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The Effect of Dietary Docosahexaenoic Acid Enrichment on the Expression of Porcine Hepatic Genes*

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ABSTRACT : To study the effect of dietary docosahexaenoic acid (DHA) enrichment on the expression of hepatic genes in pigs, weaned, crossbred pigs (30 d old) were fed diets supplemented with either 2% tallow or DHA oil for 18 d. Hepatic mRNA was extracted. Suppression subtractive hybridization was used to explore the hepatic genes that were specifically regulated by dietary DHA enrichment. After subtraction, we observed 288 cDNA fragments differentially expressed in livers from pigs fed either 2% DHA oil or 2% tallow for 18 d. After differential screening, 7 genes were found to be differentially expressed. Serum amyloid A protein 2 (SAA2) was further investigated because of its role in lipid metabolism. Northern analysis indicated that hepatic SAA2 was upregulated by dietary DHA enrichment ($p<0.05$). In a second experiment, feeding 10% DHA oil for 2d significantly increased the expression of SAA2 (compared to the 10% tallow group; $p<0.05$). The porcine SAA2 full length cDNA sequence was cloned and the sequence was compared to the human and mouse sequences. The homology of the SAA2 amino acid sequence between pig and human was 73% and between pig and mouse was 62%. There was a considerable difference in SAA2 sequences among these species. Of particular note was a deletion of 8 amino acids, in the pig compared to the human. This fragment is a specific characteristic for the SAA subtype that involved in acute inflammation reaction. Similar to human and mouse, porcine SAA2 was highly expressed in the liver of pigs. It was not detectable in the skeletal muscle, heart muscle, spleen, kidney, lung, and adipose tissue. These data suggest that SAA2 may be involved in mediation of the function of dietary DHA in the liver of the pig, however, the mechanism is not yet clear. (**Key Words** : Dietary Docosahexaenoic Acid, Pigs, Serum Amyloid A Protein, Porcine Liver, Hepatic Genes)

INTRODUCTION

Dietary polyunsaturated fatty acids (PUFA) can reduce blood lipid concentration (Rambjor et al., 1996; Harris et al., 1997; Liu et al., 2005), increase insulin sensitivity (Storlien et al., 1998; Suresh and Das, 2003), inhibit the growth of cancer cells (Rose, 1997), and inhibit expression of lipogenic genes (Kim et al., 1999) as well as increase expression of fatty acid oxidation genes (Brandt et al., 1998) to reduce body fat deposition. Dietary PUFA exert their function through expression of many genes and the gene list is increasing as research continues.

In mammals, the transcription factor, sterol regulatory element-binding protein1 (SREBP1) regulates the expression of a group of lipogenic genes, eg., fatty acid synthase (FAS) and acetyl coenzyme A carboxylase to increase lipogenic activity and fat deposition (Brown and Goldstein, 1997; Shimano, 2001). These lipogenic genes are inhibited by dietary fish oils in rodents (Xu et al., 1999; 2001). Dietary PUFA also inhibit lipogenic genes, eg., stearoyl coenzyme A reductase 1, in the adipose tissues of the rats (Jones et al., 1996). However, dietary fish oil (a mixture of fatty acids with considerable PUFA) has minimal effects on the abundance of lipogenic transcripts in porcine adipose tissue (Ding et al., 2003), indicating the effect of fatty acids on porcine gene expression *in vivo* may be different from that of rodents. Nonetheless, previous studies using high docosahexaenoic acid (DHA) algal oil in the diet demonstrated that dietary DHA enrichment suppresses expression of hepatic SREBP1 in the pig (Hsu et al., 2004; Liu et al., 2005).

Although dietary PUFA reduces body fat deposition by reducing expression of lipogenic genes and increasing expression of fatty acid oxidation genes, PUFA may also work through other pathways to regulate lipid metabolism. Therefore, in the current study, a functional genomic

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Table 1. Composition of experimental diets in experiment 1

