Optimization of in vitro conditions for bovine subcutaneous and intramuscular preadipocyte differentiation

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ABSTRACT: The objective of these experiments was to develop an in vitro cell culture system for differentiation of bovine preadipocytes, which will permit examination of differences in differentiation between intramuscular (i.m.) and subcutaneous (s.c.) bovine preadipocytes. Stromal-vascular cells from bovine i.m. and s.c. adipose depots were isolated and cultured. Clonally derived s.c. preadipocytes were used to determine the ability of insulin, bovine serum lipids, octanoate, acetic acid, dexamethasone (DEX), and troglitazone (TRO) to elicit differentiation of these cells when added to serum-free medium. Addition of 10 and 20 μL/mL of a commercially available serum lipids supplement to low-glucose Dulbecco’s modified Eagle’s medium containing 280 nM insulin increased glycerol-3-phosphate dehydrogenase (GPDH) activity (P < 0.01). Inclusion of 1.25 to 10 μM TRO to medium containing 280 nM insulin and 20 μL/mL serum lipids supplement also increased GPDH activity (P < 0.001) compared with 0 μM TRO. The combination of 280 nM insulin, 20 μL/mL serum lipids supplement, 40 μM TRO, and 0.25 μM DEX stimulated differentiation compared with the aforementioned treatment (P < 0.001). Omission of TRO or insulin from this medium reduced GPDH activity by 68% (P < 0.001), whereas removal of DEX tended to reduce GPDH activity (P = 0.06). Preadipocytes from s.c. (n = 3) and i.m. (n = 2) adipose tissues of 3 steers were used to determine the effects of TRO on differentiation using the established conditions. Forty to sixty μM TRO enhanced differentiation compared with 0 μM TRO (P < 0.02) in both depots. No depot differences in response to TRO were detected (P = 0.32). These data demonstrate that bovine preadipocytes are capable of differentiation in response to combinations of insulin, serum lipids, DEX, and TRO. Although TRO enhanced differentiation of bovine preadipocytes, no differential effects of TRO on the differentiation of s.c. and i.m. cells were detected.

Key words: bovine, differentiation, glycerol-3-phosphate dehydrogenase, preadipocyte, thiazolidinedione

INTRODUCTION

Insufficient i.m. adipose and excessive s.c. adipose are paramount beef quality challenges (Roebert et al., 2002; Smith et al., 2006). The amount of adipose in an animal is a function of preadipocyte proliferation and differentiation. However, differences in development of these cells among adipose depots in cattle are poorly understood.

Preadipocyte differentiation is a transformation from a fibroblast-like cell to a lipid-filled cell, with the expression of transcription factors, genes, and enzymes indicative of a mature fat cell (Butterwith, 1994; Grégoire et al., 1998). Limited research with bovine stromal-vascular cells (Sato et al., 1996; Torii et al., 1998) and a clonally derived preadipocyte cell line from Japanese Black cattle (Aso et al., 1995) has shown these cells to be capable of differentiation in response to adipogenic stimuli. However, limited information is available on the culture conditions for optimum bovine preadipocyte differentiation.

Wu et al. (2000) demonstrated that bovine omental stromal-vascular cells exhibit a greater relative increase in differentiation when exposed to a peroxisome
proliferator-activated receptor (PPAR)-γ agonist than s.c. cells. Given that ruminant s.c. stromal-vascular cells may have a greater intrinsic ability to produce natural PPAR-γ ligands compared with stromal-vascular cells of other depots (Soret et al., 1999; Wu et al., 2000), we hypothesized that an exogenous PPAR-γ agonist would enhance the differentiation of bovine i.m. and s.c. preadipocytes, with the relative response being greater in i.m. than s.c. cells.

The objectives of these experiments were to 1) establish a protocol for isolation of bovine preadipocytes from economically important depots; 2) establish differentiation conditions using insulin, fatty acids, serum lipids, glucocorticoids, and troglitazone (TRO); and 3) use these conditions to determine differential responses of i.m. and s.c. preadipocytes to TRO.

MATERIALS AND METHODS

Animal care was conducted according to procedures approved by the Michigan State University Committee on Animal Use and Care.

Bovine stromal-vascular cells isolated from s.c. and i.m. adipose depots were used to determine the effects of adipogenic stimuli on adipocyte differentiation. Unless otherwise stated, all reagents were of tissue culture grade and were purchased from Sigma (St. Louis, MO).

