Porcine sirtuin 1 gene clone, expression pattern, and regulation by resveratrol

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ABSTRACT: Sirtuin1 (Sirt1) is a NAD-dependent deacetylase that plays important roles in a variety of biological processes. In the current study, we examined tissue-specific and different expression pattern of porcine Sirt1 and the effect of resveratrol (RES) on expression of Sirt1 in porcine adipocytes. The full-length complementary DNA sequence of porcine Sirt1 was 4,024 bp (GenBank accession no: EU030283), with a 2,226-bp open reading frame encoding a 742-AA protein (a predicted molecular mass of 80.9 kDa; GenBank accession no. ABS29571). Comparison of the deduced AA sequence with the corresponding sequences of human, dog, cattle, and mouse Sirt1 showed 82 to 92% similarity. Furthermore, the porcine Sirt1 was highly expressed in porcine brain, to a lesser degree in spleen and white adipose tissue, and had low but detectable expression in liver. In subcutaneous adipose tissue and omental adipose tissue, expression of the porcine Sirt1 mRNA was greater in adult pigs than in young pigs ($P < 0.01$). In vitro, exposure of cultured adipocytes to 40 and 80 µM RES for 24 h increased mRNA levels of porcine Sirt1 by 47.86% ($P < 0.01$) and 91.04% ($P < 0.01$), respectively. Accordingly, lipid accumulation and NEFA release were decreased ($P < 0.05$), respectively. After cultures were treated with RES for 48 h, the mRNA level of porcine Sirt1 was increased by 103.84% ($P < 0.01$) and 148.79% ($P < 0.01$), respectively. Lipid accumulation was decreased and NEFA release was increased ($P < 0.05$), respectively. These results provide information needed for manipulating Sirt1 expression in regulating fat deposition in pigs.

Key words: cloning, gene expression, pig, resveratrol, sirtuin 1

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INTRODUCTION

Silent information regulator 2 (Sir2) is a NAD-dependent deacetylase that connects metabolism with longevity in yeast and worms (Haigis and Guarente, 2006). Mammals contain 7 homologs of yeast Sir2 [sirtuins 1 through 7] of which sirtuin 1 (Sirt1) is the closest homolog of yeast Sir2 (Frye, 2000). Sirtuin 1 plays important roles in a variety of biological processes such as stress and cytokine responses (Brunet et al., 2004; Yeung et al., 2004), proliferation and differentiation (Langley et al., 2002), apoptosis (Luo et al., 2001; Motta et al., 2004; Kitamura et al., 2005), and metabolism (Picard et al., 2004; Rodgers et al., 2005). The expression and regulation of Sirt1 by fasting (Rodgers et al., 2005; Rodgers and Puigserver, 2007), calorie restriction (Wolf, 2006; Chen et al., 2008), and nutrient availability (Kanfi et al., 2008) have been studied in human and mouse. However, little is known about the expression and regulation of the Sirt1 gene in pigs.

Resveratrol (3,5,4′-trihydroxystilbene; RES), a polyphenolic phytochemical present in grapes, peanuts, and mulberries, was reported to be an activator of Sirt1 in vivo and in vitro (Howitz et al., 2003; Picard et al., 2004; Backesjo et al., 2006). It up-regulates the expression of Sirt1 and reduces fat deposition in mice adipocytes (Picard et al., 2004). However, very little is known about the regulation of RES on expression of the porcine Sirt1 gene in porcine adipocytes.

To add to the understanding of the role of Sirt1 in lipid metabolism and provide important information for regulating fat deposition in pigs, we cloned the full-length sequences of porcine Sirt1 mRNA and investigated the tissue-specific and differential expression pattern in young and adult pigs. In addition, the effect of RES on porcine Sirt1 gene expression and lipid accumulation in porcine adipocytes was also studied.
MATERIALS AND METHODS

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

Animals

In Exp. 1, 6 female pigs (Duroc × Landrace × Yorkshire) with a mean BW of 30 kg were selected and killed. Brain, heart, liver, lung, spleen, muscle, small intestine, and white adipose tissue in right-half carcasses were collected and rapidly frozen in liquid nitrogen, and then stored at −80°C until RNA analysis. In Exp. 2, a total of 12 pigs (Duroc × Landrace × Yorkshire) were used to investigate the expression pattern of Sirt1 in young and adult pigs. Six female pigs each at 1 d (young pigs) and 168 d (adult pigs) of age were randomly selected and killed under anesthesia for samples. Omental adipose tissue (OAT), subcutaneous adipose tissue (SAT), and peritoneal adipose tissue (PAT) in right-half carcasses were collected, rapidly frozen in liquid nitrogen, and stored at −80°C until RNA analysis.

