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Insulin Inhibits the Expression of Adiponectin and AdipoR2 mRNA in Cultured Bovine Adipocytes

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ABSTRACT : Adiponectin is an adipocyte-derived protein that has a regulatory role in energy homeostasis and influences insulin sensitivity. Its effects on glucose utilization and lipid metabolism are mediated by AdipoR1 and AdipoR2. How insulin affects adiponectin gene expression and secretion is still controversial. This study was conducted to determine the expression of adiponectin, AdipRs and PPAR-γ during the differentiation of bovine preadipocytes and the effect of insulin on expression of these genes in bovine adipocytes. The bovine preadipocytes started to accumulate lipids three days after differentiation was induced, with increased expression of adiponectin, AdipoR2 and PPAR-γ mRNAs. Insulin decreased the expression of adiponectin mRNA in a dose- and time-dependent fashion, and the inhibition was detectable at insulin concentrations as low as 10 nM and as early as 2 h after addition of 100 nM insulin. Insulin also inhibited the expression of AdipoR2 mRNA at concentrations from 1 to 1,000 nM or 24 h after addition of 100 nM insulin, but did not affect the expression of AdipoR1 in bovine adipocytes. Inhibition of PI3K with LY294002 reversed the inhibition of adiponectin and AdipoR2 mRNA expression by insulin. These results suggest that insulin suppresses the expression of adiponectin and AdipoR2 at least partially via the PI3K signal pathway. (**Key Words :** Insulin, Adiponectin, Adiponectin Receptors, PI3-Kinase, Bovine Adipocyte)

INTRODUCTION

Adiponectin, also called Acrp30, GBP28, apM1 or AdipoQ, was identified first by Scherer in 1995 (Scherer et al., 1995) and then by three other groups (Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996). It is generally assumed to be a protein hormone secreted into the mammalian blood circulation exclusively by adipocytes (Hu et al., 1996). Its primary structure contains an N-terminal signal sequence, a variable domain, a collagen-like domain, and a C-terminal globular domain (Yamauchi et al., 2003). Adiponectin stimulates fatty acid oxidation, decreases plasma triglycerides, and enhances glucose metabolism by increasing insulin sensitivity (Yamauchi et al., 2002). It is also involved in the regulation of energy balance and body weight (Fruebis et al., 2001; Yamauchi et al., 2001). Its synthesis and secretion of are regulated by several factors including IGF-1 and insulin (Halleux et al., 2001) and are affected by thiazolidinediones (Bodles et al., 2006). It has been studied in a number of animal models and consistently shown to have insulin-sensitizing effects in liver and muscles (Combs et al., 2001). Plasma adiponectin levels are reduced in animal models of obesity and insulin resistance, and chronic administration of adiponectin reverses this resistance. A number of adiponectin-deficient mouse models display insulin resistance and other features of the metabolic syndrome such as dyslipidemia and hypertension (Kadowaki et al., 2006).

AdipoR1 and AdipoR2 belong to a new family of membrane receptors predicted to contain seven transmembrane domains but structurally and topologically distinct from G-protein coupled receptors (Yamauchi et al., 2003). Adiponectin binds to the extracellular C-terminal domains of AdipoRs while the cytoplasmic N-terminal domains interact with an adaptor protein. Both AdipoR1 and AdipoR2 can mediate adiponectin function and the expression of these two receptors is regulated by PPAR-γ ligands and PPAR-γ in obese patients (Chinetti et al., 2004). In mice, AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver. Adiponectin-induced activation of AMPK is

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attenuated when RNA expression of both receptors is blocked, and this impairs fatty acid oxidation and glucose uptake (Yamauchi et al., 2003).

Thus, the data obtained so far suggest that adiponectin is an important insulin-sensitizing adipocytokine. Furthermore, it appears that various factors might increase or decrease insulin sensitivity at least partly by up- or down-regulating adiponectin. The regulation of expression of adiponectin and its receptors by insulin in bovine adipocytes is little understood. In the current study, we investigated such expression during differentiation of bovine preadipocytes *in vitro* using semi-quantitative RT-PCR, and examined the effect of insulin on those genes expression in bovine adipocytes.