Ingredient	2% DHA	2% Tallow
	%	
Corn (yellow)	45.16	45.16
Soybean meal (44%)	39.70	39.70
Dried skim milk	5.00	5.00
DHA oil[a]	2.00	0
Tallow	0	2.00
Wheat bran	5.40	5.40
Limestone (pulverized)	0.83	0.83
Dicalcium phosphate	1.06	1.06
Salt (iodized)	0.25	0.25
Vitamin premix[b]	0.30	0.30
Mineral premix[c]	0.20	0.20
Antibiotics[d]	0.10	0.10
Total	100.00	100.00
Calculated values:		
Crude protein (%)	23.70	23.70
ME (Mcal/kg)	3.26	3.26
Calcium (%)	0.80	0.80
Phosphate (%)	0.65	0.65

[a] DHA oil extracted from algals and contained 44% DHA (Martek Bio).
[b] Vitamin premix provided the following vitamins per kg diet: A, 8,000 IU; D_3, 800 IU; E, 30 IU; K, 1 mg; B_1, 2.0 mg; B_2, 5.0 mg; B_{12}, 25 μg.
[c] Mineral premix provided the following minerals per kg diet: Cu, 10 mg; Fe, 100 mg; Zn, 100 mg; Mn, 10 mg; Se, 0.1 mg.
[d] Per kg antibiotics contained 22 g lincomycin and 22 g spectinomycin.

technique, suppression subtraction hybridization (SSH) was utilized to explore new target genes regulated by dietary PUFA.

MATERIALS AND METHODS

Animals and diets

The animal protocol used in the present experiment was approved by the Animal Care and Use Committee of the Animal Technology Institution in Taiwan. In Experiment 1, weaned, crossbred pigs (Landrace, Yorkshire and Duroc; 21 d old) were purchased from a commercial pig farm. They weighed 5.3±0.6 kg (mean±SE) and were fed the control diet (Table 1) for 9 days for the pigs to adapt to the diet and the environment. At 30 days of age, the pigs were fed a diet supplemented with either 2% (on as-fed basis) tallow or an algal docosahexaenoic acid (DHA) containing oil for 18 days (6 pigs per group). The calculated protein content of the experimental diets was 23%, and the calculated fat was 4.62% for the diets. Beginning at 3 days before administration of the experimental diets, pigs were fed two meals per day, one at 0700 h and the other at 1600 h. The pigs were fed to approximate the level of *ad libitum* feeding. After feeding the diets for 18 days, the pigs from each dietary group were killed 2 h after feeding to collect liver tissues for further analysis. In Experiment 2, weaned, crossbred pigs (28 days of age) were purchased from a commercial pig farm. They weighed 9.8±0.3 kg (mean±SE) and were fed the tallow-containing diet, (Table 2) for 9 days for the pigs to adapt to the diet and the environment. At 37 days of age, the pigs were fed a diet supplemented with either 10% (on as-fed basis) tallow or 10% algal DHA containing oil for 2 days. There were six pigs per treatment. The 10% DHA oil supplementation was chosen to enrich the dietary DHA to a level similar to the 40% dietary fish oil used in rodent experiments (Kim et al., 1999). The calculated protein content in the experimental diets was 22%, and the fat content was 12.62% on an as-fed basis. Beginning at 3 days before administration of the experimental diets, pigs were fed two meals per day, one at 0700 h and the other at 1600 h. The amount of feed was provided to approximate *ad libitum* feeding (according to the feed intake of the previous day). After feeding the diets for 2 days, the pigs from each dietary group were selected at random and killed at 0900 h, after the 0700 h feeding. Pigs were killed by electrical stunning coupled with exsanguination. Tissue samples were rapidly removed, wrapped in foil and frozen in liquid nitrogen to be stored at -70°C until analysis. For tissue distribution studies, 4 two-month-old Lee-Sung pigs (two females and two males) were used. Heart, longissimus muscle, liver, spleen, kidney, lung, and subcutaneous adipose tissue were dissected, frozen quickly in liquid nitrogen and stored at -70°C until RNA extraction.

