Expression Patterns of the *Cannabinoid Receptor 1* Gene in Pig Skeletal Muscle and Skeletal Satellite Muscle Cells

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Abstract: The Cannabinoid Receptor type 1 (CNR1) is a key component of the endocannabinoid system which has been reported to play a pivotal role in modulating feeding behavior and energy balance. While a large number of researches have showed clearly that CNR1 is expressed in skeletal muscle of mice and human beings, detailed analysis of *CNR1* gene expression in pig muscles and muscle cells has not been reported. Here, quantitative PCR were used to characterize the CNR1 in different muscles of pig and isolation of porcine myogenic satellite cells. Results showed that the expression of CNR1 mRNA was greatest in longissimus dorsi muscle ($p<0.01$) among that in the gastrocnemius (mix), soleus muscle (slow-type) and longissimus doris muscle (fast-type). As to age-related changes of *CNR1* gene in different pig muscles, the higher expression was found at the 30th day, then declined ($p<0.05$) in all three kinds of muscles to the 90th day. At the 150th day, the expression of CNR1 steadily decreased ($p<0.05$) in the gastrocnemius compared to the 90th day but not in longissimus doris muscle and soleus. Meanwhile the breed differences in the expression of CNR1 mRNA between Jinhua pig and Landrace were also identified. The mRNA abundance of CNR1 in Landrace was higher compared with that of Jinhua pigs and strong differences were observed in longissimus doris muscle ($p<0.05$) and soleus ($p<0.01$). *In vitro* with the differentiation of the satellite cells, the mRNA expression of the CNR1 was significantly down-regulated ($p<0.05$). The lowest mRNA expression was observed at the 9th day. These data provide a framework of *CNR1* gene expression patterns and implicate a novel gene for regulating muscle fiber types.

Key words: Cannabinoid Receptor 1 (CNR1), skeletal muscle, skeletal satellite muscle cells, pig, isolation, gastrocnemius, China

INTRODUCTION

The endocannabinoids is an endogenous amide of long-chain fatty acids. As a signal substance, it has exerted diverse biological effects on regulating energy metabolism and many other biological functions through the cannabinoid receptor type 1 (CNR1) (Cavuoto *et al.*, 2007a, b; Amoros *et al.*, 2010). CNR1 belongs to the superfamily of Gi/Go protein-coupled receptors (Matsuda *et al.*, 1990) and is mainly expressed within the central nervous system as well as peripheral tissues. In the central system, CNR1 is highly expressed in the hippocampus, cerebellum and substantia nigra (Glass *et al.*, 1997). About peripherally tissue, CNR1 also is found in adipocytes, the gastrointestinal tract, liver and skeletal muscle (Pertwee, 2001; Cota *et al.*, 2003; Osei-Hyiaman *et al.*, 2005; Silvestri *et al.*, 2011).

A larger number studies have shown that the CNR1 could regulate energy metabolism and have some relationship with obesity and other metabolic diseases (Benzinou *et al.*, 2008; Di Marzo, 2008; Schleinitz *et al.*, 2010; Milewicz *et al.*, 2011). CNR1 specific antagonists have not only been shown to inhibit appetite but also to increase peripheral energy utilization (Pagotto *et al.*,

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2006). Skeletal muscle is a contractile tissue which plays an important role in energy homeostasis and metabolic regulation (Cote *et al.*, 2007; Tedesco *et al.*, 2010). Although, CNR1 have been detected in skeletal muscle (Matias *et al.*, 2006), most of the research focused on the rodents and human beings (Cavuoto *et al.*, 2007a, b; Gustafsson *et al.*, 2008). Up to now, little was known about the expression of CNR1 in pig.

The Jinhua pig (a fatty breed) is one of the most famous local breed in China. The breed is especially noted for its superior quality in meat including high Intramuscular Fat (IMF), redder and tender meat and good taste flavor (Miao *et al.*, 2009; Yao *et al.*, 2011). Landrace pig (a leaner breed) is believed to grow faster and lower fat content (Lefaucheur *et al.*, 2002). Therefore, these two different breeds can serve as good models for studying gene expression of energy metabolism and meat quality. Moreover, skeletal satellite cells are thought to be the precursors that provide a reserve capacity to replace the differentiated in skeletal muscle (Zammit and Beauchamp, 2001). The standard approach has been established to isolate skeletal satellite cells from adult skeletal muscle by enzymatic disaggregation (Bischoff, 1974; Blau and Webster, 1981; Yablonka-Reuveni *et al.*, 1987; Qu-Petersen *et al.*, 2002; Mau *et al.*, 2008).

