Porcine adipose triglyceride lipase complementary deoxyribonucleic acid clone, expression pattern, and regulation by resveratrol

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ABSTRACT: Adipose triglyceride lipase (ATGL) was recently identified and described as a major novel triglyceride lipase in animals. In this study, we aimed to study the tissue-specific and developmental expression pattern of porcine ATGL (pATGL) and the effect of resveratrol (RES) on expression of pATGL in vitro. The full-length cDNA sequence of pATGL was 1,958 bp (accession no. EF583921), with a 1,458-bp open reading frame encoding a 486-AA protein (the predicted molecular mass of 53.2 kDa, accession no. ABS58651). Comparison of the deduced AA sequence with the bovine, mouse, rat, dog, and human adipose triglyceride lipase showed 87, 84, 83, 81, and 80% similarity, respectively. Furthermore, the pATGL was highly expressed in porcine adipose tissue, to a lesser degree in kidney, heart, and muscle, and least but detectable in brain. In s.c. adipose tissue, pATGL mRNA was low at birth (1 kg of BW) and then increased, reaching a maximal value at 20 kg of BW (approximately 8 wk old; P < 0.01). In peritoneal and omental adipose tissue, the greatest expression of pATGL was observed at 40 kg of BW (approximately 12 wk old). In vitro, exposure of cultured adipocytes to 40 and 80 μM RES for 24 h increased the mRNA levels of pATGL by 95.3% (P < 0.05) and 146.8% (P < 0.01), respectively. Accordingly, lipid accumulation was decreased by 25.7% (P < 0.05) and 60.8% (P < 0.01), respectively. When treated with RES for 48 h, the mRNA levels of pATGL were increased by 104.1% (P < 0.05) and 163.1% (P < 0.01), respectively. As expected, lipid accumulation was decreased by 9.7% (P > 0.05) and 29.0% (P < 0.05), respectively. These results add to our understanding of the role of pATGL in adipose tissue development and as a potential target for regulating fat deposition and meat quality.

Key words: adipose triglyceride lipase, cloning, gene expression, pig, resveratrol

INTRODUCTION

Adipose triglyceride lipase (ATGL), also called patatin-like phospholipase domain-containing 2, calcium-independent phospholipase A2, and desnutrin, was recently identified and described as an adipocyte-specific protein with lipid hydrolase activity in mice and humans (Jenkins et al., 2004; Villena et al., 2004; Zimmermann et al., 2004). It has been proven that ATGL is an important triglyceride (TG) hydrolase promoting the catabolism of stored fat in adipose and nonadipose tissues (Lass et al., 2006). It catalyzes the initial step in TG hydrolysis in adipocyte lipids (Kershaw et al., 2006) and in nonadipocyte cells in mammalian cells (Smirnova et al., 2006). The tissue-specific expression and the regulation of ATGL by fasting, nutritional status, and hormones (e.g., isoproterenol, insulin) have been studied in human and mouse (Kralisch et al., 2005, 2006). However, little is known about the expression and regulation of the ATGL gene in pigs.

Resveratrol (3,5,4′-trihydroxystilbene, RES), a natural polyphenolic phytochemical present in grapes, peanuts, and mulberries, has various bioactivities associated with health promotion including antiinflammatory properties, modulation of lipid metabolism, and prevention of cancer (Fremont, 2000; Pervaiz, 2003). Recently, RES has been demonstrated to reduce TG content and stimulate FFA release in the mouse 3T3-L1 adipocytes (Picard et al., 2004). However, whether RES is involved in regulation of ATGL gene expression in animal adipocytes is still unknown.

Therefore, we cloned the full-length sequences of porcine ATGL (pATGL) mRNA and investigated the tissue-specific and developmental expression pattern...
of ATGL in pigs. Furthermore, the effect of RES on the lipid accumulation and pATGL gene expression in porcine adipocytes was also studied. This work has the potential to increase our understanding of the role of ATGL in lipid metabolism and as a potential target for regulating fat deposition and meat quality.