Table 2. Composition of experimental diets in experiment 2

Ingredient	10% DHA	10% Tallow
	%	
Wheat flakes	48.75	48.75
Isolated soy protein	14.00	14.00
Whey, dried	10.00	10.00
Skim milk, dried	5.00	5.00
Wheat bran	10.00	10.00
DHA oil[a]	10.00	0
Tallow	0	10.00
$CaHPO_4$	0.70	0.70
$CaCO_3$	0.70	0.70
NaCl iodide	0.25	0.25
Vitamin premix[b]	0.30	0.30
Mineral premix[c]	0.20	0.20
Antibiotics[d]	0.10	0.10
Total	100.00	100.00
Calculated values:		
Crude protein (%)	22.19	22.19
ME (Mcal/kg)	3.42	3.42
Calcium (%)	0.75	0.75
Phosphate (%)	0.63	0.63

[a] DHA oil extracted from algals and contained 44% DHA (Martek Bio).
[b] Vitamin premix provided the following vitamins per kg diet: A, 1,750 IU; D_3, 200 IU; E, 11 IU; K, 0.5 mg; B_2, 3 mg; B_{12}, 15 μg; Folacin 0.3 mg.
[c] Mineral premix provided the following minerals per kg diet: Cu, 5 mg; Fe, 80 mg; Zn, 80 mg; Mn, 3 mg; Se, 0.25 mg.
[d] Per kg antibiotics contained 22 g lincomycin and 22 g spectinomycin.

Suppression subtractive hybridization

The SSH procedure utilized the PCR Select Kit (Clontech, Palo Alto, CA). Briefly, mRNA from each of the liver tissue treated with different dietary DHA contents (2% algal DHA oil and 2% Tallow) was reverse transcribed and double strand cDNA synthesized. The cDNA from all livers in the same treatment were pooled for the SSH procedure. After RsaI restriction enzyme digestion, the Tester DNA (cDNA from livers of 2% algal DHA oil-treated pigs) was divided into two groups and ligated with Adaptor 1 (Tester1 DNA) or Adaptor 2 (Tester2 DNA), respectively. The Driver DNA (cDNA from livers of 2% tallow-treated pigs) was not ligated with any Adaptor. Tester 1 or Tester 2 DNA was denatured at 95°C for 10 min and hybridized with denatured Driver DNA in separate tubes. After hybridization, any single stranded DNA with Adaptor 1 or Adaptor 2 represented genes expressed specifically in livers of 2% algal DHA oil-treated pigs, but not in livers of 2% tallow-treated pigs, whereas the single stranded DNA without Adaptors represented genes expressed in livers of 2% tallow-treated pigs, but not in livers of 2% algal DHA-treated pigs. The resulting two populations were pooled for a second hybridization with fresh denatured Drivers. The resulting molecules with both Adaptor 1 and 2 represent gene sequences preferentially expressed in livers of 2% algal DHA oil treated pigs. These molecules were amplified after a 14-cycle PCR using a pair of nested primer sequences from Adaptor 1 and 2. The differentially expressed gene fragments were then cloned into pGEM-T Easy TA cloning vector (Promega, Madison, WI). The resulted clones were selected for sequence analysis by a genetic analyzer (ABI 3730, Applied Biosystems, Foster City, CA). We selected 288 clones for further sequencing, differential screening, and Northern analysis to confirm the differential expression of genes between livers of 2% algal DHA oil treated pigs and livers of 2% tallow treated pigs.

Differential screening

The differential screening procedure followed that described by the PCR-Select Differential Screening Kit User manual (Clontech). Details for the screening procedure were also described by Wang et al. (2006). The DNA from forward subtraction (genes expressed in livers of 2% algal DHA-treated pigs, but not in livers of 2% tallow-treated pigs) and reverse subtraction (genes expressed in livers of 2% tallow-treated pigs, but not in livers of 2% algal DHA-treated pigs) were used as probes for the screening.

Transcript analysis

Total RNA was extracted from the tissues by the guanidinium- phenol-chloroform extraction method (Chomczynski and Sacchi, 1987); modifications were described previously (Wang et al., 2004; Yang et al., 2004; Cheng et al., 2006). The porcine 18S ribosomal RNA probe sequence was described previously (Hsu et al., 2004). The porcine serum amyloid A protein 2 (SAA2) probe sequence was from the current study (Accession no. DQ367410). Hybridization results were quantified by phosphor-image analysis as previously described (Ding et al., 2004). The densitometric value for an individual transcript in a sample lane was normalized to the densitometric value for the 18S rRNA in the same lane.

