

Effects of Lipid-Related Factors on Adipocyte Differentiation of Bovine Stromal-Vascular Cells in Primary Culture

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ABSTRACT. The effects of several factors related to lipids on bovine adipocyte differentiation were investigated in primary culture. Adipocyte differentiation was assessed by development of glycerol-3-phosphate dehydrogenase (GPDH) activity and morphological observation. Addition of triglyceride mixture (Intralipid), caprylic acid and very low-, low- and high-density lipoproteins (VLDL, LDL and HDL) stimulated bovine preadipocyte differentiation in serum-free condition. Especially, VLDL strongly increased both cell protein contents and GPDH activity, suggesting that it stimulated both proliferation and differentiation of bovine preadipocytes. Under Intralipid-induced condition, differentiation of preadipocytes from subcutaneous adipose tissues was more evident than those from omental adipose tissues. However, such depot difference was not observed in medium supplemented with indomethacin, which is a peroxisome proliferator-activated receptor (PPAR) γ agonist. This suggests that the differentiation capacity of bovine preadipocytes was different between depots and such difference is dependent on the ability to utilize lipids as endogenous PPAR γ ligands. Therefore, lipid metabolites have the stimulatory effects on bovine adipocyte differentiation *in vitro*, and lipoproteins, especially VLDL, may play an important role in development of bovine adipose tissues *in vivo*.

KEY WORDS: bovine preadipocyte, differentiation, indomethacin, lipoprotein, peroxisome proliferator-activated receptor γ .

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Adipose tissue development results from both the adipocyte enlargement and the formation of new fat cells. Recruitment of the new fat cells is largely dependent on proliferation and differentiation of adipose precursor cells (preadipocytes) present in fat deposits. Adipose tissues from animals, even in adult or aged animals, contain preadipocytes capable of proliferation and terminal differentiation throughout the life [12, 19]. In order to reveal the physiological and pathophysiological mechanisms of adipose tissue formation, various preadipocyte primary culture and cell lines, which are able to differentiate into mature adipocytes, have been established [7]. By utilizing these culture systems, many agents that accelerate adipocyte differentiation have been found. They include various hormones (e.g., glucocorticoids and thyroid hormones), growth factors (e.g., growth hormone and insulin-like growth factor-I), prostaglandins (PG; e.g., PGI₂) and drugs (e.g., thiazolidinediones) [7]. In addition, the molecular mechanism of adipocyte differentiation driven by several transcription factors including peroxisome-proliferator activated receptor (PPAR) γ [22, 23] has also been defined utilizing these culture systems. However, the adipocyte differentiation is a quite complex process and the factors affecting this process in animals have not been well defined.

In cattle, bovine intramuscular adipose precursor cell line (BIP cells) was established and the properties of ruminant preadipocytes was intensively investigated [2]. In these cells, acetate rather than glucose was preferentially used to produce fatty acids, showing the properties of lipid

metabolism in ruminant adipose tissues. Thus, volatile fatty acids are major sources of fatty acids moieties of triglycerides in ruminants [24]. On the other hand, our previous study using stromal-vascular cells from intramuscular adipose tissues demonstrated that addition of fatty acids (e.g., caprylic acids) were essential for adipocyte differentiation in primary culture [16]. From these observations, formation and utilization of lipids including triglycerides and fatty acids are considered to be important factors to induce differentiation and maturation of bovine preadipocytes into adipocytes. The cells in primary culture carry physiological host factors from the donor animals [3], indicating the advantage in studies of physiological mechanisms in comparison with established cells lines [26]. In the present study, we utilized the primary culture system of stromal-vascular cells from bovine adipose tissues to investigate the effects of lipid-related factors on bovine adipocyte differentiation. Furthermore, we compared the properties of preadipocytes from different anatomic depots in responses to such lipids.

MATERIALS AND METHODS

Chemicals: Dulbecco's Modified Eagle's Medium (DMEM) and nutrient mixture F-12 were purchased from Life Technologies, Inc (Rockville, MD, U.S.A.). Dihydroxyacetone phosphate (DHAP), reduced nicotinamide adenine dinucleotide (NADH), caprylic acid, bovine serum albumin, bovine insulin, lipoproteins from human plasma; very low-density lipoprotein (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Indomethacin and collagenase were

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from Wako Pure Chemical Industries, (Osaka, Japan). Dexamethasone (DEX), 1-methyl-3-isobutylxanthine (MIX), and biotin were obtained from Nacalai Tesque (Kyoto, Japan). Intralipid was purchased from Pharmacia AB (Stockholm, Sweden). Fetal calf serum was from Biosciences PTY Ltd. (New Southwales, Australia).