MATERIALS AND METHODS

All procedures were approved by the University of Zhejiang Institutional Animal Care and Use Committee.

Animals

In Exp. 1, six female pigs (Duroc × Landrace × Yorkshire) with a mean BW (SD = 0.57) of 30 kg were selected and killed. Brain, heart, liver, kidney, lung, spleen, muscle, small intestine, and white adipose tissue in right-half carcasses were collected and rapidly frozen in liquid nitrogen, then stored at −80°C until RNA analysis. In Exp. 2, a total of 24 pigs (Duroc × Landrace × Yorkshire) were used to investigate the developmental expression of pATGL. Six female pigs each at 1 (at birth), 20 (8 wk), 40 (12 wk), and 60 kg (16 wk) of BW were randomly selected and killed under anesthesia for samples. The ingredient and chemical compositions for the diets (as-fed basis) fed to the pigs (20 to 60 kg) were as follows: DE, 14.46 MJ/kg; crude protein, 19.50%; calcium, 0.80%; phosphorus, 0.66%; and Lys, 1.35%. Omental adipose tissue (OAT), perirenal adipose tissue (PAT) in right-half carcasses were collected, rapidly frozen in liquid nitrogen, then stored at −80°C until RNA analysis.

Cloning of Full-Length cDNA of pATGL

To obtain the full-length cDNA sequence of pATGL, rapid amplification of cDNA ends (RACE) technology was carried out to clone the 5'- and 3'-ends of pATGL by using the 5'/3'-RACE kit (Takara Biotechnology Co. Ltd., Dalian, China). Briefly, for 3'-RACE, total RNA from porcine tissues was reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, WI) using oligo(dT)18-containing anchor primer (P1, 5′-CTGATCTAGGTACCCG-GATCCCTTTTTTTTTTTTTTTTTTTTTT-3′). The PCR was performed using a combination of the adaptor primer (P2, 5′-CTGATCTAGGTACCCGGATCC-3′) and a gene-specific primer (P3, 5′-AGAGCGACATCTGC-CCGAGGA-3′) designed from the 3′-RACE sequence. The gene-specific primer P3 was designed based on the expressed sequence tag sequence of pATGL available in GenBank (DQ157429; Chen et al., 2007). The tailed cDNA was amplified by 2 rounds of PCR, as follows: 2 pATGL-specific primers (P5, 5′-GGCTGAACCTGGATGCTGGTGT-3′; and P6, 5′-GATGCTGGTGTTGTGGTACGCCGA-3′) were designed from the 3′-RACE sequence and used for outer PCR and inner PCR, respectively. The subsequent 5'-RACE product was gel-purified, cloned, and sequenced. By ligation of the 2 overlapping cDNA fragments, the full-length pATGL cDNA was obtained and deposited at GenBank (accession no. EF583921).

Sequence Analysis

Sequence alignment and analysis were carried out with the BLAST network service of the National Center for Biotechnology Information (Chen et al., 2007). The AA sequences of ATGL of other species used for comparison were downloaded from GenBank.

Cell Culture

In Exp. 3, three pigs from 5 to 7 d of age were over-dosed with sodium thiopental and exsanguinated. Subcutaneous adipose tissue was removed, and the porcine stromal-vascular (S-V) cells were prepared by methods previously published (Ramsay, 2001). The S-V cell pellet was washed with Dulbecco’s modified Eagle medium/HamF12 (DMEM/F12, Gibco Laboratories, Grand Island, NY), centrifuged, and resuspended in plating medium (DMEM/F12 + 10% fetal bovine serum), stained with Rappaport’s stain and counted on a hemocytometer. The S-V cells were seeded on 6-well (35 mm) tissue culture plates (Shanghai Sangon Co. Ltd, Shanghai, China) at a density of 2 × 10⁴ cells/cm² in complete media (DMEM/F12 + 10% fetal bovine serum + 100 U of penicillin + 100 U of streptomycin). Three replicates were performed, and each replicate consisted of a single batch of S-V cells harvested from the SAT of an individual pig. Cells were cultured at 37°C under a humidified atmosphere of 95% air:5% CO². Media were changed every 3 d. After 10 d of lipid filling, cultures were washed with DMEM/F12 and were then used for the experiment assessing the effect of RES on the gene expression of ATGL. Cells were subsequently incubated with 3 test media: complete media (control), complete media + 40 μM RES (RES40), and complete media + 80 μM RES (RES80). After incubation for 24 or 48 h, the cultures were harvested for total RNA extraction and lipids accumulation.