MATERIALS AND METHODS

Collection of bovine adipose tissue and isolation of preadipocytes

The animal protocol was approved by the Experimental Animal Care and Use Committee at Northwest A&F University. Perirenal and epididymal adipose tissues were obtained from newborn Chinese Holstein calves. The adipose tissue was rinsed with PBS containing 100 U/ml penicillin, 100 mg/ml streptomycin and 1.5 μg/ml amphotericin B, and the tissue mass was cut into approximately 1 mm^3 sections under sterile conditions and digested with type I collagenase (DMEM/F12+20 g/L BSA+1 g/L type I collagenase) for 50 min at 37°C in a shaking water bath. DMEM/F12 containing 10% FBS was then added to stop the digestion. The solution was passed through 200 μm and 80 μm nylon filters to remove undigested tissue and large cell aggregates and then centrifuged at 800×g for 5 min to separate the floating adipocytes from the pellet of preadipocytes. The pellet was washed three times with serum-free medium. Before the final washing step, the preadipocyte fraction was treated with red blood cell lysing buffer (155 mM NH_4Cl, 5.7 mM K_2HPO_4, 0.1 mM EDTA, pH 7.3) to lyse and remove red blood cells. The washed preadipocytes were resuspended in DMEM/F12 containing 10% FBS and plated at a density of 5×10^4 cells/cm^2. The S/V cells were then cultured at 37°C under a humidified 5% CO_2 atmosphere for 48 h to allow the cells to attach fully to the dish.

Cell culture and differentiation of bovine preadipocytes

After two days' preliminary incubation for proliferation (defined as day 0), the medium was removed and replaced by hormone-supplemented differentiation medium (DMEM/F12 containing 14 mM $NaHCO_3$, 25 mM glucose, 100 nM bovine insulin, 10 μg/ml transferrin, 100 nM dexamethasone, 100 U/ml penicillin, 100 mg/ml streptomycin, 1.5 μg/ml amphotericin B and 10% FBS) to induce adipogenesis. The medium was replaced every two days. To study the expression of adiponectin and adiponectin receptors during differentiation, bovine adipocytes were collected on the indicated days (0, 3, 6, and 9) and total RNA was extracted for semi-quantitative RT-PCR.

In order to explore the effect of insulin on the expression of adiponectin and adiponectin receptor genes, bovine preadipocytes were allowed to differentiate for nine days until 95% of the attached cells had visible lipid droplets. Insulin, MAPK inhibitor, PI3K inhibitor, or combinations thereof were then added and gene expression was evaluated.

Oil red O staining

Cultured bovine preadipocytes before and after induction of adipogenic differentiation were fixed with 10% formalin in isotonic phosphate buffer for 1 h and stained with 0.5% oil red O in 98% isopropyl alcohol for 30 min at room temperature. The Oil red O solution was then removed and the cells were immediately destained with 60% isopropanol for 10-20 s. The stained adipocytes were examined by phase contrast microscopy.

RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted from bovine adipocytes harvested at each time point using Trizol reagent following the manufacturer's protocol. After DNase treatment, the integrity of the RNA was determined by examining the 28S and 18S ribosomal RNA bands after electrophoresis. The RNA was quantified by spectrophotometry at 260 and 280 nm and stored at -80°C until use. Total RNA (5 μg) was reverse-transcribed with a First Strand cDNA Synthesis kit. The synthesized cDNA was amplified by PCR using paired sense and antisense primers of adiponectin, AdipoR1, AdipoR2, PPAR-γ and β-actin (Table 1). The PCR conditions included an initial denaturation at 95°C for 8 min, followed by 28 cycles of 95°C for 30 s, 52-64°C for 40 s and 72°C for 50 s, and a final extension step at 72°C for 10 min. PCR products (5 μl) were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. Images were obtained using a Gel Media System and band intensity was measured by Gel-Pro Analyzer 3.1 software. The level of β-actin was adopted as internal standard for determining the levels of the targeted mRNAs. To preclude false-positive results due to contamination by genomic DNA, samples were prepared with and without reverse transcriptase treatment, and as a negative control, RT-PCR was also performed without RNA.

