The Role of Phosphatidylinositol 3-kinase and Mitogenic Activated Protein Kinase on the Differentiation of Ovine Preadipocytes*

K. C. Choi, Y. B. Shrestha, S. G. Roh, **D. Hishikawa, M. Kuno, H. Tsuzuki, Y. H. Hong and S. Sasaki**

Dept. of Food Production Science, Faculty of Agriculture, Shinshu University, Nagano-ken 399-4598, Japan

ABSTRACT : The aim of this study was to investigate the role of phosphatidylinositol 3-kinase (PI3 kinase) and the mitogen-activating protein (MAP) kinase pathway on the differentiation of ovine preadipocytes. In order to investigate this issue, we monitored glycerol 3-phosphate dehydrogenase (GPDH) activity during differentiation with specific inhibitors of PI3 kinase and MAP kinase-Erk kinase, LY294002 and PD098059, respectively. The preadipocytes, which were obtained from ovine subcutaneous adipose tissues, were proliferated to confluence and then differentiated to adipocytes in differentiation medium with each inhibitor for 10 days. The confluent preadipocytes and differentiated adipocytes at days 3, 7 and 10 were harvested for assay of GPDH activity. LY294002 inhibited the differentiation program in dose- and day-dependent manners during 10 days of differentiation. PD098059 did not affect GPDH activity during differentiation. Furthermore, the expression of peroxisome proliferator-activated receptor γ2 (PPAR-γ2), the representative early gene of differentiation, was markedly reduced by LY294002 treatment, although PD098059 did not change it. Our results demonstrated that the activation of PI3 kinase contributes to the differentiation process of ovine preadipocytes. *(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 8 : 1199-1204)*

INTRODUCTION

It has been well known that regardless of the nature of the triggering events, a series of transcription factors, CCAATT enhancer binding protein (C/EBP) and peroxisome proliferator activated receptor (PPAR), are induced in a specific sequence during adipocyte differentiation *in vitro* (Hauner, 1990; Yeh et al., 1995; Darlington et al., 1998; Fajas et al., 1998; Reusch and Klemm, 1999). In a number of species, including humans, rodents, ruminants, and pigs (Deslex et al., 1987; Hausman, 1989; Ramsay et al., 1992; Flint and Vernon, 1993; Butterwith, 1994; Gregoire et al., 1998), the standard adipogenic cocktail contains supraphysiological concentrations of insulin, dexamethasone, triiodothyronine (T_3), and isobutylmethylxanthine (IBMX) in a serum-free medium, highlighting the involvement of insulin/IGF-1, and also glucocorticoids and T_3 in some species. From the point of view of signal transduction, these hormones activate several distinct signal transduction pathways including the phosphatidylinositol 3-kinase (PI3 kinase) and mitogen-activated protein kinase (MAP kinase) cascade. Previous reports have shown that the activation of PI3 kinase leads to the activation of c-Akt, p70S6 kinase, protein kinase C, and Rac (Franke et al., 1995; Hawkins et al., 1995; Weng et al., 1995; Ettinger et al., 1996) and is essential to insulin-induced glucose transporter, GLUT4, translocation and antilipolysis in adipocytes. Moreover, inhibition of PI3 kinase by wortmannin, a non-specific inhibitor, and by LY294002, a specific inhibitor of PI3 kinase, has been shown to block the insulin-induced differentiation of 3T3-L1 preadipocytes (Tomiyama et al., 1995; Xia et al., 1999). MAP kinase has been implicated as an important signaling mechanism involved in the differentiation of 3T3-L1 preadipocytes into adipocytes (Magun et al., 1996). Contrary to this report, effectors that activate the MEK/ERK pathway at late stages of adipogenesis are likely to block adipogenic gene expression due to a MAPK-dependent phosphorylation of PPAR (Camp and Tafuri, 1997; Chan et al., 2001). However, few reports have investigated the roles of PI3 kinase and MAP kinase on the differentiation of ovine preadipocytes.