Cells were stained with Oil Red O to determine lipid accumulation (Ramirez-Zacarias et al., 1992). Briefly, treatment cells were washed with PBS and fixed in 10% formalin/PBS for 30 min. After washing with wa-
ter, cells were stained with 0.5% Oil Red O for 10 min at room temperature, rinsed with water, and excess water was dried from the stained cells at room temperature. Morphological changes were observed by microscope and photographed. Then, 2 mL of isopropanol was added to the stained culture plates. The extracted dye was taken immediately, and its absorbance was measured at 510 nm in a spectrophotometer.

**Total RNA Extraction and Reverse Transcription**

Total RNA was isolated from the collected cells using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. All RNA was treated with RNase-free DNasel (Takara Biotechnology Co. Ltd.) to remove contaminating genomic DNA. The purity and concentration of total RNA were measured by a spectrophotometer at 260 and 280 nm. Ratios of absorption (260/280 nm) of all samples were between 1.8 and 2.0. Then, 2 μg was used for reverse transcription according to the methods of Wang et al. (2006). Briefly, total RNA (2 μg) and 2 μL of random primers (500 μg/mL) were denatured at 70°C for 5 min. The following components were added in order: 5 μL of 5× reaction buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM MgCl₂; and 50 mM dithiothreitol), 2 μL of deoxynucleoside triphosphate mix (10 mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, Deoxyguanosine triphosphate, and deoxythymidine triphosphate; Takara Biotechnology Co. Ltd.), 1 μL of M-MLV reverse transcriptase (200 U/μL, Promega, Madison, WI), 0.5 μL rRNasin ribonuclease inhibitor (Takara Biotechnology Co. Ltd.), 1 μL of M-MLV reverse transcriptase (200 U/μL, Promega, Madison, WI), 0.5 μL rRNasin ribonuclease inhibitor (Takara Biotechnology Co. Ltd.), 1 μL of M-MLV reverse transcriptase (200 U/μL, Promega, Madison, WI), 0.5 μL rRNasin ribonuclease inhibitor (Takara Biotechnology Co. Ltd.), 1 μL of M-MLV reverse transcriptase (200 U/μL, Promega, Madison, WI), and nuclease-free water to a final volume of 25 μL. The reaction was gently mixed by flicking the tube and was then incubated at 37°C for 60 min.

**Quantitative Real-Time PCR**

Quantitative real-time PCR was performed using Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Premix Ex Taq Kit (Takara Biotechnology Co. Ltd.); 18S rRNA was used as the reference gene. The following primers were used: pATGL (accession no. EF583921) TCACCAACACGGCATCCA (sense) and GCA-CATCTCTTCAGAAGCCA (antisense); 18S rRNA (accession no. AY265350) CCCACCGGAATCGAAGAG (sense) and TFGACGGAAGGCACA (antisense). The PCR system consisted of 10.4 μL of SYBR Premix Ex Taq (2×) mix, 1.0 μL of cDNA, 7.8 μL of doubled-distilled water, and 0.4 μL of primer pairs (10 μM), all in a total volume of 20 μL. Cycling conditions were 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. To compare the expression patterns in adipose tissues and adipocytes, mRNA template concentrations for 18S rRNA and the ATGL gene were calculated using the standard curve method. Standard curves were constructed using 2-fold dilutions of cDNA. The mRNA quantity of each amplicon was calculated for each standard and experimental sample. After normalization for 18S rRNA, the ATGL gene was expressed relative to the static control.