Statistical analyses

Results are shown as means±SD of three independent

Table 1. Sequences of the primer sets used for PCR

Genes	Nucleotide sequence (5'-3')	Fragment size (bp)	Tm (°C)	Accession No.
Adiponectin	Forward:TGTACCCATTCGCTTTACT Reverse: TTTCACTTTGTGCTGCTT	410	56	NM_174742
AdipoR1	Forward:AAGCACCGGCAGACAAGA Reverse:CAGAGGAGGGAGTCGTCAGTA	344	58	NM_001034055
AdipoR2	Forward:GTAGAGGCGTCTGTCCTTTCTT Reverse:GACCTTCCCAGACCTTACAAAC	169	64	NM_001040499
PPAR-γ	Forward:GAGATCACAGAGTACGCCAAG Reverse: GGGCTCCATAAAGTCACCAA	216	59	NM_181024
β-actin	Forward: GATGTGGATCAGCAAGCA Reverse: CCTTCACCGTTCCAGTTT	230	52	NM_176648

experiments. Data were analyzed by ANOVA using SPSS 13.0 to determine the significance of major treatment effects. p values <0.05 were considered statistically significant. In this study each experiment was replicated three times, using adipocytes isolated from different cattles.

RESULTS

Cell differentiation and expression of adiponectin, AdipoRs and PPAR-γ mRNAs

Bovine preadipocytes were plated at a density of 5×10^4 cells/cm^2 and reached confluence (defined as day 0) after 2 -3 days' proliferation (Figure 1A). The growth medium was then replaced with differentiation medium and the preadipocytes were induced to differentiate into mature adipocytes. Cells containing small lipid droplets visible under phase contrast microscopy appeared on day 3 (Figure 1B). They subsequently became larger and circular, the small lipid droplets fusing into large ones (Figure 1C), and ultimately differentiated into typical adipocytes (Figure 1D).

Adiponectin mRNA was not expressed on day 0 of differentiation but it increased as differentiation proceeded and reached its highest level on day 9. AdipoR1 mRNA was stably expressed throughout the differentiation period. The mRNAs for PPAR-γ, a central regulator of adipose cell differentiation, and AdipoR2 were expressed at low levels during the first 3 days but increased markedly after 6 days (Figure 2).

Time- and dose- effects of insulin on adiponectin and AdipoRs expression

Insulin markedly decreased adiponectin mRNA expression in a dose- and time-dependent fashion. Thus, significant inhibition of adiponectin mRNA was detectable at insulin concentrations as low as 10 nM ($p<0.05$), and maximal inhibition was found at 1,000 nM insulin (Figure