Animals and adipose tissues: Omental adipose tissues were obtained from 3–4 years old Holstein and Japanese Black cattle during slaughter at a local slaughter house. Omental and subcutaneous adipose tissues from the flank region of the same individual were taken from 4–5 years old Holstein cows during the abdominal surgery for depot difference experiments.

Isolation and culture of stromal-vascular cells: Dissected adipose tissues were cut into pieces and suspended (0.5 g/ml) in DMEM containing 20 mM HEPES buffer, pH 7.4, 2 mg/ml collagenase, 4 mg/ml bovine serum albumin in a sterile 50 ml plastic conical centrifuge tube. Digestion was performed for 50 min at 37°C with gentle shaking at a speed of 60 shakes per minute in a water bath. After digestion, the digest was filtered through a nylon screen with 100- μ m pores to remove large undigested tissues and some of mature adipocytes. The cell suspension was then centrifuged at approximately 1,000 \times g for 5 min at room temperature. Floating mature adipocytes and digestion medium were removed by aspiration. The sedimented cells were washed twice with 10 ml DMEM containing 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal calf serum. The main contaminating cells of the collected stromal-vascular cells were erythrocytes, which were reported to markedly decrease cell adherence and proliferation [8]. To eliminate the red blood cells, the stromal-vascular cells were treated with a erythrocyte lysing buffer (154 mM NH_4Cl , 10 mM KHCO_3 , and 1 mM EDTA). The washed cells were resuspended in the above medium and stromal-vascular cells were counted using a Thoma counting chamber. Preadipocytes were plated into 6-well plates at the density of 0.5×10^4 cells/cm² in DMEM containing 4 μ M biotin, 200 μ M ascorbate, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal calf serum (growth medium) at 37°C under 5% CO_2 atmosphere. After 24 hr, the cells were rinsed twice with phosphate buffered saline (PBS) to remove unattached cells. Cells were subsequently maintained in same medium until they reach confluence. The medium was changed every second day. At confluence (referred to as day 0), cells were treated with 10 μ g/ml insulin, 0.25 μ M DEX and 0.5 mM MIX for 48 hr in serum-free DMEM/Ham's F-12 (1:1; v/v) (differentiation medium) since no adipocyte differentiation was observed in serum-supplemented medium as previously reported [16]. At that time, caprylic acid, Intralipid, lipoproteins or indomethacin was added into medium at the concentrations as indicated in figure legends, respectively. Medium was changed every other day. During this period, confluent cells underwent an adipose conversion. Differentiated cells were harvested at indicated times for determination of GPDH activity.

Assay of glycerol-3-phosphate dehydrogenase activity:

The GPDH activity assay was performed by a spectrophotometric method for determination of the disappearance of NADH during GPDH-catalyzed reduction of DHAP under zero-order condition [9] as modified by Wise and Green [25]. Briefly, the culture medium was discarded and cultured cells were rinsed twice with ice-cold PBS. The cells from each well were scraped with a rubber policeman and then harvested into 0.3 ml of an ice-cold buffer containing 25 mM Tris/HCl, pH 7.5, 1 mM EDTA in a 1.5 ml tube. The suspension was disrupted by sonication for 5 sec at 40 watts output in a homogenizer (Sonics and Materials Inc., Danbury, CT, U.S.A.). The sonicates were centrifuged for 5 min at 15,000 rpm. Supernatant was removed into another tube carefully avoiding the lipid layer at the top of the tube for GPDH and protein analysis. The soluble protein concentration of supernatant was determined according to Lowry *et al.* [14] using bovine serum albumin as the standard.