**Data Analysis**

All experimental data are presented as means ± SEM. Comparisons were made by unpaired, 2-tailed, Student’s t-tests or 1- or 2-way ANOVA, as appropriate. Effects were considered significant at P < 0.05.

**RESULTS**

**pATGL Full-Length cDNA Clone**

Through 3′-RACE experiment, a 1,410-bp cDNA sequence was amplified. Based on the 3′-ends cDNA sequence of the pATGL, 5′-RACE experiments were carried out, and a 518-bp fragment of pATGL 5′-region was amplified. By assembling the 2 fragments of 5′-region and 3′-region, a 1,958-bp cDNA of pATGL (accession no. EF583921) was obtained, which contains 33 bp of 5′-untranslated region, 1,461 bp of coding region that encodes a 486-AA protein (accession no. ABS58651), and 464 bp of 3′-untranslated region (Figure 1). The calculated molecular mass of this protein is 53.2 kDa.

**Alignment and Sequence Analysis**

Comparison of the full-length nucleotide sequences of pATGL with the human, dog, cattle, and mouse ATGL shows 87, 87, 86, and 82% similarity, respectively. The BLAST analysis of the deduced AA sequences of pATGL with those of the other species indicated that pATGL sharing 87% similarity with Bos taurus, 84% with Mus musculus, 83% with Rattus norvegicus, 81% with Canis familiaris, and 80% with Homo sapiens (Figure 2). The N-terminal region of pATGL contains a Gly-X-Ser-X-Gly site with a putative active serine (AA 45) (Figure 2). The BLAST results also showed that the pATGL sharing 39 to 45% similarity with the other patatin-like domain-containing proteins such as adiponutrin, GS2 protein, and GS2-like protein (data not shown).

**Expression Pattern of pATGL in Pigs**

Quantitative real-time PCR was performed to determine the expression of pATGL in different tissues and in adipose tissue of pigs of different ages postnatal. Porcine ATGL was highly expressed in porcine white adipose tissue; to a lesser degree in kidney, heart, and muscle; and least but detectable in brain (Figure 3). Because white adipose tissue has the greatest expression of pATGL, we studied the expression of pATGL in porcine OAT, PAT, and SAT at different stages postnatal (Figure 4). In SAT, pATGL mRNA was very low at birth (1 kg), and then increased and reached the greatest level at 20 kg (P < 0.01), and gradually decreased
from 20 to 40 kg ($P < 0.01$). In PAT and OAT, the greatest expression of pATGL was observed at 40 kg ($P < 0.05$; Figure 4).

**Effect of RES on the Gene Expression of pATGL**

The gene expression of pATGL in adipocytes was upregulated by RES treatment (Figure 5). Compared with the control, 40 and 80 μM RES administration for 24 h increased the mRNA levels of pATGL by 95.3% ($P < 0.05$) and 146.8% ($P < 0.01$; Figure 5A), respectively. Meanwhile, RES decreased lipid accumulation in adipocytes by 25.7% ($P < 0.05$) and 60.8% ($P < 0.01$; Figure 5B), respectively. When administrated for 48 h, 40 and 80 μM RES increased the mRNA levels of pATGL by 104.1% ($P < 0.05$) and 163.1% ($P < 0.01$; Figure 5A), respectively. The RES decreased lipid accumulation by 38.5% ($P < 0.05$) and 65.7% ($P < 0.01$; Figure 5B), respectively.

**Figure 1.** The full-length cDNA sequence and the deduced AA sequence of the porcine adipose triglyceride lipase. The predicted AA sequence was presented in single-letter code. The single stop codon was indicated by an asterisk (*). The polyadenylation signals were underlined. The numbers after each line indicate the number of the nucleotide (upper number) or the amino acid (lower number). The nucleotide sequence has been deposited in GenBank under accession no. EF583921.
9.7% \( (P > 0.05) \) and 29.0% \( (P < 0.05) \), respectively (Figure 5B). In addition, RES inhibited the proliferation of the porcine adipocytes (data not shown).