Considering these paradoxical views regarding the involvement of PI3 kinase and MAP kinase cascade in the differentiation in 3T3-L1 cells, we investigated the role of PI3 kinase and MAP kinase on the differentiation of ovine preadipocytes by using the specific inhibitors, LY294002 and PD098059.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Nutrient Mixture F-12 [HAM], bovine serum albumin (Fraction V, BSA), fetal bovine serum (FBS), Insulin (porcine insulin), dexamethasone, lipid mixture (1000X), and trypsin-EDTA solution were purchased from Sigma Chemicals Co., St. Louis, MO. Collagenase (Type I) was purchased from Worthington Biochemicals Corp., Freehold,

NJ. PD098059 (Specific Inhibitor of MEK) was from Calbiochem Biosciences, Inc. LY 294002 (Specific Inhibitor of PI3 kinase), dihydroxyacetone phosphate (DHAP), T_3, and Antibiotic-antimycotic Mixed Stock Solution were purchased from Nacalai Tesque, Inc., Japan. Nicotiamide adenine dinucleotide, reduced form (NADH), was from Roche Diagnostics, Germany.

Preparation of sheep preadipocytes and differentiation experiment

Adult female Suffolk sheep weighing 56-61 Kg were used in this experiment. On the day of the experiment, animals were not fed until the samplings were completed. Subcutaneous adipose tissues were sliced and minced, and digested with collagenase solution in Krebs-Ringer bicarbonate buffer (KRB) containing 25 mM HEPES (pH 7.4), 5.5 mM glucose, 10 mM acetate, 0.2% collagenase, and 3.5% BSA for 1 h at 37°C with constant agitation (100 cycles/min). The digested tissue was filtered through a nylon mesh screen (80 mesh size) in order to separate cells from undigested tissue fragments and debris. The filtrate was collected in a sterile 50 ml centrifuge tube and centrifuged at 1,400×g for 5 min at room temperature. The top layer containing the mature adipocytes was removed and the pellet was washed twice (1,400×g, 5 min) with DMEM. The pellet was resuspended in erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$) at 37°C for 10 min, and then centrifuged at 1,400×g for 5 min at room temperature. The final pellet was resuspended in DMEM supplemented with 10% FBS and the cells were seeded in 35mm culture dishes at a density of approximately 25,000 cells/plate and incubated at 37°C in 5% CO_2 in air. The cells were grown in DMEM supplemented with 10% FBS, 10 mM sodium acetate, and 1% antibiotics mixture. The culture medium was changed every two days, allowing the cells to proliferate for 3-4 days. After the cells reached confluence, the medium was replaced with differentiation-induction medium; DMEM: Nutrient Mixture F-12 [HAM] (1:1, vol/vol) supplemented with combinations of mixed antibiotic solution, 10 mM sodium acetate, lipid mixture (1000X), T_3 (2×10^{-9} M), dexamethasone (10^{-8} M), and insulin (10^{-7} M). Cells were cultured with varying concentrations of LY294002 (10^{-9}-10^{-6} M) and PD098059 (10^{-9}-10^{-6} M) from the first day (D0) of the differentiation program to its end (D10). Differentiation medium was replaced with fresh medium every two days during the differentiation program.

Assay for glycerol 3-phosphate dehydrogenase (GPDH) activity

Cell differentiation was monitored in terms of the activity of the marker enzyme GPDH according to the method of Wise and Green (1979). GPDH assay was performed on days 0, 3, 7 and 10 of the differentiation program. The medium was discarded and the cells were briefly washed twice with ice-cold phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4); 0.5 ml of lysis buffer (100 mM triethanolamine, 2.5 mM EDTA, 0.1 mM beta-mercaptoethanol, 0.5% NP40) was added and the cells were scraped from the wells mechanically. The cell suspensions were transferred to 1.5 ml tubes and lysed by drawing the suspension in and out (8 times) of a 1 ml syringe fitted with a 25-gauze needle. The lysate was rotated for 15 min at 4°C and then centrifuged at 14,000×g for 10 min at 4°C. The 200 μl supernatant was immediately added to a cell containing 1,760 μl of assay buffer (100 mM triethanolamine, 2.5 mM EDTA, and 0.1 mM β-mercaptoethanol), 20 μl of 12 mM NADH, and 20 μl of 20 mM DHAP, and the change in absorbance at 340 nm was determined. Protein content was determined according to the Bradford method (Bradford, 1976).