Preparation of total RNA and reverse transcription-polymerase chain reaction (RT-PCR) for PPAR γ mRNA: Omental preadipocytes were grown to confluence and then induced to differentiate by treatment with 10 μ g/ml insulin, 0.25 μ M DEX and 0.5 mM MIX for 48 hr in serum-free medium supplemented with either 10 μ g/ml VLDL, 80 μ g/ml LDL or 75 μ g/ml HDL. Total RNA from each cell preparations was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany). First strand cDNA was prepared from total RNA (2 μ g) using random hexamers as primers and Super Script II reverse transcriptase (Life Technologies, Inc.) in a 20- μ l reaction mixture according to manufacturer's instruction. PCR was performed with primers 5'-GAC TTG AAC GAC CAA GTA ACT C-3' (nt 1,200–1,221) and 5'-CTC TGC TAA TAC AAG TCC TTG TAG-3' (nt 1,711–1,688), which are specific for bovine PPAR γ mRNA [21], using Taq polymerase (Qiagen) and 1 μ l of cDNA as templates. PCR condition was as follows: 30 cycles of denaturing (94°C for 30 sec), annealing (60°C for 30 sec) and extension (72°C for 1 min). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was also amplified as an internal control at the same condition for 26 cycles using GAPDH primers (5'-TGC TCC TTC TGC TGA TGC CCC CAT-3' and 5'-TCT GGG TGG CAG TGA TGG CAT GGA-3'). The PCR products were used for electrophoresis on 2% agarose gel to compare the relative signal intensity.

Statistical analysis: The data were analyzed by Student's *t*-test, or Wilcoxon-Mann-Whitney test. When the *F* value was not significant, the Student's *t*-test was used to analyze the difference between each mean value. When a significant *F* value was found, the Wilcoxon-Mann-Whitney test was used for statistical evaluation.

RESULTS

Stimulatory effects of caprylic acid and triglycerides on differentiation: Figure 1 shows the effects of lipids on adipocyte differentiation of stromal-vascular cells from

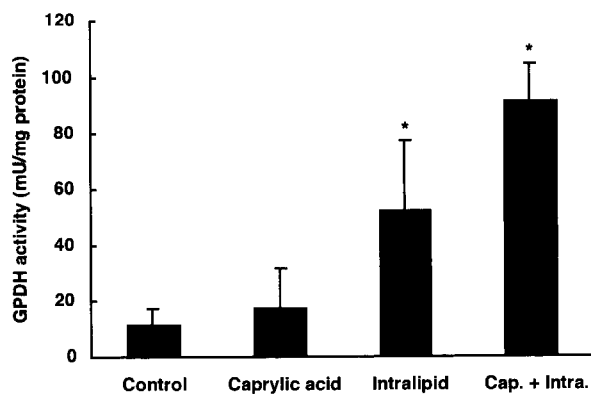


Fig. 1. Effects of caprylic acid and triglyceride (Intralipid) on differentiation of bovine preadipocytes. Omental preadipocytes were grown to confluence and then induced to differentiate by treatments with DEX (0.25 μ M) and MIX (0.5 mM) for first 48 hr in the differentiation medium supplemented with insulin (10 μ g/ml). Caprylic acid (1 mM) and/or Intralipid (2% [w/v]) were added to the differentiation medium. Cells were harvested on day 8 for GPDH activity determination. Columns show means with vertical bars indicating S.D. *Significantly different compared to corresponding control ($P < 0.01$, $n = 7$).

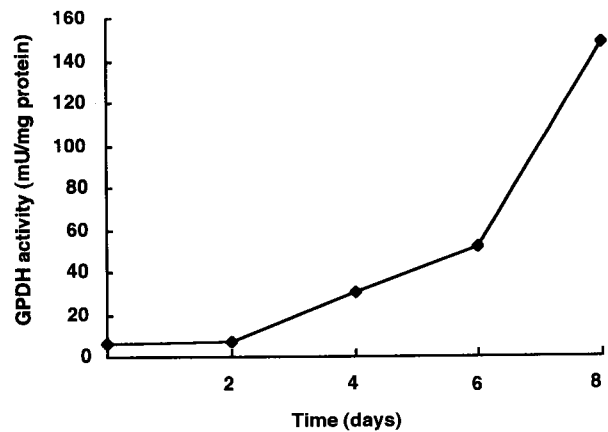


Fig. 2. Time course of bovine preadipocyte differentiation. Omental preadipocytes were grown to confluence and then induced to differentiate by treatments with DEX (0.25 μ M) and MIX (0.5 mM) for first 48 hr in the differentiation medium supplemented with insulin (10 μ g/ml) and Intralipid (2% [w/v]). At day 0, 2, 4, 6 and 8, the cells were harvested for GPDH activity determination. Each data point shows a mean value from determination in triplicate.