Stromal-vascular Cell Isolation

Subcutaneous and i.m. adipose tissues were collected from 3 Angus steers (age, 13.5 mo; HCW, 345 to 350 kg; s.c. fat thickness adjacent to the 12th rib, 12.7 to 21.6 mm). Before slaughter, steers were fed a corn-based diet for 209 to 249 d. Anabolic implants (200 mg of progesterone and 20 mg of estradiol benzoate) were administered on d 30 and 100 of the feeding period. Cells were grown to approximately 70% confluence, with subsequent cloning.

Adipose tissue samples were collected from the left side of the carcass immediately after exsanguination. Incisions were made dorsal to the 12th and 13th rib, and an approximately 10 cm³ sample containing a portion of both s.c. adipose tissue and LM was obtained. Upon collection, samples were immediately placed in a sterile ice-cold solution of PBS (171.1 mM sodium chloride, 3.4 mM potassium chloride, 10.1 mM sodium phosphate, and 1.8 mM potassium phosphate, pH 7.2) and transported to the laboratory.

Stromal-vascular cells from s.c. and i.m. adipose tissues were isolated under sterile conditions using a modification of the method described by Forest et al. (1987). Briefly, s.c. adipose tissue was separated from the LM and visible connective tissue using a hemocytometer, and resuspended in freezing media containing 2 mg/mL of collagenase (C6885, >125 collagenase digestion units/mg of solid) and 2% BSA.

Initially, samples were incubated in a 37°C water bath, with inversion of the vials at 0, 5, 10, and 15 min. Each sample was then transferred to an incubator (Lab-Line Instruments Inc., Melrose Park, IL) and further digested with shaking for 45 min at 37°C and 230 rpm. Digested material was then sequentially filtered through 1,000-, 500-, and 53-μm sterilized nylon mesh (Nitrex, Tetko, Briarcliff Manor, NY). The resultant filtrate was centrifuged at 4°C for 10 min at 800 × g, the cell pellet was suspended in DMEM, and the centrifugation was repeated. The resulting cell pellet was resuspended in growth medium comprising base medium [DMEM, antibiotic-antimycotic (100 units of penicillin, 0.1 mg of streptomycin, and 0.25 μg of amphotericin B per mL), 50 μg/mL of gentamicin sulfate, 33 μM biotin, 17 μM pantothenate, and 100 μM ascorbate], supplemented with 10% fetal bovine serum for subsequent cloning.

Preadipocyte Cloning

One gram of tissue-equivalent of cells from each depot was suspended in 4 mL of growth medium and seeded equally into 2 wells of a 6-well tissue culture plate (35-mm diam.; Corning Inc., Corning, NY). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂, and growth medium was replaced every 2 d. Cells were grown to approximately 70% confluence, washed twice with PBS, and then removed from the wells by trypsinization (0.5 g/L of trypsin and 0.02 g/L of EDTA in PBS (pH 7.2)). Cell clones were developed using a cloning-ring procedure (Doumit et al., 1993). Cells were counted, diluted, and plated at a density of 400 cells/10-cm culture plate and incubated undisturbed in growth medium for 10 to 14 d. Individual colonies were isolated using 0.5- to 1.0-cm diam. sterile glass cloning-rings dipped in silicone grease. After placement of the cloning rings, cells of a colony were washed twice with PBS, trypsinized, and transferred into one 16-mm diam. culture well containing growth medium. Isolated clones were then grown to approximately 70% confluence in 16-mm diameter and then 35 mm-diameter culture wells, with fresh media changes every 2 d. Cells were then trypsinized, counted using a hemocytometer, and resuspended in freezing media (base medium supplemented with 20% fetal bovine serum and 10% dimethyl sulfoxide). Cells were aliquoted into 1.8-mL cryogenic vials and placed in a styrofoam rack overnight at ~80°C, with subsequent storage in liquid nitrogen.

