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Constitutive Androstane Receptor is Upregulated during Porcine Primary Preadipocyte Differentiation

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Abstract: Constitutive Androstane Receptor (CAR) is one of members of the nuclear receptors superfamily which is involved in induction of drug metabolism and lipogenic pathway. In this study, the reseachers investigated whether the mRNA expression pattern of CAR in porcine preadipocyte is changed during the process of induced differentiation. Preadipocytes were aseptically taken from 3 days old piglet under general anesthesia and grown to near confluence. Postconfluent cells (Day 0) were further cultured in differentiation medium for 3 days. From day 3 onward, the cells were cultured in maintain-medium based on differentiation medium without roglitazone and IBMX until day 15. The total RNA was isolated on seven time points (i.e., days 0, 2, 4, 6, 8, 10 and 15). The mRNA expression changes of the CAR before and after porcine primary preadipocytes differentiation that acorss seven time points (i.e., days 0, 2, 4, 6, 8, 10 and 15) were measured using the quantitative RT-PCR method. In addition, the researchers also examined lipid accumulation and the mRNA expression of adpocyte differentiation markers (Such as PPARγ and RXRα) which confirmed the differentiation of preadipocytes to adipocytes. The results showed that the mRNA expression of CAR was up-regulated during porcine primary preadipocyte differentiation. There is the reason that the CAR may be involved in the process of preadipocyte differentiation and is a potential drug target for the treatment of obesity and diabetes.

Key words: Pig, Constitutive Androstane Receptor (CAR), mRNA, preadipocyte, differentiation, China

INTRODUCTION

Adipocytes play a vital role in energy homoeostasis. An understanding of the biological and physiological mechanism of adipogenesis is essential for development of new avenues for treatment and prevention of obesity-related diseases. Preadipocyte differertiation is a transformation from a fibroblast-like cell to a lipid-filled cell with the expression of transcription factors, genes and enzymes indicative of adipocyte (Grant *et al*., 2008). Many transcription factors and genes are association with adipocyte differentiation including Retinoid X Receptor α (RXRα), CCAAT Enhancer Binding Proteins (C/EBP), Adipocyte Differentiation and Determination factor- 1/Sterol Regulatory Element Binding Protein-1 (ADD-1/SREBP-1) and Peroxisome Proliferator-Activated Receptor (PPAR) γ are programmatically regulated in the process of adipogenesis (Hosono *et al*., 2005).

Constitutive Androstane Receptor (CAR), a member of the orphan nuclear receptor superfamily, mainly regulates transcription of many genes encoding cytochrome P450s and drugs transporters to induce drug metabolism (Saito *et al*., 2010). Previous reports suggest that CAR not only prevents accumulation of toxic xenobiotics and endobiotics in liver but also affects the metabolism of fatty acids, lipids and glucose (Wada *et al*., 2009). CAR can directly affect lipogenic pathways by activating Insig-1 (Roth *et al*., 2008) which is a protein of endoplasmic reticulum membrane (Yang *et al*., 2002) and plays an important role in antilipogenic process (Roth *et al*., 2008). Therefore, CAR could be a negative regulator for adipogenesis during preadipocyte differentiation. CAR can be detected in stomach, liver and intestine (Choi *et al*., 1997; Gropp *et al*., 2006; Kanno *et al*., 2004). Nonetheless, the expression changes of CAR during preadipocyte differentiation have not been explored. Moreover, pig is considered to be an attractive experimental model for human obesity-related diseases (Gurr *et al*., 1977; Larsen and Rolin, 2004). In this study, the researchers measured the mRNA expression changes of CAR acorss seven time points (i.e., days 0, 2, 4, 6, 8, 10 and 15) which coverd the key stages during the porcine primary preadipocytes differentiation using a q-PCR method. The results demonstrated that CAR mRNA abundace is up-regulated during porcine preadipocytes differentiation.

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Table 1: Primers used for real-time RT-PCR

Genes symbol	Accession no.	Organism	Primer sequence (5'-3')	Orientation	Amplicon size (bp)
*ACTB**	SSU07786	Pig	GGACTTCGAGCAGGAGATGG	Forward	233
			GCACCGTGTTGGCGTAGAGG	Reverse	
*TBP**	DQ178129	Pig	GATGGACGTTCGGTTTAGG	Forward	124
			AGCAGCACAGTACGAGCAA	Reverse	
*TOP2B**	AF222921	Pig	AACTGGATGATGCTAATGATGCT	Forward	137
			TGGAAAAACTCCGTATCTGTCTC	Reverse	
CAR	NM_001037996	Pig	TGGCATTGCGGCGAGCAAGA	Forward	201
			CAGGCACCAGGGGTGGCAAG	Reverse	
RXRα	XM_001927453	Pig	CTCGCACCGCTCCATAGCCG	Forward	223
			GGCCGGGTTCGAGAGTCCCT	Reverse	
PPARγ	NM_214379	Pig	TGGAGACCGCCCAGGTTTGC	Forward	176
			GCAGCTGCACGTGCTCTGTCA	Reverse	