**DISCUSSION**

Recently, ATGL has been cloned and further characterized as a major novel TG lipase (Jenkins et al., 2004; Villena et al., 2004; Zimmermann et al., 2004). Mouse ATGL mRNA encodes a 486-AA putative protein (predicted molecular mass of 54 kDa; Villena et al., 2004; Zimmermann et al., 2004), whereas human ATGL gene encodes a 506-AA protein with a calculated molecular mass of 56 kDa (Zimmermann et al., 2004). In the current study, the full-length cDNA sequence of pATGL, which encodes a 486-AA protein with a predicted molecular mass of 53 kDa, was cloned. Both the coding sequence and the inferred AA sequence shared substantial identity with ATGL in other species. For example, the pATGL also contained a patatin-like domain, Gly-X-Ser-X-Gly, which is also observed in ATGL and other patatin-like domain-containing pro-

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**Figure 2.** Comparisons of the mouse (m), rat (r), dog (d), human (h), cattle (c), and porcine (p) adipose triglyceride lipase (ATGL) AA sequences. Identical AA were shown in bold. The patatin-like domain Gly-X-Ser-X-Gly was boxed. Cysteine residues were marked with an asterisk (*).
proteins such as adiponutrin, GS2-like, and GS2 (Rydel et al., 2003). Furthermore, the BLAST results showed that the pATGL shared 39 to 45% similarity with these patatin-like domain-containing proteins (adiponutrin, GS2-like, and GS2; data not shown).

Adipose TG lipase specifically hydrolyses long-chain fatty acid TG and is predominantly expressed in adipose tissue. Adipose TG lipase is also expressed, to a lesser extent, in cardiac muscle, skeletal muscle, testis tissue, and other tissues (Jenkins et al., 2004; Zimmermann et al., 2004; Kershaw et al., 2006). Consistent with the previous reports in mouse and human (Jenkins et al., 2004; Zimmermann et al., 2004), the greatest level of expression of pATGL was also found in adipose tissue. In addition, the pATGL is expressed at a moderate degree in kidney, heart, and muscles. The expression level is low but detectable in the brain of pig.

Adipose TG lipase expression pattern during adipogenesis was studied in vitro. Previous studies focused on the expression of murine ATGL in mouse 3T3-L1 adipocytes at various stages of differentiation (Jenkins et al., 2004; Zimmermann et al., 2004; Kershaw et al., 2006). However, there was no report about the ATGL gene expression in animals at different stages of growth. In the current study, using the quantitative real-time technique, the expression of the ATGL gene in porcine adipose tissues at different BW was investigated. The gene expression of ATGL in porcine PAT and OAT increased with BW from birth to 40 kg and then decreased from 40 to 60 kg. Adipose TG lipase is a major TG lipase in animals. Adipose TG lipase performs the initial step in TG hydrolysis and plays a pivotal role in the lipolytic catabolism of stored fat in animals.

\[ \text{Figure 3. Relative abundance of the mRNA for porcine adipose triglyceride lipase (ATGL) in various tissues. The mRNA was determined by quantitative real-time PCR in muscle (MUS), white adipose tissue (WAT), heart (HEA), liver (LIV), kidney (KID), lung (LUN), brain (BRA), spleen (SPL), and small intestine (SIN) of 30-kg pigs. Porcine ATGL gene expression was normalized to 18S ribosomal RNA and expressed relative to ATGL expression in LIV. Each column represents the mean of 6 individual pigs ± SEM *P < 0.05, **P < 0.01 compared with expression in liver.} \]