omental adipose tissues. Addition of caprylic acid, which is effective for adipocyte differentiation in primary culture [16] or bovine intramuscular preadipocyte (BIP) cell line [2], increased the GPDH activity (17.3 ± 14.1 mU/mg protein), although it was not significantly different from control culture without caprylic acid (11.3 ± 6.2 mU/mg protein). Typically, a few lipid-accumulated cells were observed during that stage. Treatment with 2% Intralipid, which is a lipid emulsion containing phosphatidylcholine and various triglycerides, led to a significant increase in GPDH activity (52.1 ± 25.1 mU/mg protein, $P < 0.01$) and noticeable accumulation of lipid-droplets in cytoplasm. Combination of caprylic acid and Intralipid resulted in a higher increase in GPDH activity (91.3 ± 13.2 mU/mg protein, $P < 0.01$). These results indicated that differentiation of bovine omental preadipocytes was enhanced by addition of lipids. Total protein contents were rather higher in the Intralipid-induced condition (total soluble cell protein; 87.2 ± 35.9 μ g/well) than control (55.1 ± 21.1 μ g/well), indicating that decrease in cellular protein contents was not a cause of increase in specific GPDH activity (expressed as mU/mg protein).

The GPDH activity in omental stromal-vascular cells was very low (6.9 ± 2.1 mU/mg protein) at day 0, indicating that no adipocytes was contained before treatment with lipids. The GPDH activity in the presence of 2% Intralipid (Fig. 2) increased gradually from day 2 and reached the maximal level on day 8 (148.2 ± 60.2 mU/mg protein). Beyond 8 days, GPDH activity displayed a tendency to decrease (data not shown), probably due to cell detachment.

Effects of lipoproteins on differentiation and expression of PPAR γ mRNA: We investigated the effects of lipoproteins on adipocyte differentiation since vast majority of lipids

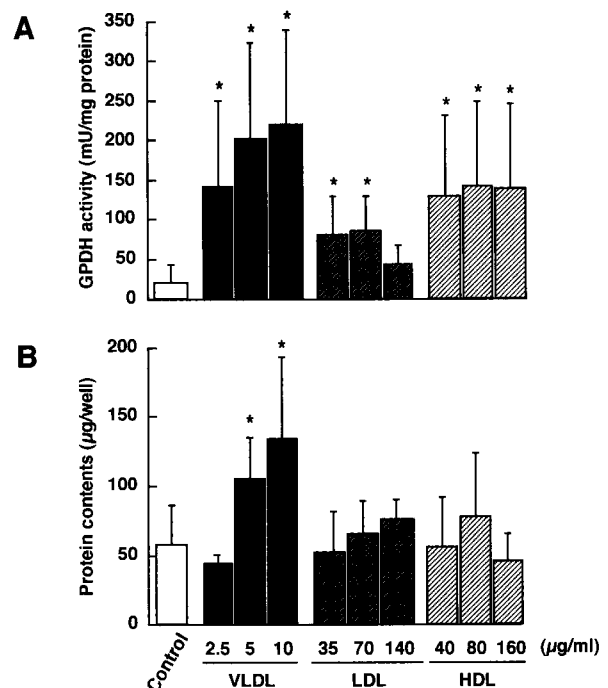


Fig. 3. Effects of lipoproteins on bovine preadipocyte differentiation. Omental preadipocytes were grown to confluence and then induced to differentiate by treatments with DEX (0.25 μ M) and MIX (0.5 mM) for first 48 hr in the differentiation medium supplemented with insulin (10 μ g/ml). Lipoproteins (VLDL, LDL or HDL) were added to the differentiation medium. Cells were harvested on day 8 for determination of GPDH activity (A) and total soluble cell protein content (B). Columns show means with vertical bars indicating S.D. *Significantly different compared to corresponding control ($P < 0.01$, $n = 6$).