*Internal control genes; *ACTB*, *β-actin*; *TBP*: TATA Box Binding Protein; *TOP2B*: Topoisomerase II-β; *CAR*: Constitutive Androstane Receptor; *RXRα*: Retinoid X Receptor alpha; PPARγ: Peroxisome Proliferator-Activated Receptor gamma

MATERIALS AND METHODS

Animals and sample collection: The male crossbred Large White x Yorkshire piglets at 3 days old were used for tissue collection. Subcutaneous dorsal adipose tissues were aseptically separated from carcass.

Primary culture and differentiation: Primary culture of porcine preadipocytes was carried out as previously described (Hai-feng *et al*., 2009). The adipose tissue was cut into pieces (~1 mm^3) and rinsed with PBS followed by the digestion with 1 mg mL^{-1} collagenase I (Invitrogen, USA) for 25 min at 37°C in a shaking water bath. After incubation, digested tissue suspension was filtered through 100 μm nylon mesh. The fraction collected by centrifugation was used as preadipocytes. The preadipocytes were washed twice with DME/Ham's F12 medium (Gibco, USA) supplemented with 10% NBS and antibiotics. The cells were then seeded in 25 cm^2 flask at a density of 2×10^5 cells cm^{-2} and cultured in growth medium containing DMEM/F12, $NaHCO_3$, 10% NBS in a humidified atmosphere of 95% air and 5% CO_2 at 37°C until 80% confluence. At 80% confluence, the medium was changed to the differentiation medium, containing 10% NBS, Dexamethasone (DEX, 100 nM, sigma), 3-Isobutyl-1-Methylxanthine (IBMX, 0.25 mM, sigma), Insulin (INS, 50 nM, sigma) and roglitazone (100 nM, sigma). After 3 days, the cells were switched to maintain-medium which based on differentiation medium without roglitazone and IBMX and changed every 3 days until day 15th. Differentiation of porcine preadipocytes was analyzed by lipid accumulation under light microscope and by real time quantitative RT-PCR for markers for adipocyte differentiation.

Total RNA extraction: Total RNA was extracted from triplicate biological replicates of primary preadipocyte at seven different time points during preadipoctyes differentiation (Cultured for 0, 2, 4, 6, 8, 10 and 15 days) using Trizol reagent (Invitrogen). Total RNA was quantified by spectrophotometer at 260 and 280 nm.

q-PCR: Total RNA was reverse-transcribed by using PrimeScipt™ RT reagent kit (TaKaRa), according to the manufacturer's instructions. Each RT-reaction served as a template in a 25 μL PCR reaction mixture containing 12.5 μL SYBR premix Ex TaqTM (TaKaRa), 0.4 μL of each primer (10 μM) and 9.7 μL H_2O. Temperature cycles were as follows: 95°C for 1 min followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec. SYBR green fluorescence was detected at the end of each cycle to monitor the amount of PCR product. At the end of each run, melting curve profiles were recorded. The primier sequences were shown in Table 1.

Data analysis: The statistical significance of variations in mRNA abundacnes was calculated by one-way repeated-measures ANOVA (n = 3) and Duncan's test using PROC GLM (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Morphological changes during porcine preadipocytes differentiation: Obviously, thers is a visible morphological change for cultured cells from fibroblast-like cell to a lipid-filled cell during this induced process. At the beginning, the cells displayed an extended fibroblast-like morphology before they were induced (Fig. 1a). Then, lipid accumulated in differentiating adipocytes was observed during preadipocyte differentiation (Fig. 1b-g). Finally, adipocytes accumulated more and larger lipid droplets compared with undifferentiated cells at the end of this induced process (Fig. 1b-g).