Full length cDNA cloning

Pig hepatic RNA was reverse transcribed (RT) using a kit, SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The transcribed single strand cDNA from pig liver was used for RT-PCR to clone the full length SAA2 with 5' (5'-GCA GCT CAG CTT CAC CAG GA-3') and 3' (5'-CTG CTC ACA GGA GGC TCA CA-3') PCR primers. These primers were based on human sequence covered sequences before start codon for the sense primer and after stop codon for the antisense primer. AccuPrime Pfx DNA polymerase (Invitrogen) was used to amplify cDNA. PCR amplification cycles were denaturation at 94°C for 20s (3 min in the first cycle), annealing at 53°C for 30s, and extension at 68°C for 1 min (5 min in the last cycle). The PCR products were cloned into a pCR-Blunt II -TOPO vector (Invrogen). Sequences of the gene molecules were determined and confirmed to be similar to SAA2 from other species.

Statistical analysis

The differences between dietary treatments were analyzed by Student's T-Test using SAS software (SAS Inst., Inc., Cary, NC) with dietary treatment as the main factor. For the tissue distribution data, ANOVA analysis was applied to detect differences among means using SAS statistical software (SAS Inst., Inc., Cary, NC). The significance level between treatments was set at 5%. The mean and SE for each transcript is presented.

RESULTS AND DISCUSSION

Differentially expressed genes from SSH

The SSH technique was utilized to explore genes that were specifically regulated by different dietary fatty acid compositions. The hepatic mRNA from experiment 1 was used. After subtraction, 288 cDNA fragments differentially expressed between livers from pigs fed with diets containing either 2% DHA oil or 2% tallow for 18 days were sequenced. They were subjected to differential screening to confirm the treatment effects on these genes. We found that 7 genes, including SAA2, vitamine D binding protein, insulin-like growth factor I, alcohol dehydrogenase-S-isoenzyme, glutathione peroxidase,

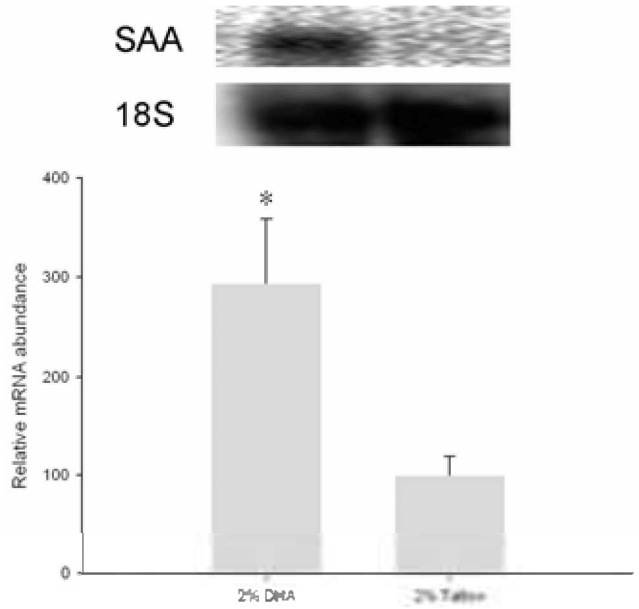

Figure 1. The effect of 2% dietary DHA oil or tallow on the abundance of porcine serum amyloid A protein 2 (SAA2) mRNA in porcine livers. Weaned, crossbred pigs were fed either a 2% tallow-added, or an algal docosahexaenoic acid (DHA) oil-added diet for 18 d (6 pigs per group). At the day of sampling, pigs from each dietary group were killed 2 h after feeding. The SAA2 and 18S ribosomal RNA (18S) were determined by Northern analysis. The SAA2 mRNA abundances are depicted relative to the control. The mRNA values were normalized to 18S rRNA content. *Asterisk* denotes a significant difference between the groups ($p<0.05$).

