Asian-Aust. J. Anim. Sci.
Vol. 22, No. 5 : 721 - 726
May 2009

www.ajas.info

Low Lysine Treatment Increases Adipogenic Potential of Bovine Intramuscular Preadipocytes

Jagadish Beloor[a], Hye Kyeong Kang[a], Cheol-Heui Yun[1], Sang Hoon Kim[2] and Yang Soo Moon*

Department of Animal Science & Biotechnology, Jinju National University, 150 Chilam-dong, Jinju 660-758, Korea

ABSTRACT : The molecular mechanism of adipocyte differentiation has been well documented. However, the effect of specific nutrients such as lysine on adipocyte differentiation is poorly understood especially in ruminant animals. Therefore, the aim of the present study was to elucidate the influence of lysine on adipocyte differentiation and adipogenic genes in cultured bovine preadipocytes. The preadipocytes were treated with different concentrations of lysine (40, 160, 320 mg/L) or troglitazone (10 μM) for 2 days and then subsequently cultured in differentiation medium until day 6. Expression levels of C/EBPα were significantly higher ($p<0.001$) in 40 and 160 mg/L lysine-treated cells compared to 320 mg/L treatment. Though there was an increasing trend in PPARγ expression levels with the decreasing lysine concentration, the results were not significant. The preadipocyte factor (pref-1), expression significantly ($p<0.001$) reduced with decreasing lysine concentration. The Oil red O staining results were better in 40 mg/L treated cells compared to 160 and 320 mg/L lysine treated cells. Our overall results indicate that insufficient supply of lysine increases the adipogenic potential in bovine intramuscular preadipocytes. (**Key Words :** Adipogenesis, Preadipocytes, Lysine, Bovine)

INTRODUCTION

Understanding mechanisms regulating fat deposition and metabolism in beef cattle is important, since the dynamics of adipose physiology is directly associated with both the quality and value of the meat (Ritchie et al., 1993; Lee et al., 2000). Intramuscular adipocyte differentiation is thought to be related to the formation of marbling (Kawada et al., 1996). Adipocyte differentiation is the switching of undifferentiated fibroblast-like preadipocytes into mature round lipid-filled unilocular fat cells (Butterwith, 1994; Hausman and DiGirolamo, 2001). Appropriate environment, gene expression, interplay between several transcription factors and hormones induces the preadipocyte conversion, many of these genes and their interaction during adipogenesis are yet unclear and not necessarily be similar to that of 3T3-L1 cells (Bluher, 2005; Fernyhough et al., 2007; Jeong et al., 2008). PPAR gamma and C/EBP alpha are the adipogenic genes that play a key role in complex transcriptional cascade during adipocyte differentiation, where as Pref-1 is highly expressed in preadipocytes and the down regulation of Pref-1 is required for adipose conversion. Nutrition is one of the potential routes to increase the intramuscular fat in animals. Recent studies have shown that diet conditions could modulate intramuscular fat content. Restriction of energy intake results in a decreased i.m. fat content (Wood et al., 1996; Candek-Potokar et al., 1998). A low protein diet results in an increased intramuscular fat when compared to adequate diet (Adeola and Young, 1989). Lysine deficient diet improves meat quality by increasing the intramuscular fat in swine (Castell et al., 1994; Kerr et al., 1995). Specific adipose depots in ruminant animals do not respond to regulatory compounds in a similar manner to that of non-ruminant animals (Fernyhough et al., 2007) hence this study was carried out to investigate the influence of lysine on adipogenic potential (adipogenic genes and adipocyte differentiation) in cultured bovine preadipocytes.

* Corresponding Author: Yang Soo Moon. Tel: +82-55-751-3262, Fax: +82-55-751-3267, E-mail: ysmoon@jinju.ac.kr

[1] Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea.

[2] Department of Biology, Kyung Hee University, 1 Hoegi-dong Dongdaemun-gu, Seoul 130-701, Korea.

[a] Jagadish Beloor and Hye Kyeong Kang have equally contributed to this work.

