The direct effects of SAA on the modification of HSL activity remain to be demonstrated. In addition to perilipin effects on lipolysis, other mechanisms to modify HSL activity (not evaluated) include cAMP concentration, protein kinase A expression and activity, and the phosphorylation status of HSL. The lipolytic rate was greater when the HSL mRNA was reduced by pSAA treatment. We speculate that even in the presence of reduced HSL protein, the activation of HSL catalytic activity by phosphorylation may be adequate to sustain the increased lipolytic rate or other lipolytic enzymes, such as adipose triacylglycerol lipase (29), or triacylglycerol hydrolase (30) may be functional. This triacylglycerol lipase is responsible for the basal lipolysis in adipose tissues (30).

The major transcription factor to induce the expression of genes related to FA β-oxidation, e.g., ACO and CPTI is PPARα (22). We found that the PPARα mRNA in adipocytes was reduced by pSAA treatment and the ACO and CPTI mRNA were reduced and increased, respectively. The reduction in PPARγ and PPARα mRNA can then be expected to reduce the expression of ACC1, as observed. The reduction of ACC1 would decrease the formation of malonyl-CoA, an active inhibitor of the CPTI activity (31). Therefore, even though pSAA treatment decreased the expression of ACO, the increased expression of CPTI coupled with the reduction of the inhibitor, malonyl-CoA, suggests an increase in FA oxidation by pSAA. Although FA oxidation rates have not been measured in porcine adipose tissue, the high expression of PPARα, ACO, and CPTI mRNA suggests there is active FA oxidation (32). In humans, the expression of these genes is low in adipose tissue but high in the liver (33).

The reduction in expression of PPARγ and its target genes, aP2, LPL, and ACC1, after pSAA treatment indicates that the SAA protein reduces the lipogenic activity. This inhibitory effect of SAA on lipogenic genes suggests that SAA may have a function in the depression of obesity.

The expression of SAA is stimulated by cytokines, such as TNFα and IL-6 (34). Also, pSAA upregulated TNFα and IL-6 in porcine adipocytes, similar to the observation in human adipose explants (17). There is evidence to show that IL-6 stimulates the oxidation of FA (35) and increases lipolysis in human adipocytes (36); our observations with porcine adipocytes are similar to both findings. Moreover, IL-6 knockout mice have adult-onset obesity that can be treated with IL-6 injection (37). Therefore, we speculate that the lipolytic and β-oxidation enhancing ability of SAA may result, at least partially, through an increase of IL-6.

The TNFα, secreted by adipocytes, stimulates lipolysis and also inhibits lipogenesis in 3T3-L1 adipocytes, resulting in a high FFA concentration in the culture medium (38). We speculate that TNFα promotes lipolysis by inhibiting the expression of perilipin through mitogen-activated protein kinases (39). In addition, TNFα inhibits the expression of HSL and LPL (40). Similarly, we found that pSAA treatment increased the expression of TNFα and concomitantly reduced the expression of HSL and LPL. It was suggested that the effect of TNFα on adipocyte dedifferentiation is to increase phosphorylation of perilipin through protein kinase A to reduce the perilipin function of protecting lipid droplets from degradation (16). Such a reduction in perilipin would then allow HSL access to lipid droplets to catalyze hydrolysis of triacylglycerol. We suggest that this mechanism was functional in our experiments with porcine adipocytes.

The expression of perilipin is regulated by PPARγ (41). The PPARγ transcription factor increases the expression of perilipin to reduce lipid droplet hydrolysis and increase lipid deposition (42). We observed that pSAA inhibited the expression of PPARγ,
providing a mechanism for the reduction in perilipin expression and the increase in lipolysis. Together, porcine SAA secreted by hepatocytes may be able to increase lipolysis andFA oxidation through the following mechanisms. First, SAA increased TNFα and reduced PPARγ expression, both of which reduced the expression of perilipin. Decreased perilipin diminished the stability of the fat droplet to allow increased lipolysis in the adipocyte. Secondly, SAA increased the expression of CPTI to increase FA oxidation. Thirdly, a reduction of PPARγ by SAA treatment reduced the expression of ACC1, resulting in the decreased production of malonyl-CoA and thus a more active CPTI and greater FA oxidation activity. It is clear that SAA can affect lipid metabolism through regulation of the expression of multiple genes related to lipid metabolism. This protein, not usually considered in schemes describing the regulation of adipocyte lipid metabolism, may be a major player in the control of fat deposition in pigs and other mammals.

**Literature Cited**


