African Journal of Biotechnology Vol. 10(20), pp. 3973-3977, 16 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2304
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Quick and sensitive determination of gene expression of fatty acid synthase *in vitro* by using real-time polymerase chain reaction amplification (PCR)

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Accepted 17 March, 2011

Obesity results from an imbalance between energy intake and energy expenditure, which leads to a pathological accumulation of adipose tissue, but the underlying mechanism at gene level, is far from being elucidated. The objective of this study was to investigate the correlation between mRNA express from fatty acid synthase (FAS) with a different glucose level in primary adipocytes by real-time polymerase chain reaction amplification (PCR), which can aid in the understanding of the mechanism of obesity *in vitro*. By using the following formula, this study was able to quantify the mRNA expression of FAS of unknown samples: Y = -3.156X + 41.21 (Y = threshold cycle, X = log starting quantity). The high concentrations of glucose group significantly improved the mRNA expression of FAS (P < 0.01) rather than 0.25 and 0% concentrations of glucose. These results provide significant data that confirm an association between different glucose level and FAS expression in preadipocytes. The glucose concentration of the high group substantially augmented the mRNA expression of FAS.

Key words: Expression, fatty acid synthase, lipid deposition, real-time polymerase chain reaction amplification (PCR).

INTRODUCTION

Excessive weight gain can be attributed to the interactions among environmental factors (dietary intake and physical activity), genetic predisposition and the individual behaviors (Campion et al., 2009; Lomba et al., 2010). Thus, genetic predisposition is an important determinant of the increasing prevalence of metabolic syndrome and associated complications such as obesity (Stone et al., 2002; Speakman, 2007; Dorn et al., 2010).

Obesity is strongly associated with the metabolic syndrome and related to many diseases such as hypertension, heart disease and diabetes. Obesity results from an imbalance between energy intake and energy expenditure, leading to a pathological accumulation of adipose tissue (Kopelman, 2000). It has a heterogeneous phenotype with the involvement of multiple genes and their interactions with non-genetic factors (Vladimir et al., 1997). The accumulation of fat is caused by hypertrophy and hyperplasia of adipocytes. Therefore, an understanding of the molecular basis of hyperplasia as well as hypertrophy would contribute to the establishment of medical treatments for preventing health risks that can cause serious illnesses and death (Hishida et al., 2007).

One explanation for the increase in the number of adipocytes is that, the uptake of exogenously derived fatty acids (FA) and *de novo* of biosynthesis of FA are increased and the hydrolysis of acylglycerols is decreased. Among the different mechanisms that could lead to fat deposition and obesity, the epigenetic regulation of gene expression has emerged in recent years as a potentially important contributor (Campion et al., 2009; Lomba et al., 2010). Recent studies have revealed that, several proteins such as fatty acid synthase (FAS), a key enzyme regulating lipid metabolism, play

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Table 1. Details of the primer pairs and cDNA concentration used for the FQ-PCR.

Name	5′ sequence 3′	Amplicon size (bp)	cDNA (μM)
Forward(FAS -F)	AGCCTAACTCCTCGCTGCAAT	196	1
Reverse(FAS -R)	TCCTTGGAACCGTCTGTGTTC		1
TaqMan probe(FAS -P)	(FAM) TCCTGCGGCATCCACGAGACCACC (Eclipse)		1.2

The cDNA concentration refers to the final concentration of cDNA equal to reverse-transcribed RNA in each PCR.

important roles in adipocyte metabolism (Lomba et al., 2010; Marie et al., 2010). The events occurring during the stages of adipocyte meta-bolism are relatively well characterized, but the molecular mechanisms underlying the stages of adipogenesis remain unknown.

This study reported a fluorescent quantitative real-time polymerase chain reaction amplification (FQ-PCR) assay for quantifying FAS gene expression at different glucose levels in primary adipocytes. The results of this study provide interesting data that may help understand the molecular mechanisms underlying the stages of adipo-genesis.

MATERIALS AND METHODS

Isolation of adipocyte precursors from pig adipose tissue

Epididymal fat pads of healthy pigs (age, 18 to 20 days; Landrace) were removed under sterile conditions and transferred to ice-cold Dulbecco's modified eagle medium containing penicillin, strepto-mycin and fungizone (Sangon Biological Engineering Technology and Services Co., Ltd, Shanghai, China). Preadipocytes were isolated as described by Janke et al. (2002).