Expression of CAR, RXRα and PPARγ in porcine preadipocytes during the process of induced differentiation: The mRNA expression changes of CAR in

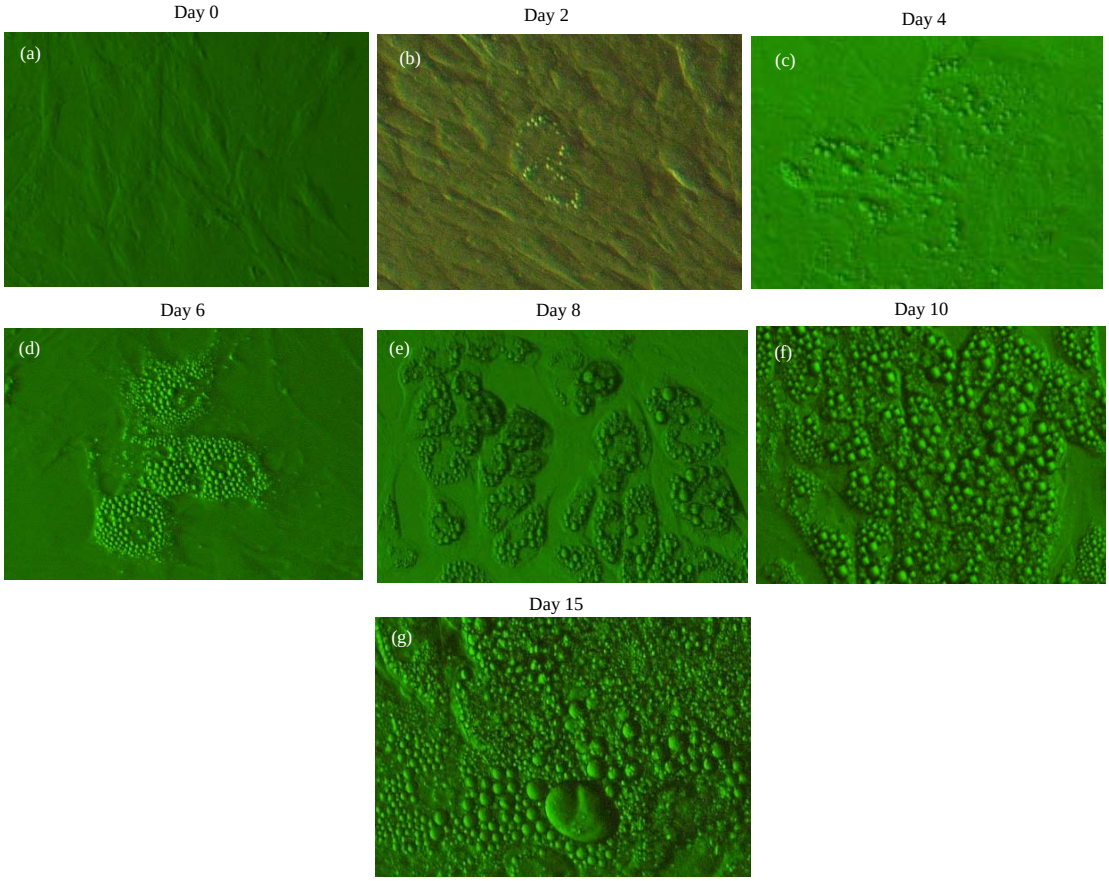

Fig. 1: Morphological changes and lipid accumulation during porcine differentiating adipocytes. The photographs of phase contrastmicroscope of porcine differentiating adipocytes at different stages of differentiation (a-g, respectively). Cells were induced to differentiate as described in material and methods. Normal differentiation of these cells is observed throughout the differentiation process as evidenced by the lipid accumulation in the cytosol of the cells

porcine preadipocytes during the process of induced differentiation were surveyed using a q-PCR approach. Before differentiation induction, CAR mRNA expression levels were almost undetectable in preadipocytes. After differentiation of porcine preadipocytes, the mRNA expression of CAR was significantly increased and reached the highest level at day 6th then the expression was decreased (Fig. 2a).

To confirm the differentiation of preadipocytes to adipocytes, the researchers examined the mRNA expression of adipocyte differentiation markers such as PPARγ and RXRα as shown in Fig. 2b. Before the differentiation induction, RXRα mRNA is expressed to some extent in preadipocytes.

After differentiation of preadipocytes, there was a significant decrease in RXRα mRNA expression level compared with the non-differentiated preadipocytes starting on the day 4 of differentiation until day 8th. Subsequently, RXRα mRNA expression level was increased from 10-15 days. The mRNA expression changes of PPARγ during porcine preadipocytes differentiation were shown in Fig. 2c.

The lowest mRNA expression of PPARγ in preadipocytes was observed before differentiation induction. Nonetheless when compared with those of non-differentiating preadipocytes, they found that the mRNA abundance of PPARγ is transcriptionally induced increased from the 2 days after induction to differentiation and reached the maximum at 10th day.