Identification of Preadipocyte Clones

Subsamples of cells from each isolated clone were suspended in 10 mL of growth medium and seeded at clonal densities (400 cells/well) in 10-cm plates to determine if the cells were adipogenic. In initial characteriza-
tions, cells were grown for 8 to 10 d to allow distinct colonies to form, then exposed to differentiation medium consisting of base medium supplemented with 8.7 nM bovine insulin, 20 mM glucose, 1 mM octanoate, and 10 mM acetic acid (EMD Chemicals Inc., Gibbstown, NJ), with addition of 0.25 µM dexamethasone (DEX) for the first 48 h of the differentiation period. Differentiation medium similar to that stated above stimulated differentiation in an i.m. preadipocyte cell line derived from Japanese Black cattle (Aso et al., 1995). After implementation of improved conditions for bovine preadipocyte differentiation in our laboratory, as more fully described later, cells were grown for 8 to 10 d and induced to differentiate in base media containing 280 nM bovine insulin, 20 mM glucose, and 10 µL/mL of bovine serum lipids supplement (Ex-Cyte, Serologicals Corp., Norcross, GA) with 48 h exposure to 0.25 µM DEX. The bovine serum lipids culture media supplement was an aqueous lipoprotein concentrate containing a mixture of fatty acids (9.3 mg/mL), cholesterol, and phospholipids, derived from bovine serum (Ex-Cyte, Serologicals Corp.).

After 10 to 12 d of exposure to differentiation media, all plates were stained with oil red O as described by Humason (1972) and visualized for the percentage of colonies containing triacylglycerol. A solution of nuclear stain was made by dissolving 1 g of giemsa into 66 mL of glycerol and 66 mL of methanol and filtering through Whatman #1 filter paper. Wells were initially aspirated of media and washed twice with PBS. Cells were then fixed with 3.7% formaldehyde (diluted from a formalin stock; Mallinckrodt Baker Inc., Phillipsburg, NJ) in PBS for 4 min. After fixation, cells were washed twice with PBS and incubated at room temperature with oil red O solution for 1 h. Residual oil red O solution was aspirated and the cells were washed twice with distilled water (15 min incubation/wash). Cell nuclei were stained by adding 1 mL of giemsa into 66 mL of glycerol and 66 mL of methanol and filtering through Whatman #1 filter paper. Wells were initially aspirated of media and washed twice with PBS. Cells were then fixed with 3.7% formaldehyde (diluted from a formalin stock; Mallinckrodt Baker Inc., Phillipsburg, NJ) in PBS for 4 min. After fixation, cells were washed twice with PBS and incubated at room temperature with oil red O solution for 1 h. Residual oil red O solution was aspirated and the cells were washed twice with distilled water (15 min incubation/wash). Cell nuclei were stained by adding 1 mL of giemsa solution to each well for 1 h, after which the cells were washed twice in distilled water. Wells were aspirated of water and stored at 4°C. Cells were visualized within 24 h of staining. Digital photographs were taken using a Nikon CoolPix 5000 digital camera (Nikon Inc., Melville, NY) fitted to a Zeiss inverted microscope (Carl Zeiss Inc., Thornwood, NY). Clones having at least 1 cell/colony stained with oil red O in ≥85% of their colonies were characterized as preadipocytes. Clones identified as preadipocytes were seeded at 1,800 cells/cm² into 10-cm diameter culture plates and sequentially propagated to approximately 70% confluence (5 to 7 d each) until reaching the eighth or ninth passage, at which the cells were used for experimental purposes.

General Procedures

Cell Culture. Stromal-vascular cells or clonally derived preadipocytes were seeded in 6-well plates at a density of 5,200 cells/cm² and incubated in growth medium at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After 24 h, plates were washed twice with PBS and fresh growth medium was added. Growth medium was replaced every 2 d until cells reached confluence. After the cells reached confluence, the plates were washed twice with PBS and experimental differentiation treatments applied. For all experiments, differentiation media were replaced with fresh media every 2 d for 10 to 12 d. Treatment media additions were present for the entire differentiation period, except DEX, which was supplemented only for the initial 48 h. All wells were washed twice with PBS before media replacement on d 2 of the differentiation period. To optimize differentiation conditions, 1 s.c. clone (MSU-SC3-C31) developed with the methods described herein and possessing a rapid growth rate and high differentiation potential was used.