In the current experiment, we wished to demonstrate the expression patterns of *CNR1* gene including age-related changes and breed difference in different muscles *in vivo*. Furthermore, the developmental CNR1 expression in isolated skeletal satellite cells was also studied. This research has the potential to increase the understanding of the role of CNR1 in skeletal muscle and provide a potential target for regulating energy metabolism and meat quality.

MATERIALS AND METHODS

All of the experiments were completed according to the guidelines for animal experiments at the National Institute of Animal health.

Animals and sample collection: Jinhua pigs and Landrace were purchased from Zhejiang Jiahua Pig Breeding Co., Ltd. The animals were raised under same conditions and were fed on commercial diet. Six Landrace (90th days) and 18 Jinhua males at the 30th, 90th and 150th day (6 individuals per stage each breed) were used to determine the CNR1 expression patterns. All pigs were selected randomly and killed after a 12 h fasting period. Three different skeletal muscles such as longissimus muscle, gastrocnemius and soleus were selected for mRNA abundance: the longissimus muscle adjacent to the

last rib were removed, gastrocnemius and soleus samples were taken from the left-half carcasses immediately after postmortem and packed into plastic bags then frozen in liquid N and stored at -80°C until further analysis.

Isolation and culture of pig skeletal satellite muscle cells: The method for isolation of pig skeletal muscle cells has earlier been described by Redshaw *et al.* (2010). Briefly, three Landrace pigs from 5-7 days of age were overdosed with sodium thiopental and exsanguinated. Longissimus muscle was removed and washed with Phosphate Buffered Saline (PBS) which was then minced and cells were dissociated by digestion with 1 g L^{-1} Pronase (Gibico) in Dulbecco's Modified Eagle's Medium (DMEM, Gibico) for 1 h at 37°C with continuous agitation. Digestion was halted by DMEM containing 20% Fetal Bovine Serum (FBS invitrogen), followed by filtration through 74 um nylon mesh filtration and centrifuged for 20 min at 1200 rpm. Cells were resuspended in Growth Media (GM invitrogen) with 20% FBS, 100 IU mL^{-1} penicillin, 100 mg mL^{-1} streptomycin. Then, the satellite cells were isolated using a preplating method. Initially cells were seeded on plastic for 6 h and then non-adhered cells were transferred to a new plastic. Cells were cultured at 37°C under a humidified atmosphere of 95 air: 5% CO_2. Media was changed every day. When the cells had grown to near 80-90% confluence they were passaged by trypsinization (0.5% trypsin in 0.25% mM EDTA). Differentiation Medium (DM) contained 2% horse serum instead of 20% fetal bovine serum. After cultured in DM for 0, 1, 3, 5, 7, 9 and 11, the cells were harvested, respectively.

RNA isolation and cDNA synthesis: The total RNA was extracted from different skeletal muscles and isolation of porcine myogenic satellite cells by Trizol reagent (Invitrogen) according to the manufacturer's protocols, then treated with RNase-free DNase I (Biolabs) to remove contaminating genomic DNA, after that RNA was further purified by RNA clean kit (Aidlab). The concentration and purity of the RNA were determined by Nano Drop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). The first cDNA was synthesized from total 0.5 ug RNA using PrimeScript Master Mix (Takara) as described in the manufacturer's protocol. The prepared cDNA was stored at -20°C.