Cells were cultured with different glucose contents

Preadipocytes were incubated at 37°C in a humidified atmosphere with 5% CO_2 as described by Janke et al. (2002). The cells were stimulated to proliferate over 24 h and then, glucose was added at various concentrations to the culture medium (glucose-free adipocyte medium, supplemented with 1 μM insulin; 0.25% glucose adipocyte medium, supplemented with 1 μM insulin and 0.5% glucose adipocyte medium, supplemented with 1 μM insulin).

Gene expression analyzed using FQ-PCR

RNA isolation and FQ-PCR

Preadipocytes (1 ml) were trypsinized with 1 ml digestive, washed once with PBS, and pelleted (380 *g*, 10 min) after glucose was added at various concentrations to the culture medium at 2, 4, 8, 16 and 32 h. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. An ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) was used for FQ-PCR. The reaction mixture was prepared using the universal PCR master mix (Applied Biosystems) according to the manufacturer's instructions. The mixture was incubated at 50°C for 5 min and at 94°C for 40 s and then, the PCR was performed by 34 cycles at 94°C for 40 s and at 59°C for 40 s, 72°C for 40 s and an additional extension period at 72°C for 2 min. Melting curve from 65 to 95°C read every 0.5°C hold 5 s. Relative standard curves were generated in each experiment to calculate the input amounts of the unknown samples. Sensitivity and specificity analysis of the assay was as described by Yang et al. (2009).

Specific RT-PCR primer and probe design

Expression analysis was performed for the genes encoding FAS published sequences at GenBank. The oligonucleotide primer set for the gene was designed using Primer Premier 5.0 software. The sequences and concentrations of primers and cDNA are shown in Table 1. The specificity of all the primers was tested by searching the homologous DNA sequences against the GenBank BLAST database.

Preparation of standard templates

The conventional RT-PCR was performed for standard RNA using the primers (FAS-F, FAS-R) mentioned earlier. The standard tem-plate was prepared as described by Yang et al. (2008).

Establishment of the FQ-PCR standard curve

Standard plasmids (1×10^9 copies/μl) were used to establish the standard curve .The Primers (FAS-F, FAS-R and FAS-P) were used for this amplification and their concentrations ranged from 1×10^8 to 1×10^2 gene copies/μl. A regression curve was constructed by plotting the threshold cycle (Ct) values versus the logarithm of the RNA copy number. Analysis of the measures was undertaken after each run to verify identical amplification efficiencies and conditions between runs.

Finally, based on the data for generating the standard curve with the software of the iCycler IQ Detection System (Bio-Rad, USA), Ct values for each sample were determined from the point at which fluorescence breached the threshold fluore-scence line (Yang et al., 2008; Yang et al., 2009).

Statistical analysis

Data were expressed as means ± SDs. Statistical significance of the differences was determined using Student's t-test. P values less than 0.01 were considered statistically significant (Kang et al., 2010).