Cloning of Full-Length Complementary DNA of Porcine Sirt1

To obtain the full-length complementary (c)DNA sequence of porcine Sirt1, rapid amplification of cDNA ends (RACE) technology was carried out according to previous studies (Shan et al., 2008) 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 Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) using oligo(dT)18-containing anchor primer (primer 1: 5′-CTGATCTAGGTTACCGATCCTTTTTTTTTTTTTTTTTTT-3′). The PCR was performed using a combination of the adaptor primer (primer 2: 5′-CTGATCTAGGTTACCGATCC-3′) and a gene-specific primer (primer 3: 5′-GTAGGAGGTGAATATGCCAAG-3′). The gene-specific primer 3 was designed based on the partial sequence of porcine Sirt1 identified by the BLAST analysis. The phylogenetic tree of the alignment sequences was constructed using the neighbor joining method and 1,000 bootstrap trials. Briefly, Sirt1 domain-containing sequences were first automatically aligned and then manually curated and saved to compute the distances and calculate the percentages of different residues between sequences. The values were then used to build the phylogenetic tree between sequences, with 1,000 bootstrap replicates performed to assess the reliability of the tree topology.

Cell Culture

In Exp. 3, SAT was collected from piglets, and porcine preadipocytes were prepared by previously published methods (Ramsay, 2001; Shan et al., 2008). The 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 medium (Dulbecco’s modified essential medium/F12 + 10% fetal bovine serum + 100 U of penicillin + 100 U of streptomycin) and cultured at 37°C under a humidified atmosphere of 95% air:5% CO₂. Medium was changed every 3 d. After 10 d of lipid filling, cultures were washed with Dulbecco’s modified essential medium/F12 and then used for an experiment assessing the effect of RES on the gene expression of Sirt1. The 3 treatments were 1) control (complete medium), 2) complete medium + 40 µM RES, and 3) complete medium + 80 µM RES. After incubating for 24 or 48 h, the cultures were harvested for total RNA extraction, lipid accumulation, and NEFA release. Cells were stained with oil-red O to determine lipid accumulation (Shan et al., 2008); NEFA release was determined according to previous reports (Kershaw et al., 2006).

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 manufacturer’s instructions. All RNA was treated with RNase-free DNase (Takara Biotechnology Co. Ltd.) to remove any trace of 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 of RNA was used for reverse transcription as described by Wang et al. (2006) and Shan et al. (2008).

Quantitative Real-Time PCR

The quantitative real-time PCR was carried out in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with a SYBR Premix Ex Taq Kit (Takara Biotechnology Co. Ltd.) and gene-specific primers; 18S ribosomal RNA was used as the reference gene. The oligonucleotide primers used for the real-time PCR were designed to span genomic introns and are available upon request. The 2−ΔΔCT (cycle threshold, CT) method was used to analyze the relative changes in each gene expression (Livak and Schmittgen, 2001). Briefly, 1 µL of cDNA template was added to each well in a 96-well reaction plate, and the transcripts of each gene were amplified in triplicate. Average CT values were calculated, and the ΔCT values relative to 18S ribosomal RNA control were computed for each gene. Subsequently, ΔΔCT was computed for each gene by subtracting the average ΔCT for the control group. The final fold differences were computed as 2−ΔΔCT for each gene. The measurements were repeated 3 times.