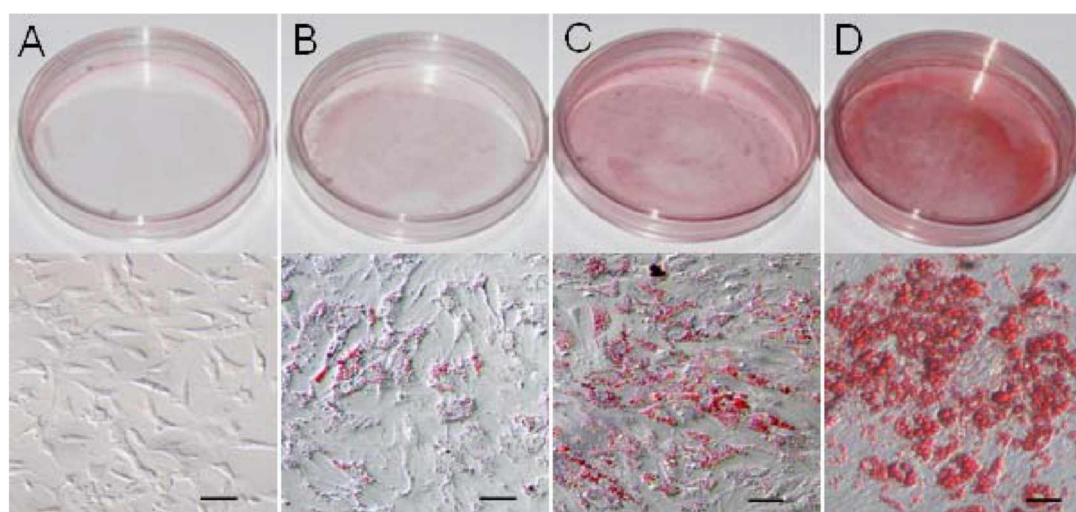

Figure 1. Morphological changes in bovine preadipocytes during differentiation. Lipid droplets were stained bright red with Oil red O. Isolated bovine preadipocytes were allowed to proliferate for 2-3 days (defined as day 0) and were then induced by differentiation medium for 9 days. Cells were stained with Oil Red O on days 0, 3, 6 and 9. (A) On day 0, the fibroblast-like preadipocytes reached confluence; (B) on day 3, the cells began to differentiate and many small lipid droplets appeared; (C) on day 6, the small lipid droplets fused into large ones; (D) on day 9, typical adipocytes with large lipid droplets predominated. Scale bar = 50 μm.

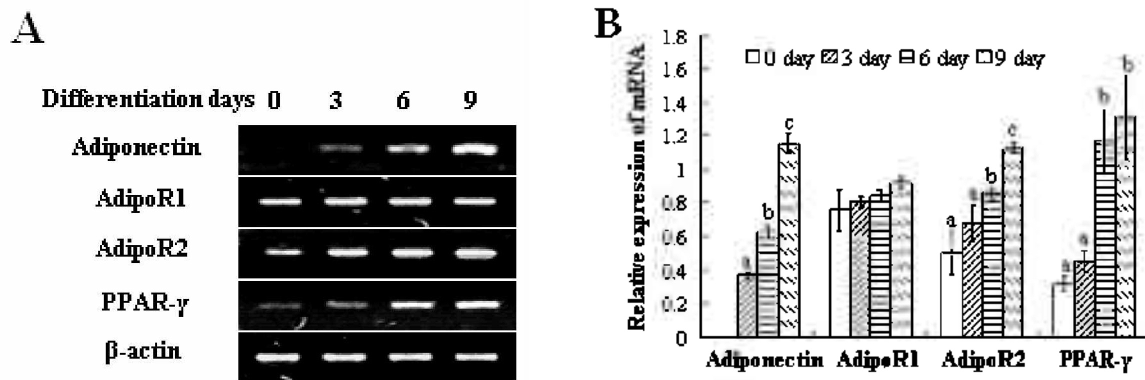

Figure 2. Expression of adiponectin, AdipoRs and PPAR-γ mRNAs during preadipocyte differentiation. Isolated bovine preadipocytes were allowed to proliferate for 2-3 days (defined as day 0) and then induced to differentiate for 9 days. Cells were harvested on days 0, 3, 6 and 9. Total RNA was extracted and subjected to semi-quantitive RT-PCR to determine the levels of adiponectin, AdipoRs and PPAR-γ mRNAs, normalized to β-actin expression as described under Materials and Methods. (A) Agarose gel electrophoresis of the PCR products at cycle 28; (B) relative levels of adiponectin, AdipoR and PPAR-γ mRNAs expression during preadipocytes differentiation. Different letters in the bars indicate significant differences ($p<0.05$).