Identification of pig skeletal satellite muscle cells: Desmin immunocytochemistry and mark genes-MyoD, *MyoG* gene expression were used to identify pig skeletal satellite muscle cells. Cells were seeded at a density of 1×10^5 cells/well in 24 well plate then cells were fixed in 4%

paraformaldehyde at 24 h of growing cell. BAD staining and characterization of satellite cells were performed by Diaminobenzidine (DAB) kit (Keygen, China) and SABC Mice IgG1/2 kit (Boster, China). The desmin antibody used was pig monoclonal antibody (1:300, Boster). Primary antibody incubations were 2 h and secondary antibody incubation was 20 min as per manufacturer's guidelines. Additional *MyoD* and *MyoG* gene expression studies used differentiated cells. Cells seeded at the same plating density in 6 well plate, once cells became 80% confluence, DM was used and cells cultured for up to 7 days with media changes every 48 h. Then the cells were collected for RNA extraction. Expression of MyoD and MyoG were determined by conventional PCR and gel electrophoresis. The following primers were used: 18S (accession AY265350) CCCACGGAATCGAGAAAGAG (sense) and TTGACGGAAGGGCACCA (antisense); MyoD (accessionU12574.1) TGCGTATTCTCAACCCCTC (sense) and AGTATGCAAGGGTGGAGTGG (antisense), MyoG(accessionNM_001012406.1)CTTCTACCAGGAA-CCCCACTTCT (sense) and GTCCC-CAGCCCC-TTATCTTC (antisense). PCR reactions were prepared using (Sangon, China) and the cycling parameters were: 95°C 2 min, 30-35 cycles of 95°C 10 sec, 60°C 30 sec and final elongation at 72°C for 10 min and then all products were resolved on 1.5%.

Quantitative Real time PCR (RT-PCR): RT-PCR was performed using Applied Biosystem 7500 Fast real time PCR system (Applied Biosystems, Foster city, CA) and SYBR premix ExTaq™ (Tli RNaseH Plus) (Takara). *CNR1* gene was selected to analyze the RNA abundance and 18S was used as the reference gene. The primers were designed and synthesized by Takara Co., Ltd. and primers sequences are shown as follow. About 18S (accession-AY265350) CCCACGGAATCGAGAAAGAG (sense) and TTGACGGAAGGGCACCA (antisense); CNR1 (accessionXM_003121292.2) TACAAGGAGAAT GACGAGAA (sense) and TGGAGGATAACGCACAGT (antisense). The PCR System was composed of 1 μL cDNA sample, 0.4 μL sense primer (10 μM), 0.4 μL anti-sense primer (10 μM), 0.4 μL Rox, 10 μL SYBR premix and 7.8 μL water (Rnase, DNase-free) to a total volume of 20 μL. The PCR was run for 2 min at 95°C, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. The melting curve was analysed to verify the specificity of the reactions. The relative target gene expression was evaluated according to previous publications of the research group (Guo *et al.*, 2011). About $2^{-\Delta\Delta CT}$ Method was used to determine mRNA expression levels and the target gene expressions were normalized to reference 18S rRNA gene expression.

Statistics: All data are expressed as the mean±SEM and mean values were compared by unpaired 2-tailed student's t-tests or ANOVA using SPSS16.0 as appropriateness. Differences were considered to be significant at p<0.05. All experiments were repeated in three times.

RESULTS AND DISCUSSION

CNR1 mRNA expression is greatest in longissimus muscle: The different skeletal muscles such as longissimus dorsi muscle (primarily fast-type) and gastrocnemius (mixed) as well as soleus (primarily slow-type) were dissected from 90 days old male Jinhua pigs. All of these muscles were used for RT-PCR. The levels of CNR1 mRNA were significantly greater in the longissimus dorsi muscle than in the gastrocnemius which was significantly greater than that analyzed in the soleus (Fig. 1).

Developmental expression patterns of CNR1 in different muscles: The developmental expression pattern of *CNR1* gene in different muscles of Jinhua pig at age of 30, 60 and 150 days were evaluated and the results of relative expression of CNR1 were shown in Fig. 2. The expression levels of *CNR1* gene showed decreasing trend in different muscles with growing. In details, the expression of *CNR1* gene was at a high level at 30 days in three kinds of muscles. At the 90th day, the levels of CNR1 mRNA were significantly lower than that of 30 days in different
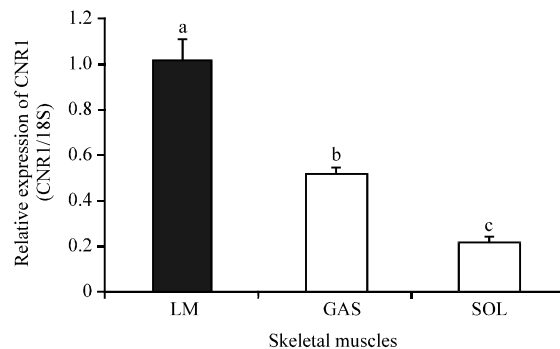