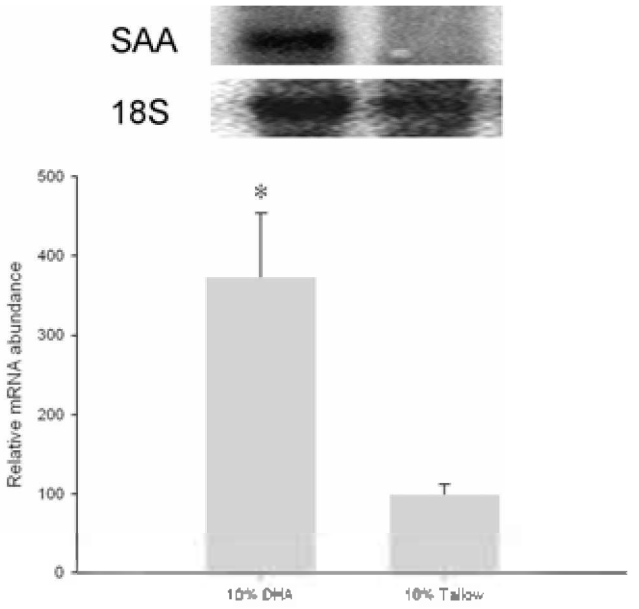

Figure 2. The effect of 10% dietary DHA oil or tallow on the abundance of porcine serum amyloid A protein 2 (SAA2) mRNA in porcine livers. Weaned, crossbred pigs were fed either a 10% tallow-added, or an algal docosahexaenoic acid (DHA) oil-added diet for 2 d (6 pigs per group). At the day of sampling, pigs from each dietary group were killed 2 h after feeding. The SAA2 and 18S rRNA were determined by Northern analysis. The SAA2 mRNA abundances are depicted relative to the control. The mRNA values were normalized to 18S ribosomal RNA (18S) content. *Asterisk* denotes a significant difference between the groups ($p<0.05$).

superoxide dismutase 1, and one novel gene (sequence not found in GenBank database of NCBI; http://www.ncbi.nlm.nih.gov/) were differentially expressed. Because SAA2 may have great importance in lipid metabolism, we further analyzed its expression by Northern analysis to confirm that hepatic SAA2 was upregulated by the 2% dietary DHA treatment (Figure 1). When the hepatic SAA2 mRNA abundance was measured in Experiment 2, acute treatment with 10% dietary DHA also significantly increased the expression of SAA2 when compared with the tallow treatment (Figure 2, $p<0.05$). The combined data indicate that acute and chronic dietary DHA enrichments upregulated the expression of hepatic SAA2 in pigs.

Demonstrated effects of SAA on lipid metabolism indicate that SAA has high affinity for cholesterol and can enhance cholesterol transport into HepG2 cells (Liang and Sipe, 1995) and rabbit neonatal aortic smooth muscle cells (Liang et al., 1996). The SAA is also able to decrease triacylglycerol (TG), cholesterol and phospholipid levels in smooth muscle cells in rabbits (Schreiber et al., 1999). We demonstrated that both acute and chronic dietary DHA treatment significantly increased the expression of SAA2 mRNA in the liver of pigs. Previous experiments showed that 10% dietary DHA treatment for two days significantly reduced the porcine plasma TG and cholesterol concentrations (Liu et al., 2005). Whether this reduction of plasma TG and cholesterol results from the increased expression of SAA2 requires further investigation. Dietary DHA decreases the expression of SREBP1 mRNA in porcine livers (Hsu et al., 2004; Liu et al., 2005) and reduces plasma TG (Liu et al., 2005). Thus, the reduction in porcine plasma TG may be partially due to a reduction of lipogenic activity by lowering SREBP1 expression.

Tissue distribution of porcine SAA2

The SAA2 transcript was highly expressed in the liver of pigs (Figure 3). It was not detectable in the adipose tissue, skeletal muscle, heart muscle, spleen, kidney, or lung of the pig. The SAA2 is also highly expressed in human and mouse liver (Ramadori et al., 1985; Whitehead et al., 1992; Urieli-Shoval et al., 1998). However, the SAA2 is also detectable in human kidney and spleen (Whitehead et al., 1992; Urieli-Shoval et al., 1998), and in the lung of the mouse (Meek and Benditt, 1986; Ramadori et al., 1985). Although we could not detect SAA2 mRNA in subcutaneous adipose tissue of the pig, Sjoholm et al. (2005) demonstrated that SAA2 was highly expressed in human omental adipose tissue. Overall, the data suggest species