Received January 16, 2009; Accepted March 22, 2009

MATERIALS AND METHODS

Preparation of intramuscular preadipocytes

Intramuscular adipose tissue of longissimus dorsi

Table 1. Primers used for semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Gene	Primer	Sequence (5'-3')	Size	Tm (°C)
pref-1	Forward	CTTCATGGACAAGACCTGCAG	330 bp	55
	Reverse	TGCTCAGATCTCCTCCTCG		
C/EBP alpha	Forward	ATGGAGTCGGCCGACTTCTAC	1.2 kb	58
	Reverse	CGCGCAGTTGCCCATGGCCTT		
PPAR gamma	Forward	ATGGGTGAAACCCTGGGAGAT	1.4 kb	50
	Reverse	ATACAAGTCCTTGTAGATTTC		
Beta-actin	Forward	GCCACGAGGCCCAGAGCAAG	230 bp	52
	Reverse	GGGGCCACACGCAGCTCATT		

muscle was obtained from female Korean native cattle during routine slaughtering. Upon collection, samples were immediately placed in a 39°C solution of PBS (171.1 mM sodium chloride, 3.4 mM potassium chloride, and 10.1 mM sodium phosphate, and 1.8 mM potassium phosphate, pH 7.2) and transported to laboratory. Preadipocytes were isolated under sterile and 39°C conditions until specified using modified Aso's method (Aso et al., 1995). Briefly, intramuscular adipose tissue was separated from the muscle and visible connective tissue. All the samples were then cut into approximately 2-mm^3 sections. For each depot, samples of approximately 2 g were aliquoted into 50-ml conical tubes and digested in 6 ml of Dulbecco's modified Eagle's medium (DMEM; 5.5 mM glucose; Invitrogen Corp.) containing 2 mg/ml of collagenase and 2% bovine serum albumin at 39°C for 60 min. The digested tissue was filtered sequentially through a 100 μm and 40 μm stainless steel mesh, centrifuged for 10 min at 800 g and the pellet was collected. The pellet was resuspended in culture medium; DMEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

Cell culture

The filtered cells were seeded in 6-multiwell or dishes at density of 1×10^5 cells/ml. After 24 h, attached cells were washed twice with HBSS and fresh medium was added. Culture medium was changed every second day, allowing the cells to proliferate to confluence. The lysine (40, 160, 320 mg/L), or troglitazone (10 μM) containing RPMI media along with 1% antibiotics, 17 μM pantothenate, 1 mM carpylic acid, 200 μM ascorbic acid, 33 μM biotin, 10 mM acetic acid, 10 ng/mL insulin, 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine.was treated for initial 2 days and then replaced with differentiation- induction medium containing DMEM, 10% FBS, plus antibiotics, 17 μM pantothenate, 1 mM carpylic acid, 200 μM ascorbic acid, 33 μM biotin, 10 mM acetic acid, and 10 ng/ml insulin after every 2 days. Phase-contrast photomicrographs of the fibroblast-like cells were taken from bovine preadipocyte at confluent (day 0) and at day 2, 4, and 6 of culture.

Total RNA extraction

Total RNA was extracted from lysine and troglitazone treated cells using Trizol reagent (Invitrogen Life Technologies). RNA was isolated on day 2, 4 and 6 of the treatment.

Semi-quantitative RT-PCR analysis of C/EBP alpha, PPAR gamma and pref-1

Semi-quantitative RT-PCR was performed as previously described (Hong et al., 2005) to measure the levels of C/EBP alpha, PPAR gamma and pref-1 and beta-actin mRNAs. The gene specific primers, annealing temperature, are shown in Table 1. PCR products were resolved on a 1.2% agarose gel. The DNA was visualized by ethidium bromide staining and analyzed using gel viewer software and EasyDoc Gel documentation system (EasyDoc, Korea). The mRNA levels were corrected using the transcription level of the beta-actin gene as an internal standard.

Oil red O staining

Cytoplasmic lipid droplets were stained with Oil red O on day 6. Briefly, cells were rinsed three times in PBS and then fixed in 10% (vol/vol) formaldehyde for 10 min. After being washed twice with PBS, cells were stained for 30 min at 37°C in freshly diluted Oil red O (Sigma Chemical Co.) Solution (six parts Oil red O stock and four parts H_2O; Oil red O stock solution is 0.5% Oil red O in isopropanol), followed by further washing with PBS.

Statistical analysis

An analysis of variance was performed on all the variables measured using the general linear model (GLM) procedure of the SAS statistical package (SAS Institute, 1999). The Duncan's multiple range test was used to determine differences between treatment means.