Semi-quantitative RT-PCR analysis of PPAR-γ2 mRNA

Total RNA was extracted from primary cultured preadipocytes and differentiated adipocytes pooled in 6 well culture plates. Semi-quantitative RT-PCR was performed as previously described (Roh et al., 2001). RT followed by PCR amplification was employed in order to measure levels of ovine PPAR- γ2 mRNA. Primers specific for PPAR-γ2 are: PPAR-γ2 forward primer (5'-TGGGTGAAACTCTGG GAGAT-3) and PPAR-γ2 reverse primer (5'-CCATAGTG GAAGCCTGATGC-3). Based on preliminary experiments focusing on the climbing phase of PCR products in relation to PCR cycle numbers, PCR with 29 cycles with a 57°C annealing temperature for the PPAR-γ2 (product size of 454 bp) was performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; product size of 452 bp), the housekeeping gene, was amplified as an internal control with 24 cycles at a 55°C annealing temperature. The relative levels of ovine PPAR-γ2 mRNA to GAPDH mRNA were calculated and reported in the figures.

Statistical analysis

The data shown are the means (±SEM) of three or four experiments from each different sheep. Comparisons were tested by ANOVA, followed by Fisher's protected least significant difference as a post-hoc analysis. All analyses were performed using the SAS program (SAS/STAT Version 6, SAS Institute, Cary, NC, USA).

RESULTS

Figures 1 and 2 clearly showed that the GPDH activity of the differentiated preadipocytes increased markedly with
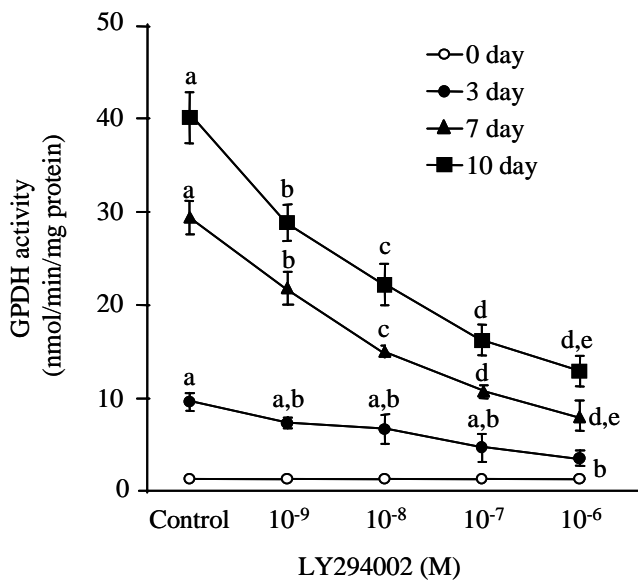
Figure 1. Effect of LY294002 on the GPDH activity during 10 days of differentiation on preadipocytes derived from ovine subcutaneous adipose tissue. The data represent the mean±SEM of 3 separate experiments. [a, b, c, d, e]: Different letters within each day represent significant difference (p<0.05).
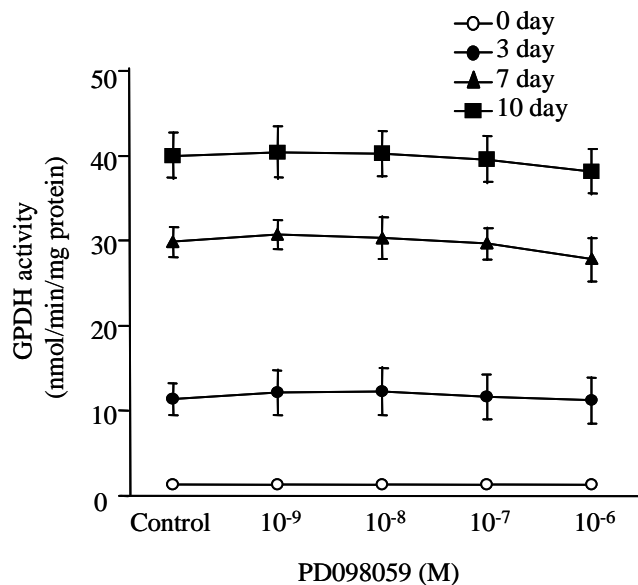