Fig. 1: Expression of CNR1 in pig different skeletal muscle. Longissimus dorsi muscle, gastrocnemius and soleus were taken from 90 days old Jinhua pig. These muscles will be used for RT-PCR analysis of *CNR1* gene expression. About 6 mRNA samples of each type of muscle were conducted for 3 times and normalized to 18S rRNA expression. The data represented the change in mRNA expression relative to the LM which was arbitrarily defined as 1. Means with different superscripts differ (p<0.01)
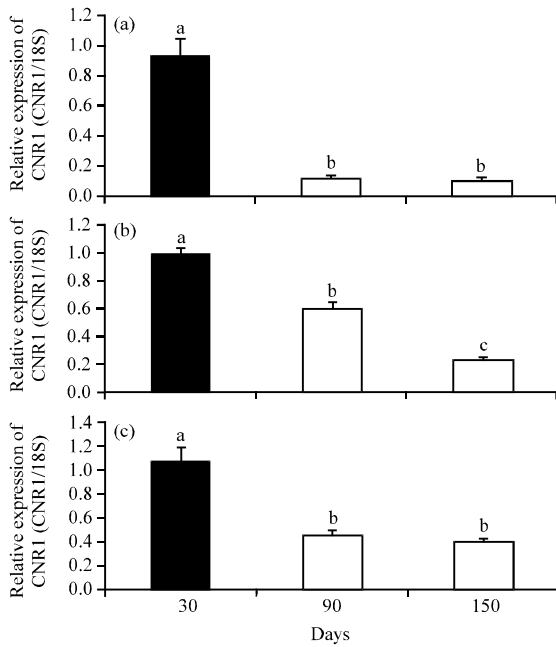
Fig. 2: The patterns of *CNR1* gene mRNA levels in different muscles of Jinhua pigs at 30, 90 and 150 days. Longissimus dorsi muscle, gastrocnemius and soleus were gathered from 30, 90, 150 day olds Jinhua pig. RT-PCR was performed to analysis of gene expression. The result of developmental expression patterns in a) longissimus dorsi muscle; b) in gastrocnemius and c) in soleus. Six mRNA samples each type of muscle per stage were conducted 3 times and normalized to 18S rRNA expression. The data represented the change in mRNA expression relative to the 30 days which was arbitrarily defined as 1. Means with different superscripts differ (p<0.05)
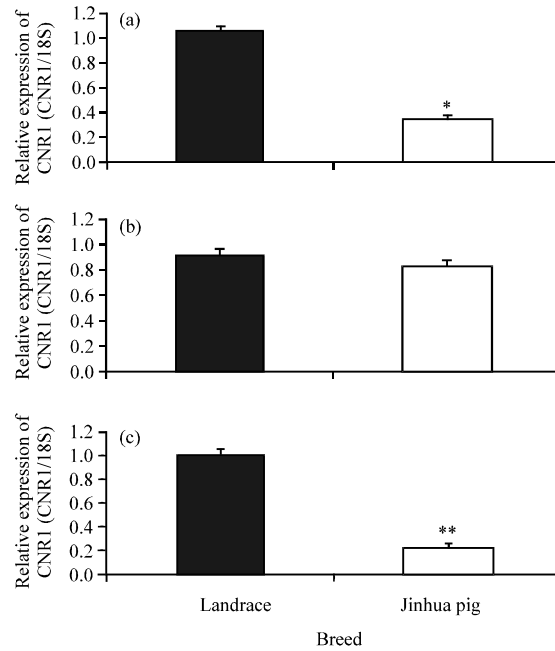
Fig. 3: Effect of breed on the gene expression of CNR1; a) in longissimus dorsi muscle; b) in gastrocnemius and c) in soleus) six Landrace (90 days) and 6 Jinhua (90 days) pigs were selected for analysis of gene expression using real time quantitative PCR. Relative gene expression was normalized to 18S rRNA. The data represented the change in mRNA expression relative to the Landrace which was arbitrarily defined as 1.*p<0.05, **p<0.01

muscles and then steadily declined at the 150th days. But there were not significant differences between that of 90 and 150 days in longissimus dorsi muscle and soleus.