RESULTS AND DISCUSSION

This experiment was performed to prove the hypothesis that lysine; an essential amino acid when provided in an insufficient quantity makes the preadipocytes in order to conserve energy get converted to energy storing fat cell. Our results are the first *in-vitro* results to demonstrate that low lysine treatment increases the adipogenic potential and triggers better adipogenesis compared to its higher

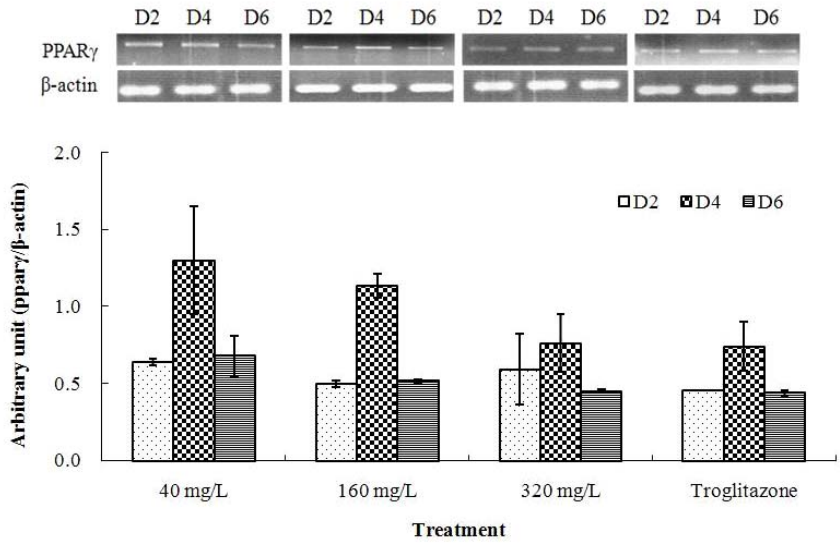

Figure 1. The mRNA levels of PPAR gamma in cells treated with different concentrations of lysine. Upper panel, representative agarose gel stained with ethidium bromide is showing amplified PPAR gamma on day 2, 4, 6 for different treatments of lysine. Lower panel, data were normalized using beta-actin mRNA levels and expressed as arbitrary units on y-axis. The data here represents the means±SEM of three independent experiments.

concentration treatments in bovine i.m. preadipocytes.

Preadipocyte differentiation and conversion to a viable adipocyte is a complex process accompanied by co-ordinated change in cell morphology, hormone sensitivity, and gene expression (Kokta et al., 2004; Fernyhough et al., 2007). Our present study and the previous studies (Lee et al., 2000; Tanaka et al., 2001) show that the external factors like lysine, Zinc and steroidal hormones can increase the adipogenic potential. In this study the fibroblast like bovine preadipocytes was confirmed to proliferate and differentiate to lipid filled adipocytes with similar cell physiology and morphology reported in rodent adipocyte models (Sugihara et al., 1987; Ntambi et al., 2000).These changes were the result of the potential action of several transcription factors including PPAR gamma and C/EBP alpha which play a regulatory role in the process (Spiegelman and Flier, 1996; Fernyhough et al., 2005). Hence we checked the expression levels of PPAR gamma, C/EBP alpha and a preadipocyte factor pref-1 to measure the adipogenesis in cells treated with different concentrations of lysine and troglitazone.

Lysine is an essential amino acid and an important component of a protein diet. Though previously (Adeola and Young, 1989) it has been shown that low protein diet results in increased intramuscular fat but the exact metabolic action of lysine for adipogenesis is yet to be understood. Troglitazone is a ligand for PPAR gamma and a potent activator of adipocyte differentiation program and thus was used to compare the adipogenic potentials with that of lysine treatment.

Though all the treatment could induce the adipogenesis but the expression levels of C/EBP alpha, PPAR gamma and pref-1 were different among the treatments. Though the expression levels of PPAR gamma showed increasing trend in low lysine (40 mg/L) followed by 160 and 320 mg/L of lysine treatments (Figure 1) but the results did not show significance when subjected to statistical analysis. Our results coincides with the previous results of Katsumata et al. (2007) that shows low lysine diet increases the expression of PPAR gamma in swine muscle cells. The expression levels of C/EBP alpha was significantly higher ($p<0.001$) in 40 and 160 mg/L compared to 320 mg/L lysine treated cells on day 2 of the treatment but did not show any significant difference on the other days of the treatment (Figure 2). The expression of the preadipocyte marker pref-1 (Figure 3) reduced significantly ($p<0.001$) and it was the least in 40 followed by 160 mg/L and lastly by 360 mg/L treatment.