Statistical 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

Porcine Sirt1 Full-Length cDNA Clone

In the 3′-RACE experiment, a 2,431-bp cDNA sequence was amplified. Based on the 3′ ends of the cDNA sequence of the porcine Sirt1, 5′-RACE experiments were carried out, and a 1,072-bp fragment of the porcine Sirt1 5′-region was amplified. By assembling the fragments of the 5′ and 3′ regions, a 4,024-bp cDNA of porcine Sirt1 (accession no. EU030283) was obtained, which contained 15 bp of 5′-untranslated region (UTR), 2,226 bp of coding region that encodes a 742-AA protein (accession no. ABS29571), and 1,780 bp of 3′-UTR (Figure 1). The calculated molecular mass of this protein is 80.9 kDa. The 3′-UTR was shown to contain the eukaryotic polyadenylation consensus signal ATTAAA downstream of the termination of translation signal TAA (Figure 1; Fitzgerald and Shenk, 1981; Voelter-Mahlknecht and Mahlknecht, 2006).

Alignment and Phylogenetic Tree Analysis

Comparison of the full-length nucleotide sequences of porcine Sirt1 with the human, dog, cattle, and mouse Sirt1 showed 90, 92, 92, and 84% similarity, respectively (data not shown). The BLAST analysis of the deduced AA sequences of porcine Sirt1 with those of the other species indicated that Sirt1 shared 87% similarity with Homo sapiens, 82% with Mus musculus, 92% with Canis familiaris, and 91% with Bos taurus (Figure 2). The alignment and phylogenetic tree analysis of the Sirt1 of different species suggested that the porcine Sirt1 is more closely linked to mammals than microbes.
The porcine Sirt1 also contained a Sirt1 core domain, which has been observed in other sirtuin proteins (data not shown). There are several short motifs of conserved amino acids present within the Sirt1 core domain; these include GAGXSXXXGIPDFR, TQNID, CHGSF, CXXC-(18–20)X-CXXC, which is a zinc-finger domain (Frye, 2000; Finnin et al., 2001), FGE, GXSLKV, and LIN (Figure 2).

Expression Pattern of Porcine Sirt1 in Pigs

Quantitative real-time PCR was performed to determine the expression of porcine Sirt1 in different tissues and in adipose tissue of young (1 d) and adult pigs (168 d). Porcine Sirt1 was highly expressed in brain, to a lesser degree in spleen and adipose tissue, and was expressed at a low but detectable level in liver (Figure 3).
In SAT (Figure 4A) and OAT (Figure 4B), expression of porcine Sirt1 mRNA was greater in the adult pigs than in the young pigs ($P < 0.01$), whereas in PAT, expression of the porcine Sirt1 mRNA was not greater in the adult pigs compared with the young pigs ($P > 0.01$; Figure 4C).

**Effect of RES on Gene Expression of Porcine Sirt1**

The gene expression of porcine Sirt1 in adipocytes was upregulated by RES treatment (Figure 5). Compared with the control, administration of 40 and 80
µM RES for 24 h increased the mRNA levels of porcine Sirt1 by 47.86% \((P < 0.01)\) and 91.04% \((P < 0.01; \text{Figure 5A})\), respectively. Meanwhile, RES decreased lipid accumulation in adipocytes by 25.70% \((P < 0.05)\) and 60.80% \((P < 0.01; \text{Figure 5B})\), respectively. When administered for 48 h, 40 and 80 µM RES increased the mRNA levels of porcine Sirt1 by 103.84% \((P < 0.01)\) and 148.79% \((P < 0.01; \text{Figure 5A})\), respectively. Treatment with RES for 48 h decreased lipid accumulation by 9.7% \((P > 0.05)\) and 29.0% \((P < 0.05)\), respectively (Figure 5B). In addition, RES treatment increased \((P < 0.05)\) NEFA release in the treatments for both 24 and 48 h (Figure 5C).