The breed differences of CNR1 mRNA levels in different muscles: We analyzed the expression of CNR1 in longissimus dorsi muscle, gastrocnemius and soleus of Landrace and Jinhua pig. In short, the mRNA abundance of CNR1 in Landrace was higher compared with that of Jinhua pigs. In addition, notable differences were observed in longissimus dorsi muscle and soleus (Fig. 3a and c). But the CNR1 expression in gastrocnemius did not differ significantly between the 2 breeds.

Myogenic characterization of pig skeletal satellite muscle cells: Satellite cells were isolated from longissimus dorsi muscle based on their adhesion properties. These cells were subsequently used for Myogenic characterization. Characterization via Desmin immunocytochemistry confirmed myogenic origin. In addition, expression of marker gene (both MyoD and MyoG) was used for characterization. Cells isolated were capable of fusing and forming myotubes which stained positive for Desmin (Fig. 4a) and detected MyoD, MyoG expression (Fig. 4b and c). This indicated that the cells were pig skeletal satellite muscle cells.

Developmental expression patterns of CNR1 in pig skeletal satellite muscle cells: Pig skeletal satellite muscle cells were isolated and cultured in GM and induced to differentiate by switching to DM. Total RNA was extracted from proliferating (0 day) or differentiated cells at 1, 3, 5, 7, 9 and 11 days after switching to DM and assayed by RT-PCR. The mRNA expression of the CNR1 was significantly down-regulated in cells differentiated. The lowest mRNA expression was observed at the
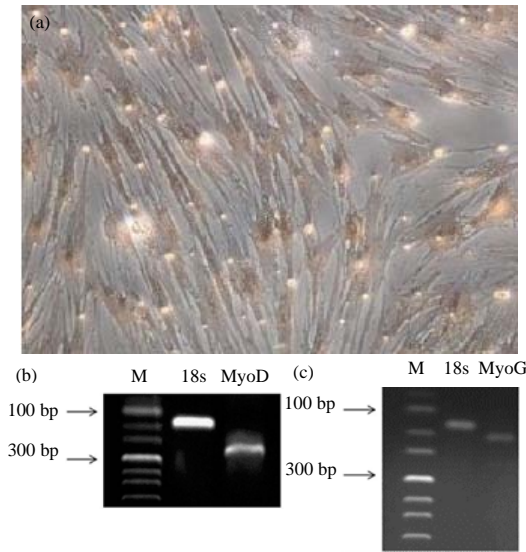
Fig. 4: Identification of porcine skelatal muscle satellite cells. Satellite cells from longissimus dorsi muscle expressed Desmin and myogenic genes *MyoD*, *MyoG*. Growing cells (24 h) were selected for Desmin expression analysis and differentiated cells (7 days) choose for MyoD, MyoG expression using conventional PCR. a) Immunohistochemistry of Desmin (100x); b) PCR analysis of MyoD mRNA expression; c) PCR analysis of MyoG mRNA expression and differentiated cells

Fig. 5: CNR1 mRNA decreased during differentiation of skeletal satellite muscle cells Total RNA was extracted from proliferating cells (prolif) or cells differentiated for 1, 3, 5, 7, 9 and 11 days. Relative mRNA expression of CNR1 was assayed by RT-PCR and normalized to 18S rRNA expression. The data represented the change in mRNA expression relative to the Prolif which was arbitrarily defined as 1. Means with different superscripts differ ($p < 0.05$)

9th day. In addition, CNR1 mRNA expression were significantly greater at that of 3 and 11 days compared with that of 1, 5, 7 and 9 days (Fig. 5).