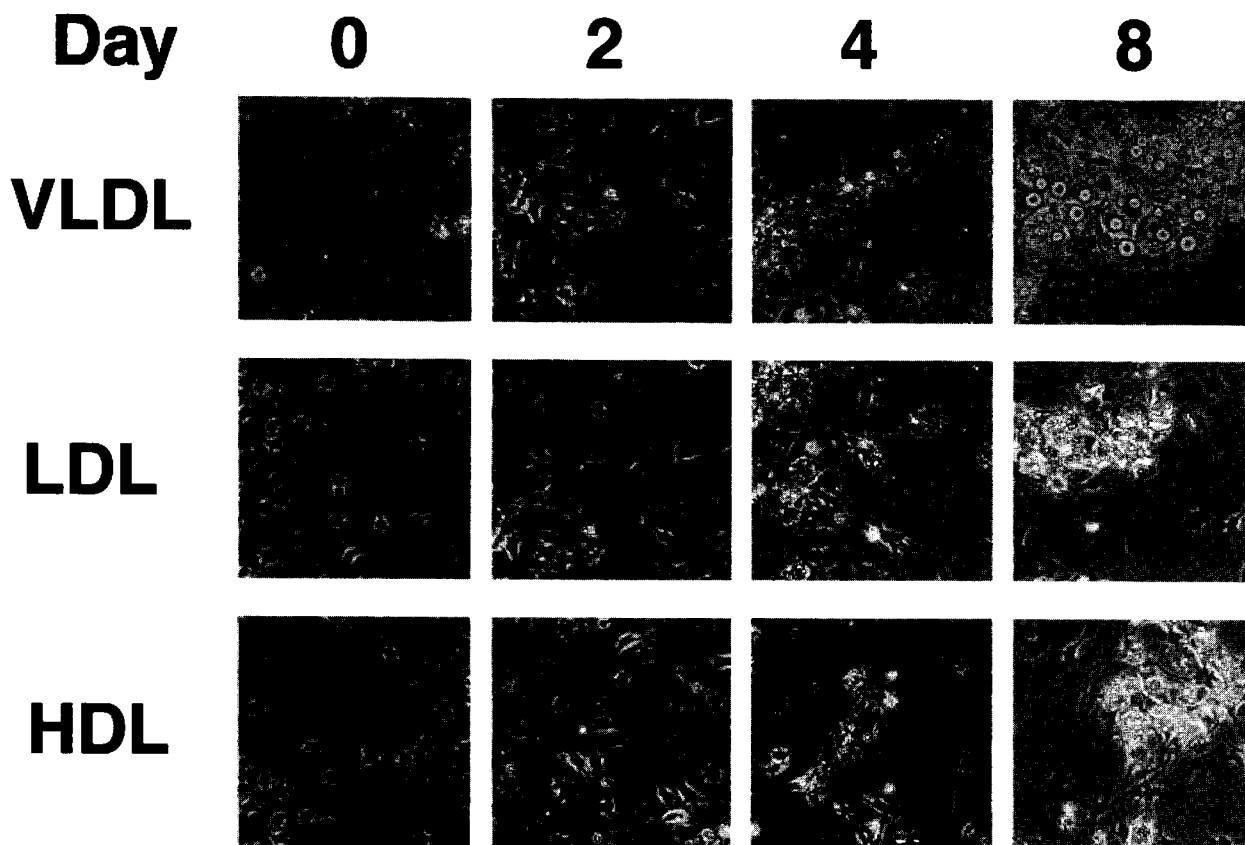


Fig. 4. Morphological changes at different periods of bovine preadipocyte differentiation stimulated by lipoproteins. Phase-contrast photomicrographs of confluent preadipocytes (day 0) and differentiated preadipocytes were taken during differentiation at different times as indicated. Omental preadipocytes were grown to confluence and then induced to differentiate by treatments with DEX (0.25 μ M) and MIX (0.5 mM) for first 48 hr in the differentiation medium supplemented with insulin (10 μ g/ml) and VLDL (10 μ g/ml), LDL (80 μ g/ml) or HDL (75 μ g/ml).