**DISCUSSION**

Mammalian Sirt1 NAD-dependent protein deacetylase, the closest homolog of Sir2, regulates diverse biological processes including metabolism (Rodgers et al., 2005), apoptosis (Motta et al., 2004; Kitamura et al., 2005), cell cycle and differentiation (Langley et al., 2002), insulin-signaling pathways (Yang et al., 2006), and possibly aging (Chua et al., 2005). To date, full-length cDNA sequences of Sirt1 genes have been cloned and further characterized in humans (Frye, 1999; Gray and Ekström, 2001; Voelter-Mahlknecht and Mahlknecht, 2006), mice (Imai et al., 2000; Sakamoto et al., 2004), and other animals (Matsushita et al., 2005). Human Sirt1 mRNA has an open reading frame of 2,447 bp, which yields a 747- AA protein with a predicted molecular mass of 81.7 kDa (Voelter-Mahlknecht and Mahlknecht, 2006), whereas the mouse Sirt1 encodes a 737- AA protein (Imai et al., 2000; Sakamoto et al., 2004), and the chicken Sirt1 encodes a 757- AA protein (Matsushita et al., 2005). In the current study, the full-length cDNA sequence of porcine Sirt1, which encodes a 742- AA protein with a predicted molecular mass of 80.9 kDa, was cloned. The inferred AA sequence shared substantial identity with Sirt1 in other species.

The sirtuin protein family contains a core domain that consists of a series of conserved sequence motifs (Frye, 2000; Huhtiniemi et al., 2006). Like other Sir2-like proteins, porcine Sirt1 also contains the Sir2-like protein homology domain, which includes a series of conserved sequence motifs including GAGXXGIPDFR, TQNID, CHGSF, CXXC-(18–20)X-CXXC, FGE, GXSLKV, and LIN. The sequence CXXC-(18–20)X-CXXC includes the zinc-coordinating Cys residues, which are present in all classes of Sir2-like enzymes (Finnin et al., 2001; Huhtiniemi et al., 2006). The zinc atom is tetrahedrally coordinated by the thiols of 4 Cys residues (365, 368, 389, and 392) in porcine Sirt1. The other conserved residues such as GAGXXGIPDFR, TQNID, and CHGSF are involved in creating the binding pocket (Huhtiniemi et al., 2006). These results indicated that the porcine Sirt1 was evolutionally conserved and may be involved in the regulation of various biological processes such as adipogenesis, endocrine signaling, and glucose and lipid metabolism (Huhtiniemi et al., 2006).

Sirtuin 1, the most studied of the 7 sirtuin family members, is highly expressed in several adult tissues such as brain, heart, and skeletal muscle (Huhtiniemi et al., 2006). Human Sirt1 is expressed in a variety...
Figure 5. Effect of resveratrol (RES, 40 or 80 µM) on A) the abundance of mRNA for sirtuin 1 (Sirt1), B) lipid accumulation, and C) NEFA release. Porcine Sirt1 expression was determined by quantitative real-time PCR, normalized to that of 18S ribosomal RNA, and expressed relative to porcine Sirt1 expression in control. The NEFA release was normalized to total cellular protein and expressed relative to the control group. Each column represents the means ± SEM from 3 replicates; *P < 0.05, **P < 0.01.
of tissues, and high levels of mRNA expression have been observed in adult brain and testis (Frye, 1999; Michishita et al., 2005). Mouse Sirt1 is predominantly expressed in lung, and to a lesser extent in testis, ovary, spleen, thymus, and heart (Sakamoto et al., 2004). In a calorie-restriction rat model, greater expression of Sirt1 was also found in brain, visceral fat pads, kidney, and liver (Cohen et al., 2004). However, the expression pattern of porcine Sirt1 has been little studied (Bai et al., 2008). In the current study, we quantitatively examined the expression levels of porcine Sirt1 in various tissues of pigs by quantitative real-time PCR. Consistent with the previous reports (Frye, 1999; Cohen et al., 2004; Michishita et al., 2005; Bai et al., 2008), porcine Sirt1 mRNA was widely expressed in multiple pig tissues. In addition, a greater level of expression of porcine Sirt1 was also found in brain, spleen, and adipose tissue.