When CNR1 was first determined and cloned (Devane *et al.*, 1988; Matsuda *et al.*, 1990), it was thought to be only expressed in brain (Herkenham *et al.*, 1990). But subsequent research revealed that the CNR1 was expressed not only in the brain but also in the peripheral cells and organs such as lungs, liver, kidneys, gastrointestinal tract, adipose tissue and skeletal muscle (Pertwee, 2001; Bensaid *et al.*, 2003; Casu *et al.*, 2003; Pagotto *et al.*, 2006). Until recently, expression of CNR1 had only been reported in skeletal muscle, muscle cells or myotubes of rodent, human or frog (Cavuoto *et al.*, 2007a, b; Kim *et al.*, 2008; Eckardt *et al.*, 2009). In this study, researchers confirmed the expression of CNR1 in different skeletal muscles (longissimus dorsi muscle, gastrocnemius and soleus) of Landrace and local Jinhua pig as well as in skeletal satellite muscle cells. To be the best of the knowledge this is the first investigation of CNR1 expression in pig muscle and pig muscle cells.

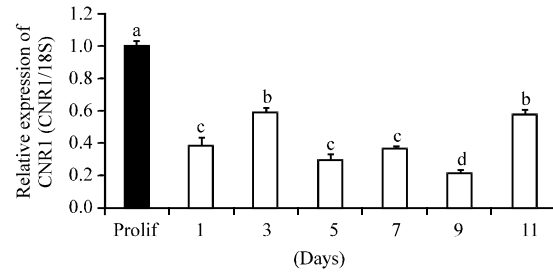Researchers further investigated the expression patterns of CNR1 in different muscles at different stage in pigs. As is known, the different muscles are composed of different skeletal muscle fibers and have different biochemical and biophysical characteristics (Kim *et al.*, 2008). Earlier studies suggested that expression of CNR1 was significantly higher in fast muscle compared with slow muscle (Eckardt *et al.*, 2009). In current study, researchers compared the CNR1 expressions in longissimus dorsi muscle, gastrocnemius and soleus, the results also showed CNR1 mRNA expression was greatest in fast-type (longissimus dorsi muscle). The developmental expression patterns of CNR1 in different muscles in general, showed the downtrend with age-increasing. These results were consistent with who found that the CNR1 mRNA expression was significantly down-regulated with age-increasing in mice gastrocnemius. Interestingly, we observed that after 90 days, the expression of CNR1 mRNA declined slower both in fast-type and slow-type muscle. But in mixed-type (gastrocnemius), expression of CNR1 mRNA decreased steadily.

The local Jinhua pig which is noted for its high-quality of meat, scarlet muscle and higher IMF contents. By contrast, the Landrace pig is a lean breed with greater growth but lower meat quality. The erarlier studies had shown obvious differences in the intramuscular fat content, drip loss, redness, muscle metabolic enzymes and muscle fiber types between Jinhua and Landrace (Shan *et al.*, 2009; Guo *et al.*, 2011). The breed differences of *CNR1* gene was not reported in earlier study. This study found the strong differences in longissimus dorsi muscle and soleus. But no difference was significant between the 2 breeds in gastrocnemius. The data of this study may suggest that CNR1 take part in controlling muscle fiber types.

Pig skeletal satellite muscle cells, a kind of adult muscle stem cells which reside outside the fibre plasmalemma but within the basal lamina and mediate capacity for skeletal muscle fibers hypertrophic growth and regeneration in the post-natal animal (Redshaw *et al.*, 2010). Zhao have investigated the CNR1 mRNA during differentiation of C2C12 skeletal muscle cells. The results showed that significantly up-regulated 24, 72 and 96 h post differentiation. In contrast, this study found CNR1 mRNA expression was significantly down-regulated after cultured in DM. The differences may be due to different cells. In addition, during differentiation, CNR1 mRNA expression were significantly greater at 3 and 11 days compared with that of 1, 5, 7 and 9 days. These results suggested that CNR1 might play a role in the functional of proliferating and differentiation in pig skeletal satellite muscle cells.

CONCLUSION

In this study, researchers have demonstrated clear expression patterns of *CNR1* gene in different muscles of pig. *In vitro*, how did the *CNR1* gene expression change with skeletal satellite muscle cells differentiation was also evident. These results revealed new insights into the *CNR1* gene in pigs. In addition, the results suggested that CNR1 might be involved in regulating muscle fiber types and differentiation of skeletal muscle cells in pig.

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