3B). An inhibitory effect on adiponectin mRNA expression was determined as early as 2 h after treatment with 100 nM insulin. Maximal inhibition was observed when bovine adipocytes were cultured in insulin-containing medium for 24 h ($p<0.05$) (Figure 3D). Furthermore, an inhibitory effect on AdipoR2 mRNA was observed at insulin concentrations from 1 to 1,000 nM (Figure 3B) and 24 h after adding 100 nM insulin (Figure 3D). In contrast, insulin made no significant differences to the expression of AdipoR1 mRNA in bovine adipocytes.

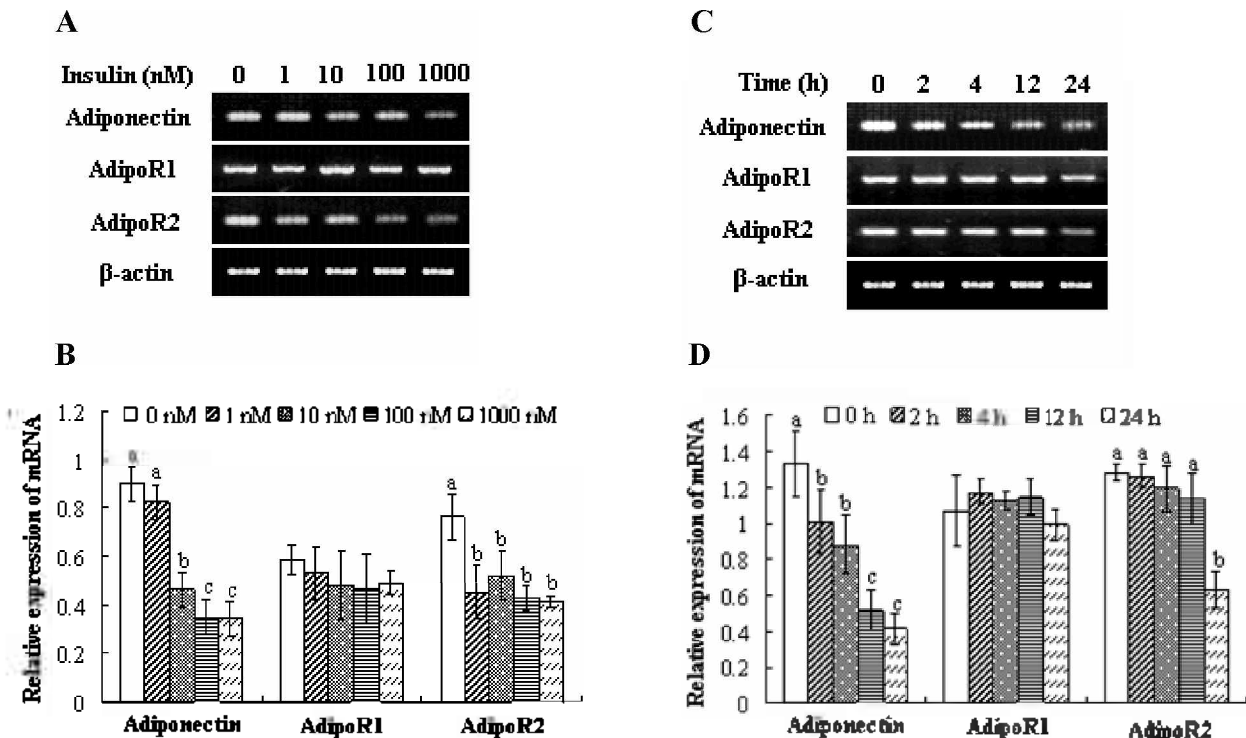

Figure 3. Dose- and time-effects of insulin on adiponectin and AdipoRs expression. To study these effects, 9-day-differentiated adipocytes were washed with PBS and serum-starved for 6 h, then various concentrations (0, 1, 10, 100 and 1,000 nM) of insulin were added for 24 h, or 100 nM insulin was added for different times (0, 2, 4, 12 and 24 h). Total RNA was extracted and subjected to semi-quantitive RT-PCR to determine adiponectin and AdipoRs mRNA levels, normalized to β-actin expression as described under Materials and Methods. (A, C) Agarose gel electrophoresis of the PCR products at cycle 28. (B, D) Relative levels of adiponectin, AdipoR1 and AdipoR2 mRNA expression. Different letters in the bars indicate significant differences ($p<0.05$).