In this study, they present the mRNA expression changes of CAR during preadipocytes differentiation. The striking differences for CAR mRNA expression were observed before and after preadipocyte differentiation.

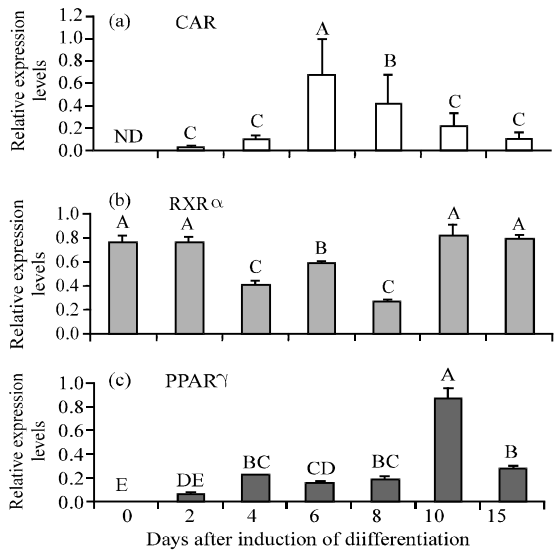

Fig. 2: a) mRNA expression of CAR; b) RXRα and c) PPARγ in porcine preadipocytes during the process of induced differentiation; one-way repeated-measures ANOVA (n = 3), Duncan's test. Values are means±SEM. Means with the same letter are not significantly different (p>0.05). ND denotes undetectable expression

They tentatively suggest that the CAR maybe has a functional role in the process of porcine differentiation. It is well establish that the role of CAR in the regulation of expression of numerous gene involved in metabolism of drugs such as cytochrome P450s (Thomas *et al*., 2003; Handschin and Meyer, 2003). Furthermore, CAR can directly affect lipogenic pathways (Roth *et al*., 2008). The molecular mechanisms of CAR regulation in lipogenic process maybe refers to activation of Insig-1. Insig-1 is a protein of Endoplasmic Reticulum (ER) membrane and plays an important role in the control of triglyceride and cholesterol biosynthesis (Yang *et al*., 2002). When Insig proteins are activated, Scap-Srebp complex can be retained in the ER membrane thereby preventing expression of Srebp-dpendent target gene involved in the synthesis of cholesterol, fatty acid and triglycerides (Roth *et al*., 2008; Shimano *et al*., 1999). Thus, CAR acts as a negative regulator of lipid metabolism.

During preadipocytes differentiation many transcription factors including PPARγ and RXRα are sequentially activated to regulate the adipogenic gene expression cooperatively (Lin *et al*., 2007). In this study, the expression changes of these genes were examined at different time points before and after differentiation. RXRα is a member of the nuclear receptor superfamily and is involved in numerous metabolic processes. This receptor can forms heterodimers with different nuclear receptor partners like PPARγ, PXR and CAR to regulate the transcription of target genes (Xu *et al*., 2005). Similar to previous reports (Ding *et al*., 1999), they also found RXRα mRNA transcripts were detected before and after preadipocytes differentiation. Nonetheless, the mRNA expression pattern of RXRα is markedly distinct with other genes measured in the present study. Notably, the mRNA abundacne of RXRα was firstly decreased and then increased. On the contrary, mRNA abundacne of CAR and PPARγ were firstly increased and then decreased. The PPARγ was considered as a master transcriptional regulators of adipogenesis and drive adipocyte-specific gene expression (Ji *et al*., 2010). The protein and gene expression of PPARγ are linked to adipocytes differentiation (Hausman, 2000, 2003). PPARγ and RXRs form a transcriptionally active complex and involves the induction and stimulation of fat-specific genes including adipocyte fatty acid binding Protein (aP2), lipoprotein lipase, Glycerol-3-Phosphate Dehydrogenase (GPDH) and CD36/fatty acid translocase in mammals (Kersten *et al*., 2000). In accordance with the previous reports (Hai-feng *et al*., 2009), the researchers also found that PPARγ mRNA is transcriptionally induced obviously increased after induction to differentiation in this study. The presents of biomarker genes as well as the evidences of morphological changes conformed that the preadipocytes was differentiated to bina fide adipocytes.

CONCLUSION

These results showed that the mRNA expression of CAR was up-regulated during porcine primary preadipocyte differentiation. The resaerchers reason that the CAR may be involved in the process of preadipocyte differentiation and is a potential drug target for the treatment of obesity and diabetes.

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