Figure 2. Effect of PD098059 on the GPDH activity during 10 days of differentiation on preadipocytes derived from ovine subcutaneous adipose tissue. The data represent the mean±SEM of 3 separate experiments.

the days of differentiation in differentiation medium alone. Treatment of LY294002 during the differentiation of preadipocytes significantly inhibited GPDH activity in dose- and day-dependent manners (Figure 1). The GPDH activity was significantly blocked by all concentrations of LY294002 during 10 days of differentiation (p<0.05). All
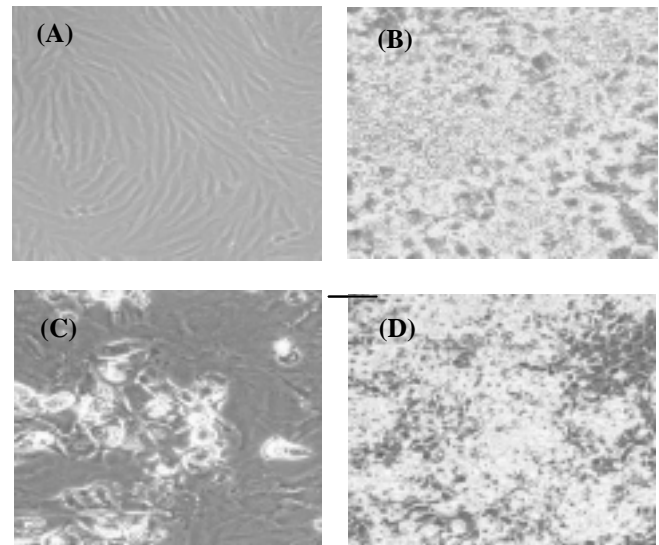

Figure 3. Morphological observations of differentiated ovine preadipocytes (×150). (A) confluent preadipocytes, (B) differentiated adipocytes with differentiation medium alone for 10 days, (C) differentiated adipocytes cultured with LY294002 (10^{-6} M) for 10 days, (D) the differentiated adipocytes cultured with PD098059 (10^{-6} M) for 10 days. Scale bar=100 μm.

doses of LY294002 significantly inhibited GPDH activity for 7 and 10 days compared with control (p<0.05). 10^{-6} M LY294002 at a high dose inhibited this activity for only 3 days (p<0.05). However, the treatment at 10^{-7} M, 10^{-8} M, and 10^{-9} M doses for 3 days showed a decrease with no significant difference. Furthermore, a significant decrease of lipid drop by 10^{-6} M of LY294002 treatments was seen for 10 days, as observed via microscope as depicted in Figure 3C. Figure 2 shows the effect of PD098059 on the differentiation of preadipocytes. PD098059 at any concentration did not affect the differentiation program for 10 days. Figure 3D shows that the lipid drop was not affected by 10^{-6} M of PD098059 treatments. Figure 3 shows the morphological changes during 10 days of differentiation of 10 days in preadipocytes.