Inhibition of PI3K and MAPK signal pathways

Two major signal transduction pathways mediate the regulation of gene expression by insulin, one through MAPK and the other through PI3K (Saltiel et al., 2001). Nine-day-differentiated bovine adipocytes were serum-starved for 6 h and then pretreated for 1 h with the MAPK inhibitor PD 98059 at 50 μM, or the PI3K inhibitor LY 294002 at 10 μM, or combinations thereof. Insulin (100 nmol/L) was then added and incubation was continued for 24 h. Compared with controls, treatment with 100 nM insulin ±PD98059 significantly decreased the expression of adiponectin and AdipoR2 mRNA ($p<0.05$). However, these mRNAs were more highly expressed when the PI3K signal pathway was inhibited by treatment with insulin+LY294002, insulin+LY294002+PD98059, or LY294002 alone ($p<0.05$) (Figure 4A and 4C). In contrast, there was no significant change in AdipoR1 mRNA expression after treatment with insulin, LY294002, PD98059 or combinations (Figure 4B). Therefore, the data suggest that insulin decreases the expression of adiponectin and AdipoR2 mRNAs in bovine adipocytes mainly through the PI3K signal pathway.

DISCUSSION

Understanding the mechanisms regulating fat deposition and metabolism in beef cattle is important, since the dynamics of adipose physiology are directly associated with both the quality and the value of the meat (Ritchie et al., 1993). Adipogenesis is defined as the proliferation and differentiation of adipose lineage cells, and the initial incorporation of lipids, to form lipid-assimilating adipocytes (Fernyhough et al., 2007). Adipogenesis within specific adipose depots in ruminants may be under somewhat different regulation from that experienced by