The results were compared with that of the troglitazone, though the gene expression trend were similar in troglitazone and lysine treated cells but the levels of expression of C/EBP alpha were significantly higher and the pref-1 level was significantly lower in troglitazone treated cells indicating that troglitazone had better capacity to induce adipogenesis compared to lysine (Figures 2 and 3).

The Oil red O staining results (Figure 4) of the accumulated lipid droplets in the cells treated with different lysine concentrations clearly show higher number of lipid droplets in the 40 mg/L treated cells compared to that of the 160 and 320 mg/L.

Though the underlying mechanism of adipogenesis in this case in unclear and yet to be studied but the PPAR

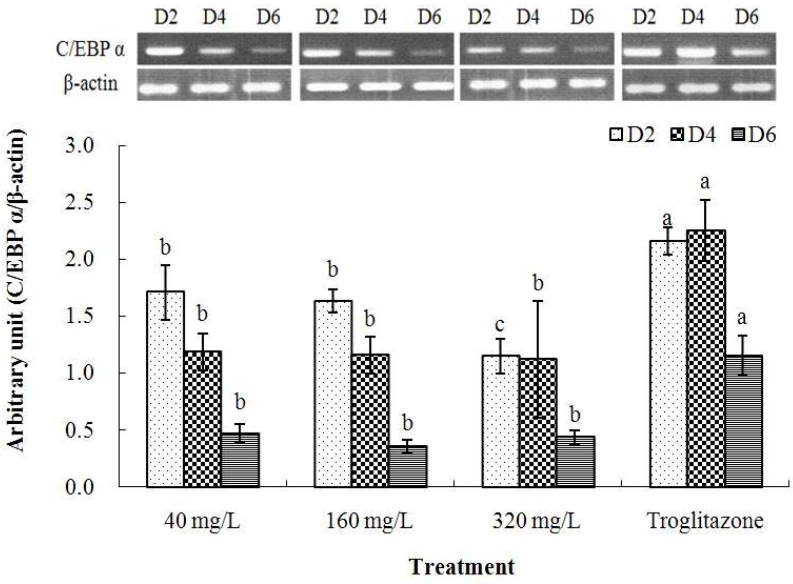

Figure 2. The mRNA levels of C/EBP alpha in cells treated with different concentrations of lysine. Upper panel, representative agarose gel stained with ethidium bromide is showing amplified C/EBP alpha on day 2, 4, 6 for different treatments of lysine. Lower panel, data were normalized using beta-actin mRNA levels and expressed as arbitrary units on y-axis. The data here represents the means±SEM of three independent experiments. The bars representing the same day of different treatments with different superscripts are significantly different with $p<0.001$.

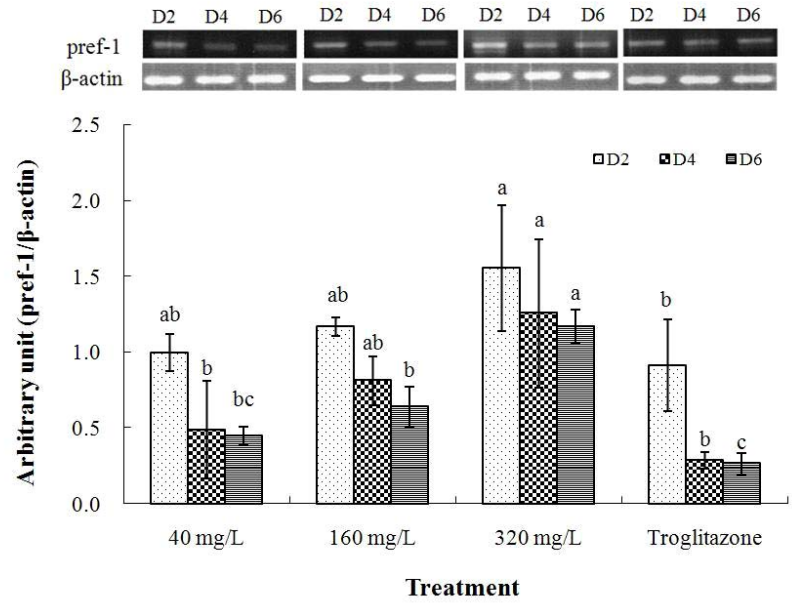

Figure 3. The mRNA levels of pref-1 in cells treated with different concentrations of lysine. Upper panel, representative agarose gel stained with ethidium bromide is showing amplified pref-1 on day 2, 4, 6 for different treatments of lysine. Lower panel, data were normalized using beta-actin mRNA levels and expressed as arbitrary units on y-axis. The data here represents the means±SEM of three independent experiments. The bars representing the same day of different treatments with different superscripts are significantly different with $p<0.001$.