such as triglycerides and cholesterol are supplied to the cells as lipoproteins *in vivo*. Figure 3A shows the effects of VLDL, LDL and HDL on induction of GPDH activity during differentiation. Each of them could promote the differentiation of bovine preadipocytes and VLDL exhibited the greatest stimulatory effect (220.1 ± 102.1 mU/mg protein) on differentiation in comparison with LDL (88.2 ± 42.0 mU/mg protein) or HDL (143.1 ± 92.1 mU/mg protein). Differentiation seemed to reach the maximal levels of GPDH activity by addition of 80 μ g/ml for HDL and 70 μ g/ml for LDL. On the other hand, VLDL (2.5, 5 or 10 μ g/ml) stimulated the development of GPDH activity in a dose-dependent manner. These results were essentially parallel to the accumulation of intracellular lipid droplets under microscopic observation (Fig. 4). Microscopic observation showed that proportion of differentiation in the presence of VLDL attained approximately 70%, which was clearly higher than that in the presence of LDL or HDL. In these experiments, increase in total cell protein contents was also stimulated by addition of VLDL in a dose-dependent manner (total soluble cell protein contents at 10 μ g/ml VLDL; 135.0 ± 57.9 μ g/well vs control; 58.8 ± 27 μ g/well), suggesting that VLDL possessed some stimulatory effects

on cell growth after confluence (Fig. 3B). Since specific GPDH activity was also higher in the presence of VLDL (Fig. 3A), these results indicated that VLDL could induce both proliferation and differentiation of bovine preadipocytes. PPAR γ , which is a key regulator for adipocyte differentiation, is expressed during differentiation in several preadipocyte cell lines and some preadipocytes in primary culture [7, 22]. We, therefore, examined the expression of PPAR γ mRNA in differentiating cells by RT-PCR. As shown in Fig. 5, mRNA of PPAR γ was already present in confluent preadipocytes at low level (day 0) and the band intensities of PCR products became evident until day 8 in lipoprotein-stimulated cultures. This demonstrated that PPAR γ expression increased during differentiation induced by lipoproteins. The cells differentiated with VLDL exhibited gradual increase in PPAR γ expression from day 2 to day 8. On the other hand, expression of PPAR γ became evident from day 2 and maintained the similar level for LDL and HDL, but slightly decreased at day 8 for HDL. Thus, the signal intensities at day 8 were the highest in VLDL treated cells, demonstrating that VLDL is the most effective to promote bovine adipocyte differentiation through the induction of PPAR γ expression.

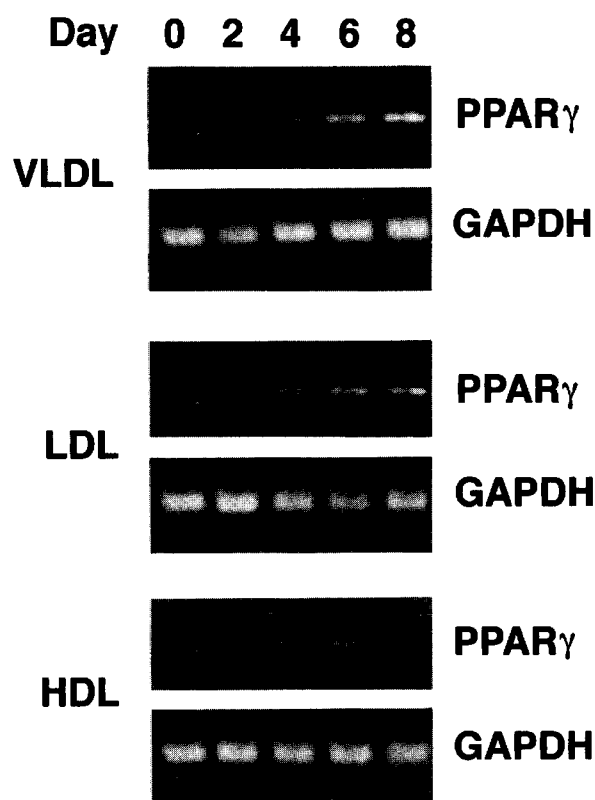


Fig. 5. The expression of PPAR γ mRNA at different periods of bovine preadipocyte differentiation stimulated by lipoproteins. Omental preadipocytes were grown to confluence and then induced to differentiate by treatment with DEX (0.25 μ M) and MIX (0.5 mM) for first 48 hr in the differentiation medium supplemented with insulin (10 μ g/ml) and VLDL (10 μ g/ml), LDL (80 μ g/ml) or HDL (75 μ g/ml). Total mRNA was extracted from cells at the indicated times. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with a pair of bovine PPAR γ specific primers. PCR products were used for electrophoresis on 2% agarose gel to compare the relative signal intensity. Figure shows the representative patterns from three independent cultures.