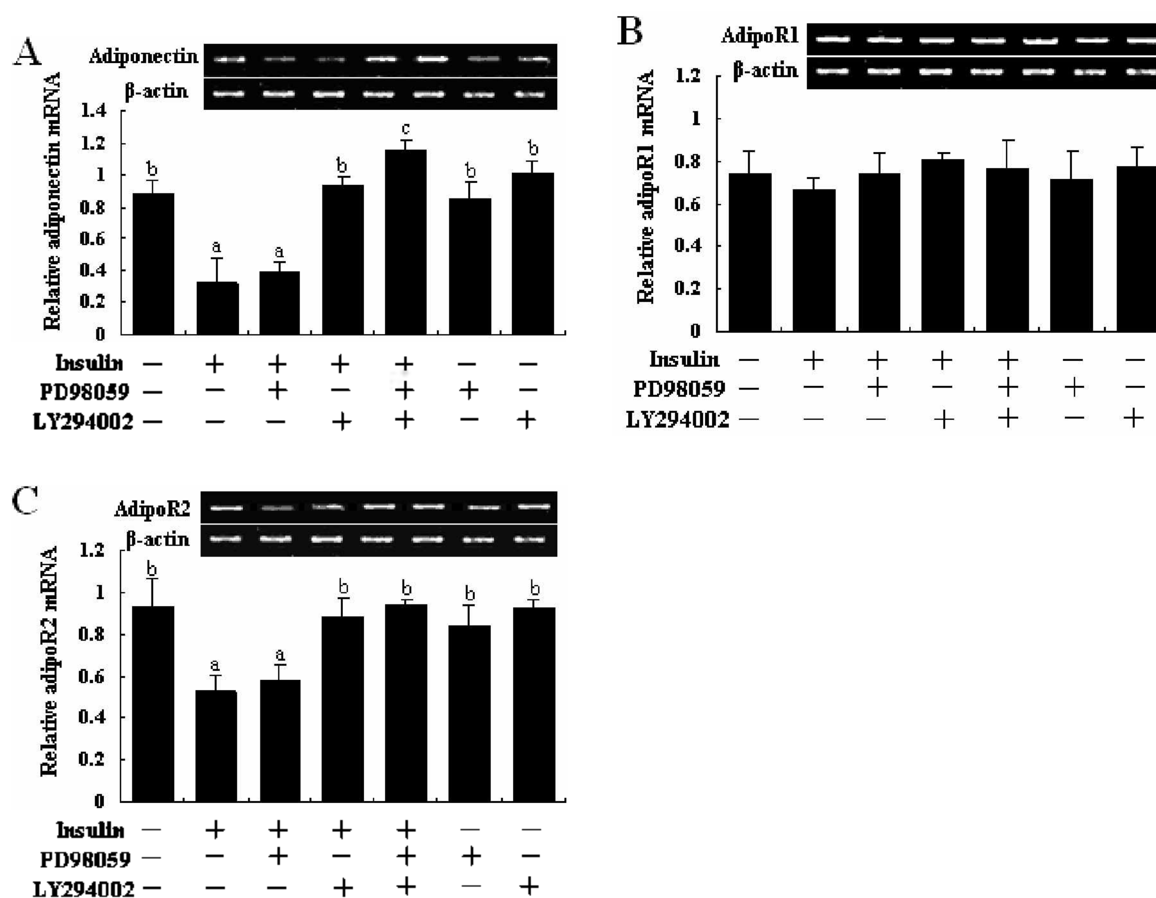

Figure 4. Inhibition of PI3K and MAPK signal pathways. To determine whether either PI3K or MAPK has a role in the regulation of adiponectin and AdipoRs gene expression by insulin, 9-day-differentiated bovine adipocytes were serum-starved for 6 h and then pretreated for 1 h with the MAPK inhibitor PD 98059 at 50 μM, or the PI3K inhibitor LY 294002 at 10 μM, or combinations thereof. Insulin (100 nmol/L) was then added and incubation was continued for 24 h. Total RNA was extracted and subjected to semi-quantitive RT-PCR to determine the adiponectin and AdipoR mRNA levels, normalized to β-actin expression as described under Materials and Methods. (A), (B), (C) show the relative expression levels of adiponectin, AdipoR1 and AdipoR2 mRNAs, respectively. An agarose gel electropherogram of the PCR products at cycle 28 is inserted in each case. Different letters in the bars indicate significant differences ($p<0.05$).

3T3-L1 cells. In the present work, bovine adipocytes were used as an *in vitro* model to study the expression of adiponectin, AdipoR1 and AdipoR2 during adipocytes differentiation and the effect of insulin on this expression.

It is important to note that the levels of most adipose tissue-derived products such as leptin, tumor necrosis factor-α, resistin and interleukin-6 are increased in obesity. In contrast, adiponectin levels decrease. Adiponectin is very specifically expressed in adipose tissue and forms several different complexes in the adipocytes before being secreted into the serum (Pajvani et al., 2003). Studies have suggested that high molecular weight adiponectin may be biologically active and critical for enhancing insulin sensitivity (Pajvani et al., 2004). The expression of both AdipoR1 and AdipoR2 genes is down-regulated in adipocytes overexpressing adiponectin (Fu et al., 2005), and adiponectin down-regulates its own production and the expression of AdipoR2 in transgenic mice (Bauche et al., 2006). These results suggest that adiponectin may act in an autocrine or paracrine fashion to regulate the function of its receptors in adipose tissue.