RESULTS

Standard curve of the established FQ-PCR

The standard curve of FQ-PCR was constructed using

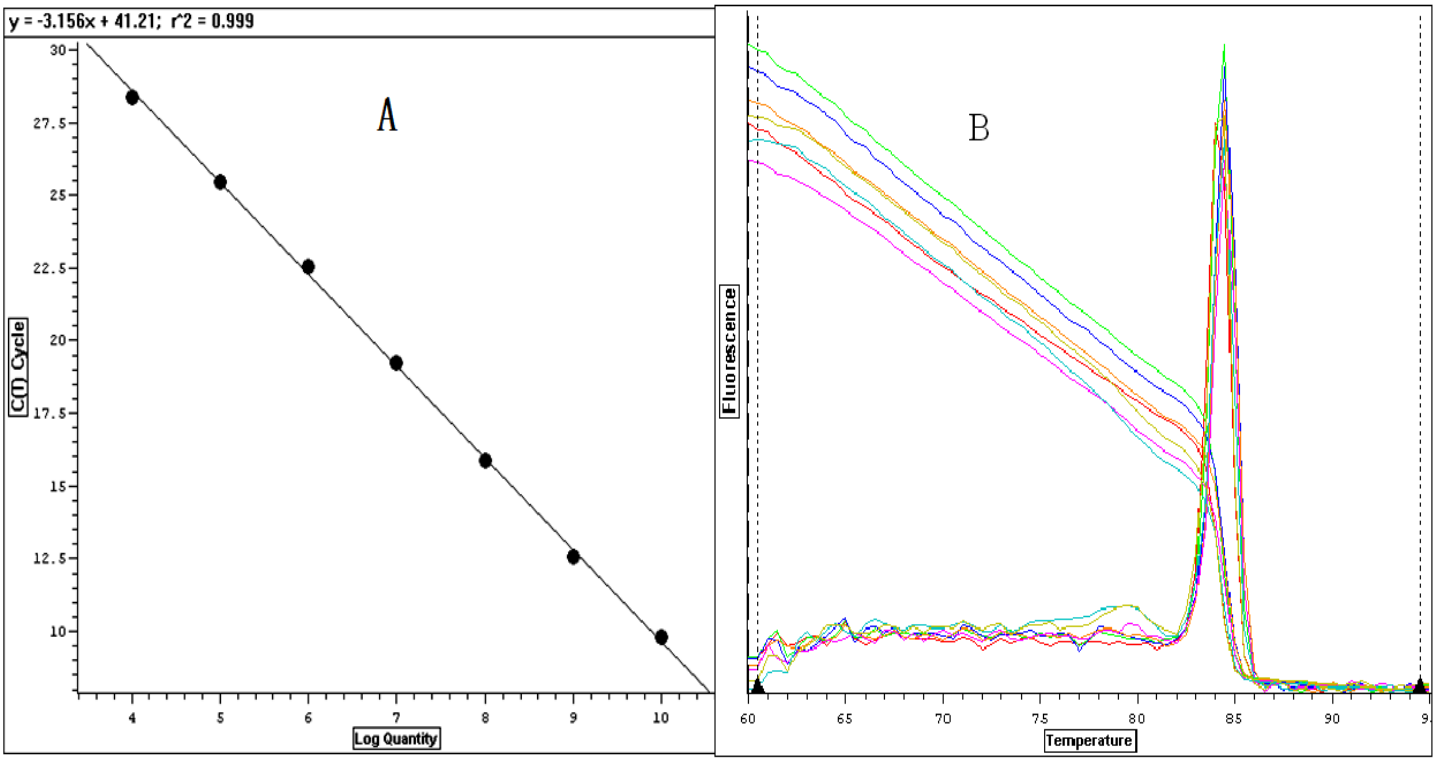


Figure 1. FAS gene FQ-PCR standard curve graph. A, standard curve of FAS by the FQ-PCR: B, dissociation curve for FAS. Ten-fold dilutions of standard DNA ranging from 1×10^8 to 1×10^2 copies/µl were used, as indicated in the x-axis, whereas the corresponding cycle threshold (Ct) values are presented on the y-axis. Each dot represents the result of triplicate amplification of each dilution. The correlation coefficient and the slope value of the regression curve were calculated and are indicated.

serially diluted plasmids with tenfold dilution, ranging from 10^8 to 10^2 copies as templates. The correlation coefficients of the standard curve was 0.9998 and the PCR efficiencies was greater than 0.95 (Figure 1). The high PCR reaction efficiencies indicated that, the FQ-PCR assay was suitable for genomic DNA quantification. The good linearity between DNA quantities and fluorescence values (Ct) indicated that, the assay was well suitable for quantitative measurements. By using the following formula, this study could quantify the mRNA expression of FAS of unknown samples: Y = -3.156X + 41.21 (Y = threshold cycle, X = log starting quantity).

Sensitivity, specificity analysis of the established FQ-PCR

Ten-fold dilutions of the standard templates were tested by the established FQ-PCR assay to evaluate the sensitivity of the system and the detection limit was found to be 1.0×10^1 copies/reaction (data no shown).