Depot difference in bovine adipocyte differentiation: Preadipocytes from omental and subcutaneous adipose tissues were used to investigate whether preadipocytes from different sites display disparities in adipocyte differentiation (Fig. 6). These preadipocytes were induced to differentiate in the medium supplemented with triglyceride mixture (Intralipid) or indomethacin, which is a PPAR γ ligand [13]. When the effect of Intralipid on preadipocytes were examined, strong stimulation was observed in subcutaneous cells in comparison with omental cells (subcutaneous cells; 220.0 ± 84.1 mU/mg proteins vs omental cells; 94.7 ± 31.3 mU/mg proteins). This result demonstrated that capability of adipocyte differentiation was different between anatomic depots in bovine adipose tissues. In order to further characterize this depot difference, we examined the effect of indomethacin. In this condition, omental cells also exhibited higher GPDH activity and depot difference was not

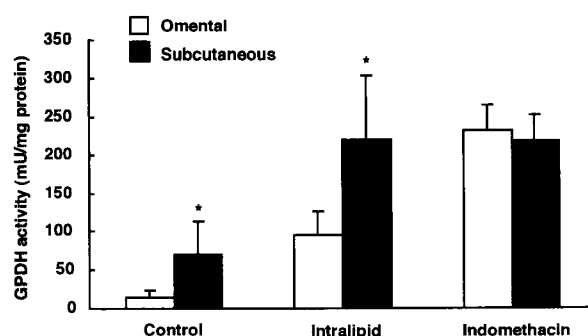


Fig. 6. Depot-specific differences in GPDH activity. Both subcutaneous and omental preadipocytes from the same individuals were grown to confluence and then induced to differentiate by treatments with DEX (0.25 μ M) and MIX (0.5 mM) for first 48 hr in control differentiation medium (without lipids), 2% Intralipid-supplemented medium or 100 μ M indomethacin-supplemented medium in the presence of 10 μ g/ml insulin, respectively. Cells were harvested on day 8 for determination of GPDH activity. Columns show means with vertical bars indicating S.D. *Significantly different compared to omental preadipocytes ($P < 0.01$, $n = 6$).

observed (subcutaneous cells, 220.0 ± 84.1 mU/mg protein; omental cells, 231.8 ± 32.7 mU/mg protein). These results demonstrated that the omental cells could also undergo adipose conversion when PPAR γ was activated exogenously.

DISCUSSION

In the present study, we demonstrated the effects of lipids on adipocyte differentiation of bovine preadipocytes in primary culture. Preadipocytes isolated from bovine adipose tissues could differentiate into adipocytes by treatment with caprylic acids and/or triglyceride mixture (Fig. 1). This suggests that some lipids themselves or their metabolites are possible to induce the differentiation of bovine preadipocytes. The results that caprylic acid and triglyceride mixture induced GPDH activity additively (Fig. 1) suggest that they stimulate adipocyte differentiation through diverse pathways. Some kind of fatty acids, such as long-chain unsaturated fatty acids, have recently been shown to be ligands for PPAR γ [4, 10]. These facts may be responsible for the adipogenic effects of caprylic acid and triglycerides *in vitro*. Although caprylic acid can be incorporated into cells and oxidized to acetyl-CoA in mitochondria, this product must be further acylated to form long-chain fatty acids. On the other hand, triglycerides (Intralipid) can be simply metabolized to produce various long-chain fatty acids by the action of lipase, such as lipoprotein lipase. Thus, such diversity in metabolic pathway to produce long-chain fatty acids as endogenous PPAR γ ligands may be a cause of difference in effects between caprylic acid and triglyceride mixture.