The present data show that preadipocytes from perirenal and epididymal adipose tissue in newborn Chinese Holstein calves can be differentiated into mature adipocytes *in vitro* with concomitant increase in the expression of adipogenesis-related (PPAR-γ) and adipocytokine (adiponectin and AdipoRs) genes. PPAR-γ belongs to the nuclear hormone receptor superfamily and is a central regulator of adipose cell differentiation. It is constitutively expressed in bovine perirenal adipocytes, as reported previously (Ohyama et al., 1998), and possibly enhances the transcription of genes that regulate the conversion of preadipocytes into mature adipocytes. Bovine adiponectin has been isolated from serum and cloned from adipose tissue (Sato et al., 2001). In this study, the expression of adiponectin and AdipoR2 mRNAs increased in a differentiation-dependent fashion, whereas AdipoR1 mRNA was stably expressed throughout the differentiation period. The result is similar to that reported in swine (Liu et al., 2008). The data suggest that expression of adiponectin and AdipoR2 is associated with bovine adipocyte differentiation. The major adiponectin transcript in bovine adipocytes is approximately 2.5 kb (Mohamed et al., 2007) and there are at least two additional transcripts, as in the swine and rat adiponectin genes (Bensaid et al., 2003; Jacobi et al., 2004), whereas mouse adipose tissue expresses a single transcript (Hu et al., 1996).

Human adiponectin is abundant in plasma with concentrations ranging from 5 to 30 μg/ml, accounting for approximately 0.01% of the total plasma protein (Maeda et al., 2001). This is three orders of magnitude higher than the concentrations of most other hormones (Arita et al., 1999). Plasma adiponectin also has a rapid turnover (Pajvani et al., 2003). The expression and secretion of adiponectin are inhibited by tumor necrosis factor-α, interleukin-6 and dexamethasone (Fasshauer et al., 2003). The effects of insulin on adiponectin gene expression and secretion are still controversial (Scherer et al., 1995; Halleux et al., 2001; Bluher et al., 2002; Fasshauer et al., 2002; Motoshima et al., 2002; Seo et al., 2004). In this study, we used various concentrations of insulin (0, 1, 10, 100 and 1,000 nM) to treat 9-day-differentiated bovine adipocytes for 24 h and 100 nM insulin to treat them for different times (0, 2, 4, 12 and 24 h). Insulin significantly decreased the expression of adiponectin mRNAs in time and dose-dependent fashion, with inhibition detectable at concentrations as low as 10 nM, or as early as 2 h after addition of 100 nM insulin. Insulin also inhibited the expression of AdipoR2 mRNA at concentrations from 1 to 1,000 nM, or 24 h after adding a 100 nM dose, but did not affect the expression of AdipoR1. The data suggest that either the AdipoR2 is a more important receptor mediating adiponectin function in bovine adipocytes, or that it is more sensitive to insulin than AdipoR1 in differentiated bovine adipocytes.

In recent years, a better understanding of the signaling pathways by which insulin mediates its metabolic effects has been obtained. Thus, it has been shown that the activated phosphorylated insulin receptor binds and phosphorylates insulin receptor substrate proteins and Shc, which in turn bind differentially to various downstream signaling proteins (Virkamaki et al., 1999). Important signaling molecules downstream of IRS proteins and Shc include MAPK and PI3K (Saltiel et al., 2001). In the present study, we used insulin signaling pathway inhibitors (LY294002 and PD98059) to block these signaling molecules. The results demonstrated that inhibition of PI3K with LY294002 reverses the inhibitory effect of insulin on the expression of adiponectin and AdipoR2 mRNAs. A recent study indeed showed that adiponectin enhances insulin-stimulated IRS-1 tyrosine phosphorylation and Akt phosphorylation (Wang et al., 2007). It also revealed that activation of the serine/threonine kinase 11/AMPK/TSC1/2 pathway alleviates the p70S6 kinase-mediated negative regulation of insulin signaling (Wang et al., 2007). These data suggest that insulin at least partially suppresses the expression of adiponectin and AdipoR2 via the PI3K pathway. Direct insulin-induced inhibition of AMPK activity through the PI3K/Akt pathway in the heart (Kovacic et al., 2003) provides a potential mechanism by which adiponectin increases insulin sensitivity in adipocytes.

In summary, the present experiment has shown that insulin inhibits the expression of the insulin-sensitizing adipocytokine adiponectin and adiponectin receptor2 in

bovine adipocytes. It has also provided evidence that insulin exerts this effect at least partially via PI3K signaling pathway.

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