\[ \text{Figure 4. Effect of BW on the relative abundance of mRNA for porcine adipose triglyceride lipase (ATGL) in adipose tissues (s.c. adipose tissue, peritoneal adipose tissue, and omental adipose tissue) of pigs. Porcine ATGL expression was determined by quantitative real-time PCR and was normalized to 18S ribosomal RNA and expressed relative to porcine ATGL expression at 1 kg. Each column represents the mean of 6 individual pigs ± SEM *P < 0.05, **P < 0.01.} \]
adipose tissue (Haemmerle et al., 2006; Schoenborn et al., 2006). In human and mouse, ATGL contributes to adipocyte lipolysis and participates in TG-specific hydrolysis (Kralisch et al., 2005; Kim et al., 2006). The upregulation of ATGL enhances the lipolysis and prevents TG accumulation in adipocytes (Zimmermann et al., 2004). It is believed that expression of ATGL is related to the lipolysis ratios in adipose tissue. Evidence demonstrated that the lipolysis ratios in adipose tissue decreased with age (Mersmann, 1998). Thus, expression pattern of ATGL, which increased and maximized in young pigs and then decreased at heavier BW, was partly consistent with the lipolysis ratios.

Adipose tissue expression of ATGL is reciprocally regulated by nutritional status and by other factors such as hormones. In mice, ATGL is upregulated by fasting and downregulated by refeeding (Jenkins et al., 2004; Kershaw et al., 2006). In addition, adipose expression of ATGL was increased by insulin deficiency and decreased by insulin replacement in streptozotocin-induced diabetic mice (Kershaw et al., 2006). Expression of ATGL was also increased in fat-specific insulin receptor knockout mice (Kershaw et al., 2006). In 3T3-L1 adipocytes, isoproterenol (Kralisch et al., 2005), insulin (Kralisch et al., 2005; Kershaw et al., 2006), and tumor necrosis factor α (Kralisch et al., 2005; Kim et al., 2006) administration reduced ATGL expression in a dose- and time-dependent manner. One more recent study demonstrated that ATGL gene expression also could be regulated by rosiglitazone (Shen et al., 2007). In the current study, we found that RES significantly elevated the expression of pATGL and stimulated the lipid mobilization in porcine adipocytes in the treatments for both 24 and 48 h.

Resveratrol, a polyphenol abundant in grape skins and juice, has various pharmacological effects including protection of cells from lipid accumulation, chemoprevention, immunomodulation, antiproliferation, and promotion of differentiation (Pervaiz, 2003). In vitro studies showed that RES reduces TG content and stimulates FFA release in mice 3T3-L1 adipocytes (Picard et al., 2004). Based on the previous reports and the results of the current study, it was concluded that RES stimulates the gene expression of pATGL and the upregulation of ATGL gene expression and subsequently enhances the lipid mobilization in porcine adipocytes.

Resveratrol is the activator of Sirtuin1 (Sirt1, Picard et al., 2004; Backesjo et al., 2006). The activation of Sirt1 represses peroxisome proliferator-activator receptor γ (PPARγ) by docking with its cofactors nuclear receptor co-repressor and SMRT silencing mediator of retinoid and thyroid hormone receptors (Picard et al., 2004). Peroxisome proliferator-activated receptor γ agonist stimulates lipolysis by increasing the lipolytic potential, including the expression levels of the genes encoding ATGL (Festuccia et al., 2006). Evidence showed that the ATGL is subject to transcriptional control by PPARγ-mediated signals (Kim et al., 2006).

Combining the results of the current study and previous documentation, we speculate that RES initially activates the expression of Sirt1, and the expressed Sirt1 subsequently inhibits the action of PPARγ. Ultimately, PPARγ-mediated signals increase the ATGL gene expression. Of course, the precise model of RES action on the ATGL gene expression needs further studies.

In conclusion, the full-length of pATGL was cloned and sequenced in the current study. Meanwhile, the tissue-specific and development expression and the RES regulation of the pATGL gene were examined. These results reveal new insights into manipulation of pATGL expression in regulating lipid metabolism and improving meat quality.

**LITERATURE CITED**

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