gamma, C/EBP alpha, pref-1 gene expression levels and the Oil red O staining results when put together clearly indicate that the low lysine (40 mg/L) can induce better adipogenic gene expression and adipocyte differentiation, thus increasing the adipogenic potential compared to the higher concentration (320 mg/L) of lysine in bovine i.m preadipocytes. Our results further more get the support from the previous (Gondret and Lebret, 2002) *in-vivo* findings that reduction of protein content in the feed diet increases the intramuscular fat in porcine. Generally adipose depot in

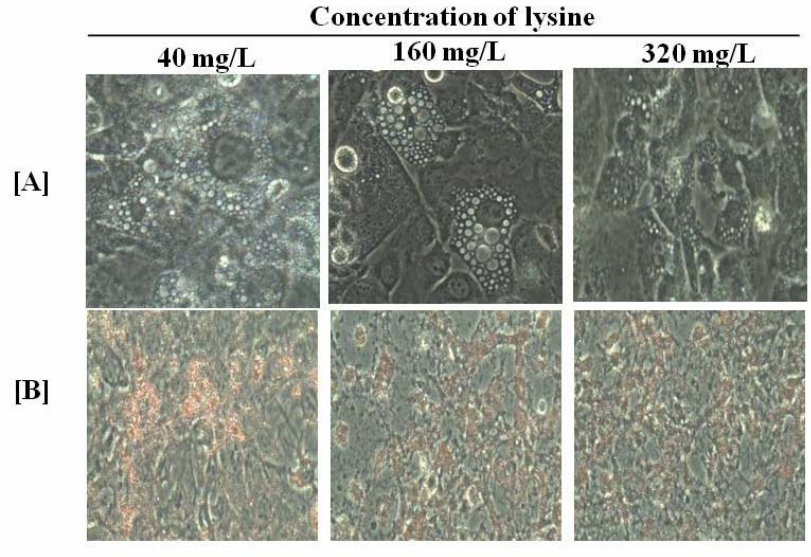

Figure 4. Differentiation of bovine intramuscular preadipocytes at day 6. Cells were fixed and stained with Oil red O to detect oil droplets. A. Unstained cells. B. Stained cells. Magnification of each picture is 200×.

ruminant and non-ruminant animals responds differently to regulatory molecules (Fernyhough et al., 2007) but here when we compare out results of bovine (ruminant) with previous findings in swine (non-ruminant) shows no difference and proves that low lysine treatment or diet increases adipogenesis.

The present study provides evidence that insufficient supplying of an essential amino acid like lysine increases the adipogenic potential. Thus directly finds its application in increasing the i.m. fat content and helps in meat marbling. The study opens a gate for further studies like understanding the mechanism of adipogenesis in this case.

ACKNOWLEDGMENTS

This work was supported by grants from the Basic Research Program of the Korea Science & Engineering Foundation (R01-2006-000-10884-0), the Korea Research Foundation Grant (KRF-00066) and Regional Animal Industry Center of Jinju National University.

REFERENCES

Adeola, O. and L. G. Young. 1989. Dietary protein-induced changes in porcine muscle respiration, protein synthesis and adipose tissue metabolism. J. Anim. Sci. 67:664-673.

Aso, H., I. Abe, K. Nakajima, T. Ozutsumi, Yamaguchi and Y. Takamori. 1995. A preadipocyte clonal line from bovine intramuscular adipose tissue, Biochem. Biophys. Res. Commun. 213:369-375.

Bluher, M. 2005. Transgenic adipocyte differentiation, animal models for the study of adipose tissue biology. Best Pract. Res. Clin. Endocrinol. Metab. 19:605-623.

Butterwith, S. C. 1994. Molecular events in adipocyte development. Pharmacol. Ther. 61:399-411.

Candek-Potokar, M., L. Lefaucheur, B. Zlender and M. Bonneau. 1998. Effect of age and/or weight at slaughter on longissimus dorsi muscle: biochemical traits and sensory quality in pigs. Meat Sci. 48:287-300.