Aging is one of the greatest risk factors for metabolic complications such as obesity, glucose intolerance, and type 2 diabetes (Chang and Halter, 2003; Moller et al., 2003). Sirtuin 1 plays important roles in the response to metabolism, longevity, and aging (Engel and Mahlknecht, 2008). Previous studies showed that the expression of Sirt1 changed with aging (Alcendor et al., 2007; Ferrara et al., 2008). The expression of Sirt1 in hearts of old monkeys was greater than that in young animals (Alcendor et al., 2007). In rats, aging induced a decrease in Sirt1 activity in the heart (Ferrara et al., 2008). Other studies demonstrated that myocardial Sirt1 expression was not different between young and old rats (Cross et al., 2008). However, there are no reports about porcine Sirt1 gene expression in pigs at different stages of growth. In the current study we investigated the expression of Sirt1 gene in porcine adipose tissues in young and adult pigs using quantitative real-time PCR. The expression of porcine Sirt1 in SAT and OAT of the adult pigs was greater than that of the young animals, whereas in PAT, there was no difference between the young and adult animals. These findings provide new insight into the age-dependent regulation of Sirt1 expression in mammals.

Sirtuin 1 is regulated by nutritional status (Bordone and Guarente, 2005; Moynihan and Imai, 2006) and by other factors such as nicotinamide, sirtinol, and splittomicin (Bedalov et al., 2001; Bitterman et al., 2002; Araki et al., 2004). Fasting and caloric restriction increased Sirt1 levels in tissues such as muscle, brain, liver, and fat (Rodgers et al., 2005; Ramsey et al., 2008). In the current study, we found that RES increased the expression of porcine Sirt1, stimulated NEFA release, and further decreased lipid accumulation in porcine adipocytes.

Resveratrol, a natural polyphenolic phytochemical, has various bioactivities including immunomodulation, antiproliferation, and modulation of lipid metabolism (Pervaiz, 2003). Treatment with RES decreases triglyceride content and stimulates FFA release in mice 3T3-L1 adipocytes (Picard et al., 2004). Recent studies demonstrated that RES activates the expression of Sirt1 and reduces fat deposition (Howitz et al., 2003; Picard et al., 2004; Backesjo et al., 2006; Kim et al., 2008). The activation of Sirt1 promotes fat mobilization in 3T3-L1 adipocytes (Picard et al., 2004). Based on the results of our study and previous documentation, we concluded that RES activated the expression of Sirt1 and subsequently promoted lipid mobilization in porcine adipocytes.

Sirtuin 1 plays a key modulatory role in animal fat deposition, is involved in adipogenesis, and could promote fat mobilization in white adipocytes (Picard et al., 2004; Picard and Guarente, 2005; Wolf, 2006). Sirtuin1 regulates several transcription factors that govern fat metabolism, including peroxisome proliferator-activator receptor γ (PPARγ; Picard et al., 2004), forkhead-box transcription factors (Kobayashi et al., 2005; Yang et al., 2005), and adiponectin (Qiao and Shao, 2006; Qiang et al., 2007). The activation of Sirt1 promotes the fat mobilization by repressing PPARγ, one of the transcriptional factors in fat storage (Picard et al., 2004). Sirtuin 1 binds directly to the PPARγ-negative cofactors, nuclear receptor co-repressor or silencing mediator of retinoid and thyroid hormone receptors, and functions as a negative regulator of PPARγ (Picard and Guarente, 2005). Evidence showed that PPARγ-mediated signals could transcriptionally control gene expression of adipose triglyceride lipase (ATGL; Kim et al., 2006). Our previous study indicated that RES elevated the expression of ATGL and stimulated the lipid mobilization in porcine adipocytes (Shan et al., 2008). Combining the results of the current study and previous reports, we speculate that RES initially activates the expression of Sirt1, and the activated Sirt1 subsequently increases ATGL gene expression through PPARγ-mediated signals. However, the precise model of RES action on Sirt1 gene expression and lipid mobilization needs further study.

In conclusion, the full length of porcine Sirt1 was cloned and sequenced in the current study. In addition, the tissue specificity and differential expression between young and adult pigs, and the RES regulation of porcine Sirt1 gene were examined. These results add to our understanding of the role of porcine Sirt1 in lipid mobilization and as a potential target for regulating fat deposition in pigs.

**LITERATURE CITED**