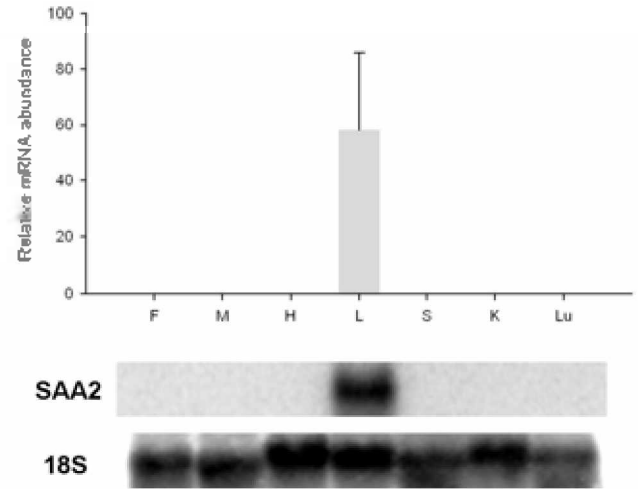

Figure 3. Tissue distribution of porcine serum amyloid A protein 2 (SAA2) mRNA in pigs. Total RNA from each tissue was extracted, Northern blots were prepared, and were quantified by phosphor-imagine analysis. The data represent the mean±SE for RNA from 4 pigs. Tissues are A: subcutaneous adipose tissue, M: skeletal muscle, H: heart, L: liver, K: kidney, S: spleen, and Lu: lung. 18S = 18S ribosomal RNA.

variation in expression of SAA2 in tissues. The very high level of SAA2 expression in porcine liver, coupled with the enhanced expression of this gene under DHA treatment suggests a role of hepatic SAA2 in mediating the function of DHA in pigs.

Full length cDNA of SAA2

The porcine SAA2 full length cDNA (Accession no. DQ367410) was cloned from RT-PCR of mRNA from porcine liver and the sequence was compared to those for the human and mouse. The homology between pig and human SAA2 cDNA (Accession no. NM_030754) was 77% and between pig and mouse (Accession no. NM_011314) was 70% (Figure 4). The amino acid sequence of this protein was deduced from the full length cDNA sequence of porcine SAA2. The homology between pig and human SAA2 was 73% and between pig and mouse was 62% (Figure 5). The data indicate that even though there is considerable homology between these species, a great deal of difference is present. Of particular note is a deletion of eight amino acids (amino acid number 88 to 95) in the human compared to the porcine SAA2. This deletion is a

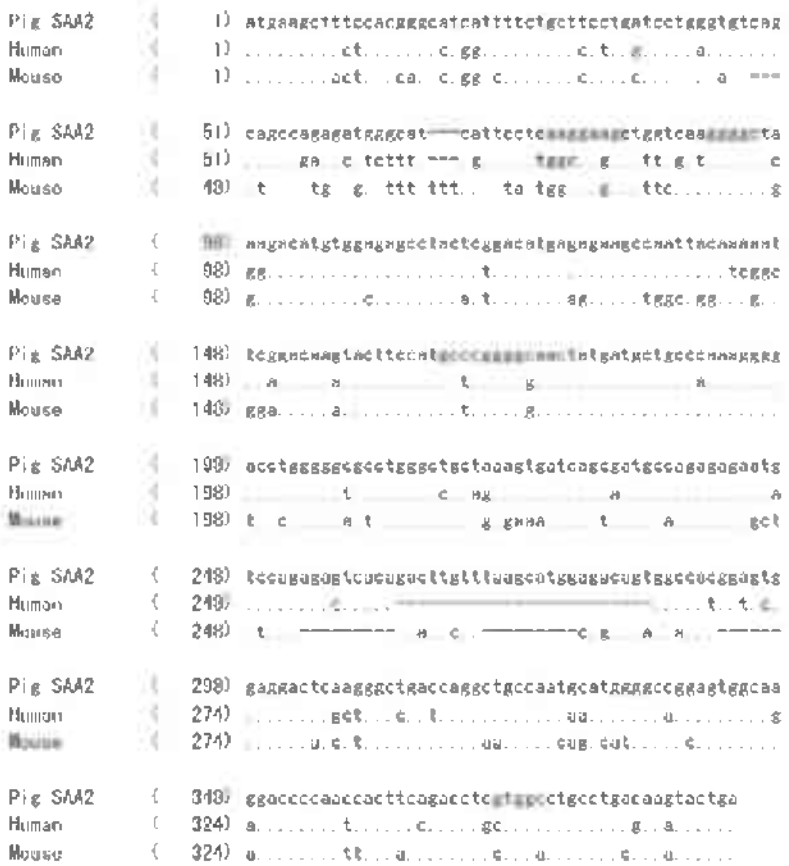

Figure 4. Full length cDNA sequence for the pig serum amyloid A protein 2 (SAA2; Accession no. DQ367410). This full length open reading frame was obtained by RT-PCR of mRNA from porcine liver. The dots indicate the same nucleotide. The homology between pig and human SAA2 cDNA (Accession no. NM_030754) was 77% and between pig and mouse DNA (Accession no. NM_011314) was 70%.