Castell, A.G., R. L. Cliplef, L. M. Poste-Flynn and G. Butler. 1994. Performance, carcass and pork characteristics of castrates and gilts self-fed diets differing in protein content and lysine: Energy ratio. Canadian J. Anim. Sci. 74:519-528.

Fernyhough, M. E., D. L. Helterline, J. L. Vierck, G. J. Hausman, R. A. Hill and M. V. Dodson. 2005. Dedifferentiation of mature adipocytes to form adipofibroblasts: more than just a possibility. Adipocytes 1:17-24.

Fernyhough, M. E., E. Okine, G. J. Hausman, J. L. Vierck and M. V. Dodson. 2007. Invited review: PPAR-gamma and GLUT-4 expression as differentiation markers for preadipocyte conversion to become an adipocyte. Domest. Anim. Endocrinol. 33:367-378.

Gondret, F. and B. Lebret. 2002. Feeding intensity and dietary protein level affect adipocyte cellularity and lipogenic capacity of muscle homogenates in growing pigs, without modification of the expression of sterol regulatory element binding protein. J. Anim. Sci. 80:3184-3193.

Hausman, D. B. and M. DiGirolamo. 2001. The biology of white adipocyte proliferation. Obes. Rev. 2:239-254.

Hong, Y. H., Y. Nishimura, D. Hishikawa, H. Tsuzuki, H. Miyahara, C. Gotoh, K. C. Choi, D. D. Feng, C. Chen, H. G. Lee, K. Katoh, S. G. Roh and S. Sasaki. 2005. Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. Endocrinol. 146:5092-5099.

Jeong, H. Y., M. S. Lee, H. Kim, Y. H. Park, D. Yoon, S. J. Moon, A. Hosoda, H. D. Kim, S. Saeki and M. J. Kang. 2008. Cloning, expression, and regulation of bovine cellular retinoic acid-binding protein-2 (CRABP-2) during adipogenesis. Asian-Aust. J. Anim. Sci. 21:1551-1558.

Katsumata, M., M. Matsumoto, S. Kobayashi and Y. Kaji. 2007.

Reduced dietary lysine enhances proportion of oxidative fibers in porcine skeletal muscle. Anim. Sci. J. 79:347-353.

Kawada, T., Y. Kamei and E. Sugimoto. 1996. The possibility of active form of vitamins A and D as suppressors on adipocyte development via ligand-dependent transcriptional regulators. Int. J. Obes. Relat. Metab. Disord. 20:52-57.

Kerr, B. J., F. K. McKeith and R. A. Easter. 1995. Effect on performance and carcass characteristics of nursery to finisher pigs fed reduced crude protein, amino acid-supplemented diets, J. Anim. Sci. 73:433-440.

Kokta, T. A., M. V. Dodson, A. Gertler and R. A. Hill. 2004. Intercellular signalling between adipose tissue and muscle tissue. Domest. Anim. Endocrinol. 27:303-331.

Lee, S. C., H. J. Lee, D. W. Kim and K. I. Han. 2000. Effect of hormones on proliferation of stromal vascular cells from Hanwoo cattle adipose tissue. Asian-Aust. J. Anim. Sci. 13:161-166.

Ntambi, M. and Y. C. Kim. 2000. Adipocyte differentiation and gene expression, J. Nut. 130:3122-3126.

Ritchie, H. D., S. R. Rust, R. A. Merkel and W. G. Bergen. 1993. Getting rid of excess fat is not an easy task. Feedstuffs 13.

SAS. 1999. SAS/STAT Software for PC. Release 6.11, SAS Institute, Cary, NC, USA.

Spiegelman, B. M. and J. S. Flier. 1996. Flier, Adipogenesis and obesity: rounding out the big picture. Cell 87:377-389.

Sugihara, H., N. Yonemitsu, S. Miyabara and S. Toda. 1987. Proliferation of unilocular fat cells in primary culture. J. Lipid Res. 28:038-1045.

Tanaka, S., E. Takahashi, T. Matsui and H. Yano. 2001. Zinc promotes adipocyte differentiation *in vitro*. Asian-Aust. J. Anim. Sci. 14:966-969.

Wood, J. D., S. N. Brown, G. R. Nute, F. M. Whittington, A. M. Perry, S. P. Johnson and M. Enser. 1996. Effects of breed, feed level and conditioning time on the tenderness of pork. Meat Sci. 44:105-112.