Our results also revealed that lipoproteins, especially VLDL, had stimulatory effects on bovine adipocyte

differentiation (Figs. 3A and 4). In addition, VLDL supported cell growth after confluence, suggesting that VLDL also stimulated preadipocyte proliferation (Fig. 3B). Process of adipocyte differentiation involves several distinct steps, that is growth arrest at confluence, mitotic clonal expansion and following expression of adipocyte genes [7]. This suggests that preadipocytes increase their numbers after confluence during adipocyte differentiation. The results that stimulatory effects of VLDL on both differentiation and proliferation may reflect the process of clonal expansion during bovine adipocyte differentiation. In addition, the expression of PPAR γ , which is a key regulator expressed at high levels in mature adipocytes [17, 22, 23], was also stimulated by lipoproteins during differentiation (Fig. 5). These results suggest that lipoproteins possess stimulatory effects on the adipogenesis in bovine preadipocytes mediated by expression of PPAR γ . Induction of PPAR γ was also higher in VLDL-stimulated cells when compared at day 8, supporting the results that this form of lipoprotein was the most effective for adipocyte differentiation. The major function of plasma lipoproteins is to transport lipid from the sites of absorption or production to sites of utilization [11]. Since some lipids were adipogenic for bovine preadipocytes as described above, the effects of lipoproteins may indicate that certain adipogenic lipids are supplied to preadipocytes as lipoprotein components. LDL and HDL possess lipogenic effects on 3T3-L1 cells and human preadipocytes [20] and mitogenic properties in vascular smooth muscle cells [6]. However, the greater effects of VLDL by addition of smaller amount (up to 10 $\mu\text{g/ml}$) than the others (35–160 $\mu\text{g/ml}$) suggest that VLDL may be optimum carriers of adipogenic lipids for bovine preadipocytes. VLDL particles are different from other lipoproteins in lipid composition (higher triglyceride content) and apolipoprotein components [15]. This suggests that some lipid- or protein-components specific for VLDL may be appropriate for supplying some adipogenic lipids to bovine preadipocytes. Taken together, VLDL is a remarkable adipogenic factor for bovine preadipocytes and availability of VLDL may be one of the most important factors for adipose tissue development in cattle.

On the other hand, we found depot difference in response to triglyceride mixture as the differentiation stimuli (Fig. 6). Thus, the differentiation capacity of preadipocytes from omental adipose tissues was lower than that of subcutaneous cells in response to triglyceride mixture. In contrast, indomethacin, which is a direct ligand of PPAR γ [11], could induce adipocyte differentiation of omental preadipocytes, indicating that the difference is likely to be dependent on the ability to utilize triglycerides as the differentiation stimuli. Since metabolites of triglycerides and/or fatty acids would be included in natural ligands of PPAR γ , the depot difference observed in this culture system may also be found *in vivo*. In rats, preadipocytes from perirenal adipose tissues possess a higher ability than those from other depots [5], suggesting that these cells carry intrinsic differences in capacity of differentiation. On the other hand, studies with

human [1] and ovine cells [18] indicated that subcutaneous preadipocytes were differentiated with a higher GPDH activity than omental preadipocytes. Especially, responses to lipids and exogenous PPAR γ ligands in ovine cells are very similar to our observations in bovine preadipocytes (Fig. 6). Although the precise mechanisms of the depot difference remains to be determined, the ability to produce endogenous PPAR γ ligands may be different between the sites as proposed in ovine cells [18] since direct activation of PPAR γ with indomethacin could induce marked adipocyte differentiation of the omental cells (Fig. 6). We previously reported that bovine intramuscular preadipocytes underwent adipose conversion by addition of caprylic acid [16] in contrast to the present results that the omental preadipocytes did not show significant adipose conversion (Fig. 1). Although we could not exclude the possibility of interindividual difference, this discrepancy may also be due to depot difference, implying that omental cells have less ability to differentiate into adipocytes in lipid-induced conditions than intramuscular cells. Such differences are intriguing, but another experiment using same cattle is required to evidence the difference between these depots.

In conclusion, we demonstrated that triglycerides and lipoproteins stimulated differentiation of bovine preadipocytes and VLDL was the remarkably effective adipogenic factor that promote both proliferation and differentiation of bovine preadipocytes in primary culture. Moreover, Such responses to lipid-related factors were different between anatomic depots. These results suggest that availability of adipogenic lipoproteins and the ability to produce endogenous PPAR γ ligands may play important roles in the development of bovine adipose tissues *in vivo*.

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