As the expression of PPAR-γ2 is significantly increased in differentiated adipocytes, it has been widely used as an index of differentiation (Tontonoz et al., 1994). In order to clarify if there is any change of PPAR-γ2 expression with treatment of LY294002 and PD098059 in the process of preadipocyte differentiation, the levels of PPAR-γ2 mRNA in the preadipocytes and during differentiation at days 3, 7 and 10 were analyzed (Figure 4). The level of PPAR-γ2 mRNA was not detected in confluent preadipocytes, and the expression of PPAR-γ2 was significantly increased during differentiation and reached its maximum at days 10 of differentiation (p<0.05). Differentiated adipocytes cultured with LY294002 (10^{-6} M) for 10 days showed a significant decrease on the level of PPAR-γ2 mRNA compared with
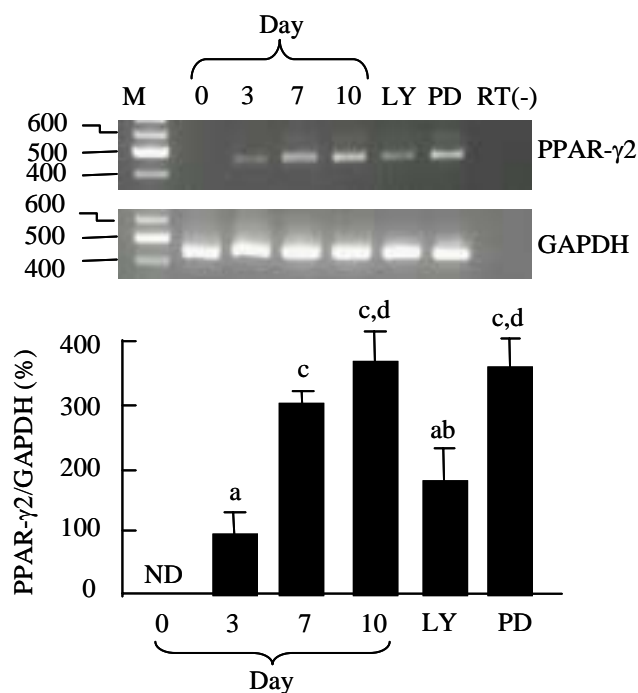
Figure 4. The level of PPAR-γ2 mRNA in confluent preadipocytes and differentiated adipocytes. LY indicates the differentiated adipocytes cultured with LY294002 (10^{-6} M) for 10 days. PD indicates the differentiated adipocytes cultured with PD098059 (10^{-6} M) for 10 days. Ethidium bromide-stained agarose gel shows the amplified PPAR-γ2 (454 bp) and GAPDH (452 bp) with molecular markers from differentiation day and treatments indicated at the top. The RT-PCR results shown are representative of 3 separate experiments using the same protocol. M shows the molecular size ladders (650, 500, 400 bp). The column represents the mean±SEM of 3 separate experiments. [a, b, c, d]: Different letters within treatment represent significant difference ($p<0.05$). ND: No dected

control cells in the presence of only differentiated medium ($p<0.05$). However, this expression was not changed by PD098059 treatment (10^{-6} M) for 10 days.

DISCUSSION

This experiment showed that inhibition of PI3-kinase by treatment of LY294002 blocked the differentiation process of ovine preadipocytes as measured by its marker enzyme, GPDH activity, and the level of PPAR-γ2 mRNA. GPDH activity was most significantly inhibited the differentiation with LY294002 at dose of 10^{-6}-10^{-9} M against the controls. These results are in concordance with the results obtained with LY294002 and wortmannin in 3T3-L1 preadipocytes (Okada et al., 1995; Tomiyama et al., 1995; Xia et al., 1999), which demonstrates the regulatory mechanisms of PI3 kinase on the differentiation program. Precipitation of PI3 kinase with antibodies to phosphotyrosine was observed to