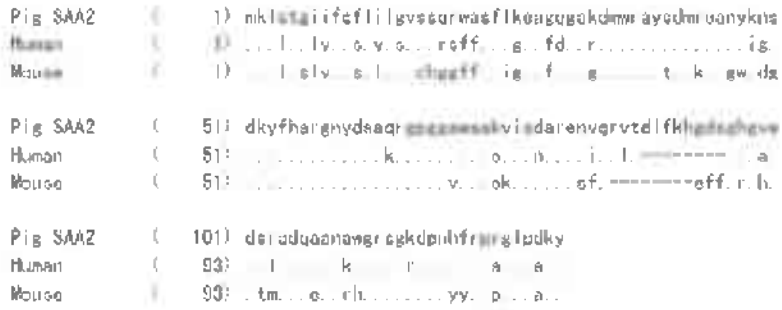

Figure 5. The deduced amino acid sequence for the pig serum amyloid A protein 2 (SAA2; Accession no. DQ367410). The amino acid sequence of this protein was deduced from the full length cDNA sequence (Figure 3) of porcine SAA2. The dots indicate the same amino acid. The homology between pig and human SAA2 (Accession no. NP_110381) was 73% and between pig and mouse SAA2 (Accession no. NP_035444) was 62%.

specific characteristic for the acute inflammation reaction in the human SAA (Whitehead et al., 1992). Therefore, the function of porcine SAA2 may be different from that in the human (Whitehead et al., 1992) and mouse (Lowell et al., 1986).

The SAA is a family of apo-lipoproteins that response to inflammation factors, such as interleukin-1 (IL1), IL6 or infection (Husby et al., 1994). In humans, there are three genes in this family, i.e., SAA1, SAA2, and SAA4 (Whitehead et al., 1992); the SAA1 and SAA2 belong to the acute-phase reactants and are used as markers for inflammation (Whitehead et al., 1992; Steel et al., 1993; de Beer et al., 1995). However, in rodents, the SAA3 is the acute-phase reactant in this family (Ramadori et al., 1985). The SAAs that have a deletion of eight amino acids in their proteins are referred to acute inflammation reaction proteins. This octapeptide encoded by non-acute-phase reactant type SAA gene has a glycosylation site (NSS) that is differentially used to generate the 14 kDa unglycosylated and 19 kDa glycosylated forms of SAA in human serum (Whitehead et al., 1992).

The SAA proteins are also associated with lipid metabolism (Steinmetz et al., 1989; Liang and Sipe, 1995; Liang et al., 1996). It has been found that SAA decreases lipogenic activity of smooth muscle cells in a time and dose dependant fashion (Schreiber et al., 1999). Dietary DHA treatment reduces porcine plasma TG concentration (Liu et al., 2005) and reduces expression of the mRNA for the hepatic lipogenic transcription factor, SREBP1. It was hypothesized that the reduction in SREBP1 was the major mechanism through which dietary DHA reduces lipogenic gene expression (Hsu et al., 2004; Liu et al., 2005). Because of the role of SAA2 in downregulating the expression of lipogenic genes, we speculate that increased SAA2 may partially contribute to the mediation of the inhibitory effect of dietary DHA on porcine hepatic lipogenic genes. It has also been demonstrated that SAA proteins are involved in cholesterol metabolism (Liang and Sipe, 1995; Liang et al., 1996) by enhancing cholesterol efflux (van der Westhuyzen et al., 2005). Therefore the increase of SAA2 after DHA treatment may enhance the metabolism of cholesterol and thus reduce the plasma cholesterol concentration, as previously observed (Liu et al., 2005).

In conclusion, the current study utilized the SSH technique to successfully discover a gene, SAA2 that is highly expressed after dietary DHA enrichment. This is the first research to demonstrate that hepatic SAA2 is responsive to dietary DHA. The data suggest that the DHA-mediated increase in porcine hepatic SAA2 expression may provide at least part of the mechanism leading to DHA-induced decreases in plasma TG and cholesterol.

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