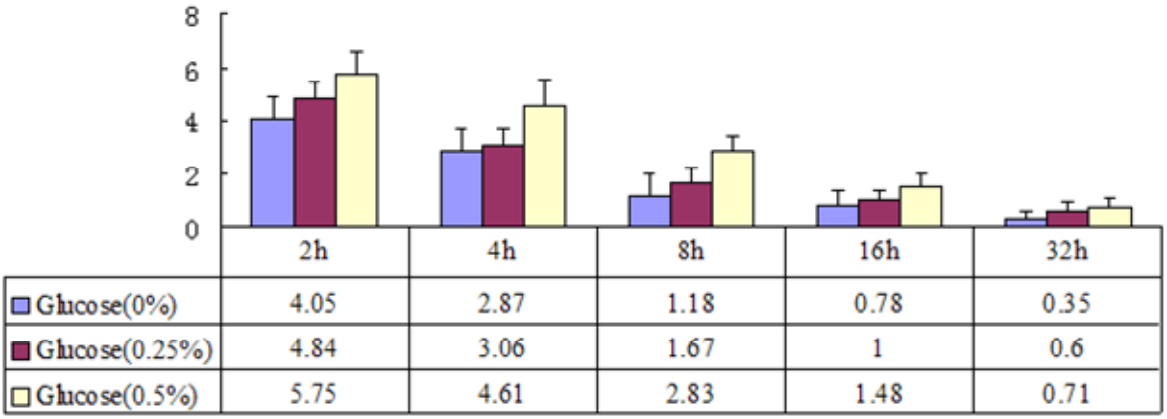
The test was performed using DNA from standard RNA and several other bacteria as templates to examine its specificity; the result of this analysis showed that, none of the bacteria yielded any amplification signal, suggesting that the established FQ-PCR assay was highly specific (data no shown).

Dynamic changes in FAS gene expression in adipocyte

The dynamic changes in FAS gene expression in adipocytes after glucose was added at various concentrations to the culture medium were intermittently determined over a 32 h period by FQ-PCR. The study results revealed that, the copy numbers of FAS of samples peaked at 2 h when the glucose concentration was 0, 0.25 and 0.5%, they were 4.05 ± 0.86, 4.84 ± 0.62, 5.75 ± 0.88 (log copies/µl), respectively. The glucose concentration of the high group substantially augmented the mRNA expression of FAS ($P < 0.01$) (Figure 2).

DISCUSSION

FQ-PCR has become a potentially powerful alternative for quantifying mRNA expression because it is simpler, more rapid, more reproducible and more sensitive than other methods (Shimazu et al., 2009; Brym et al., 2007; Calikoglu et al., 2006). In this study, the applicability of FQ-PCR for the quantification of FAS mRNA expression



	2h	4h	8h	16h	32h
Glucose(0%)	4.05	2.87	1.18	0.78	0.35
Glucose(0.25%)	4.84	3.06	1.67	1	0.6
Glucose(0.5%)	5.75	4.61	2.83	1.48	0.71

Figure 2. Dynamic changes in FAS gene expression in adipocytes with different glucose concentration in different time.

was clearly established by showing that, it has remarkable sensitivity and high-throughput potential, which are beyond the scope of other assay methods.

FAS catalyze the last step in the fatty acid biosynthesis and thus, it is believed to be a major determinant to generate fatty acids by *de novo* lipogenesis (Dorn et al., 2010). It is clear that some genes would show an increase or decrease in response to glucose. The aim of this study was to analyze the expression of FAS, which is a central enzyme of lipogenesis with a different glucose level. This study revealed a significant correlation of FAS expression with the high degree of glucose in primary pig preadipocytes *in vitro*. Surprisingly, the copy numbers of FAS peaked at 2 to 8 h at various concentrations of glucose. At 0.5% concentration, glucose significantly augmented the mRNA expression of FAS ($P < 0.01$) when compared with 0.25 and 0% concentrations. These results finding is just an initiation of FAS in obesity to be elucidated. Further studies are required to unravel the relationship between nutrient and hormone and other gene such as hormone-sensitive lipase.

Conclusion

In conclusion, the study results confirm the association between different glucose levels and FAS expression in preadipocytes. At 0.5% concentrations, glucose significantly augmented the mRNA expression of FAS ($P < 0.01$) rather than at concentrations of 0.25 and 0%. However, further research is needed to validate this conclusion.

ACKNOWLEDGEMENTS

This study was supported by The Chongqing Natural Science Funds (No. 2008BA1013); The Chongqing Natural Science Funds (No. 2009BA1078).

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