be transiently increased during differentiation between day 4 and day 6 (Sakaue et al., 1998). In addition, the inhibition of PI3 kinase by LY294002 treatment induced the reduction of PPAR-γ2 mRNA. Although PPAR-γ is an important transcriptional regulator during adipocyte differentiation, other transcription factors, such as C/EBP alfa, beta, delta, and sterol regulatory element binding protein 1 (SREBP1), also contribute to this process in 3T3-L1 cells (Wu et al., 1996; Hwang et al., 1997). Thus, transcriptional control of the corresponding genes appears to be independent of PI3 kinase in cell lines. Studies carried out on cell lines show that treatment with adenovirus vector encoding p85 and p110 N in 3T3-L1 and 3T3-F442 cells inhibited differentiation but did not affect expression of PPAR-γ at either the mRNA or protein level (Sakaue et al., 1998). However, a controversial finding has been reported indicating that LY294002 (40 μM) blocked the induction of PPAR-γ mRNA expression during the initiation of adipogenesis and the differentiation itself in 1246 cells (adipogenic cell line; Xia et al., 1999). This difference in results might be due to the different cell lines used by these research groups, and explained by reports showing that pharmacological inhibitors attenuate the activity of enzymes other than PI3 kinase (Brunn et al., 1996; Toker et al., 1997). However, our results lie in accordance with these above reports demonstrating the involvement of PI3 kinase in cell differentiation. Furthermore, Akt, also known as protein kinase B, one of the downstream effectors of PI3 kinase (Franke et al., 1995), when overexpressed into 3T3-L1 preadipocytes, caused spontaneous differentiation with increased expression of mRNA of adipocyte P2 (aP2) or C/EBP alfa (Magun et al., 1996). It has been suggested that since activation of PI3 kinase was observed exclusively at a specific stage of adipogenesis in cell lines (Sakaue et al., 1998; Xia et al., 1999), there is a possibility that expression of constitutively active Akt at a stage when PI3 kinase is not normally activated modulates the transcriptional control. However, this requires further investigation of the mechanisms of PI3 kinase on the adipocyte-specific transcription factors that are expressed during the initiation of the differentiation program, such as the C/EBP and aP2, in order to fully understand the role that it appears to play in sheep.

Our study showed that the inhibition of the MAP kinase cascade during differentiation, at pharmacological concentrations, did not seem to have any effect on the differentiation as compared with the controls. However, it is not known at this stage of this research whether the insulin-induced adipogenesis is at all mediated by its IR/IRS-1 or Shc/Ras/Raf/MAP kinases in the differentiation of ovine preadipocytes. If it is found to be mediated, this means that insulin-induced adipogenesis might involve another pathway, which does not activate MAP kinase, but might

activate a protein kinase somewhere upstream of the IR/IRS-1 or Shc/Ras/Raf/MAP kinases pathway as has been described in other cell-type studies (Robert, 1992; Winston et al., 1995; Font et al., 1997). These reports suggest that the activation of Raf-1 in differentiating 3T3-L1 cells is completely dissociated from the activation of MAP kinase by insulin. This would mean that at least two separate signals emerge from Ras after insulin stimulation, regulating growth or differentiation depending on the state of the cell or the presence of other environmental factors. Nevertheless, these mechanisms must be examined in detail in sheep before any comparisons with the above mentioned reports can be made. In differentiating ovine preadipocytes, the MAP kinase pathway seems not to be important. Therefore, even though there exist inconsistent views concerning the role of MAP kinase in differentiation in cell lines, this study suggests that MAP kinase does not appear to have a role or may not be necessary in the differentiation of ovine preadipocytes. This is clearly confirmed by our result which shows no change of PPAR-γ2 mRNA in differentiated adipocytes cultured with PD098059 for 10 days.

In conclusion, our result clearly demonstrated that PI3 kinase mediates its role on the differentiation of preadipocytes derived from ovine subcutaneous adipose tissues substantially from the early stages of program. However, the MAP kinase pathway does not seem to be involved in the differentiation of preadipocytes.

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