Glycerol-3-phosphate Dehydrogenase Activity Assay. Cell differentiation was quantified biochemically by measuring glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity using a modification of the method described by Adams et al. (1997). Cells were washed twice with ice-cold PBS. Cells from 2 wells per treatment were harvested by scraping and combined in a total volume of 200 µL of ice-cold Tris (pH 7.4) containing 1 mM EDTA and 50 µM dithiothreitol (extraction buffer), then transferred into prechilled 1.5-mL microcentrifuge tubes. Each sample was then disrupted by sonication 3 times at 40 W (3 s bursts with 1 min of cooling on ice between bursts) using a Sonifier-Cell Disrupter 350 (Branson Sonic Power Co., Danbury, CT). Samples were then centrifuged at 16,000 × g for 15 min at 2°C. Solutions containing 50 µL of the resulting supernatant and 150 µL of assay buffer were assayed in duplicate within 30 min for GPDH activity. The final concentration of the supernatant and assay buffer solution was: 100 mM triethanolamine-HCl (pH 7.4), 2.5 mM EDTA, 50 µM dithiothreitol, 0.8 mM dihydroxyacetone phosphate, and 0.317 mM NADH. Each reaction was initiated by the addition of assay buffer to the supernatant in 1 well of a 96-well plate (Immulon 1B, Fisher Scientific, Hampton, NH). Extraction buffer (50 µL) served as the reagent blank. To obtain the reaction rate, the ΔA_{405} was recorded at 15-s intervals for 6 min at 30°C using a spectrophotometer (Versamax Tunable Microplate Reader, Molecular Devices, Sunnyvale, CA). All reactions measured were linear for at least 220 s. The GPDH activity was calculated from the linear range and expressed as nanomoles of NADH oxidized·min⁻¹·mg of protein⁻¹.

Protein Quantification. Aliquots (20 µL) of supernatant were assayed in duplicate to determine protein concentration using the bicinchoninic acid assay (BCA Protein Assay Reagent Kit, Pierce, Rockford, IL). Samples were mixed with 200 µL of assay reagent, incubated for 30 min at 37°C, and absorbance at 562 nm was determined. Protein concentration was determined using BSA as a standard.
Experiment 1

This experiment was conducted to determine the effect of increasing concentrations of serum lipids on preadipocyte differentiation. Three independent experiments were performed using differentiation media consisting of base medium supplemented with 280 nM bovine insulin, 20 mM glucose, and 0, 5, 10, 20, or 40 μL/mL of bovine serum lipids supplement. Protein concentrations and GPDH activities were measured after a 12-d differentiation period.

Experiment 2

To evaluate the adipogenic effects of TRO, a thiazolidinedione PPAR-γ agonist, 2 independent experiments were performed using differentiation media consisting of base medium supplemented with 280 nM bovine insulin, 20 mM glucose, 20 μL/mL of serum lipids supplement, and 5, 10, 20, 40, or 60 μM TRO (Calbiochem, La Jolla, CA). Troglitazone was solubilized in dimethyl sulfoxide at a stock concentration of 22.7 mM before addition to media. Addition of dimethyl sulfoxide to differentiation media alone did not significantly affect differentiation (data not shown). Protein concentrations and GPDH activities were measured after a 10-d differentiation period.

Experiment 3

This experiment was designed to examine the contribution of individual serum-free media components to preadipocyte differentiation. Three independent experiments were performed with differentiation media treatments consisting of base medium with 20 mM glucose, supplemented with: 280 nM bovine insulin, 0.25 μM DEX, 1 mM octanoate, and 10 mM acetic acid (TRT 1); 280 nM bovine insulin, 20 μL/mL of serum lipids supplement, and 0.25 μM DEX (TRT 2); 280 nM bovine insulin, 20 μL/mL of serum lipids supplement, 0.25 μM DEX, and 40 μM TRO (TRT 3); 280 nM bovine insulin, 20 μL/mL of serum lipids supplement, and 40 μM TRO (TRT 4); or 20 μL/mL serum lipids supplement, 0.25 μM DEX, and 40 μM TRO (TRT 5). Protein concentrations and GPDH activities were measured after a 12-d differentiation period.

Experiment 4

The effects of TRO on differentiation of s.c. and i.m. stromal-vascular cells from 3 steers were compared. Differentiation media consisted of base medium supplemented with 280 nM bovine insulin, 20 mM glucose, 20 μL/mL of serum lipids supplement, and 5, 10, 20, 40, or 60 μM TRO. Protein concentrations and GPDH activities were measured after a 12-d differentiation period.

Statistical Analysis

Data were analyzed using the mixed model procedure (PROC MIXED) of SAS (SAS Inst. Inc., Cary, NC) as appropriate for completely randomized designs. Experiments 1, 2, and 3 were conducted within a s.c. and i.m. stromal-vascular cells derived from both the s.c. and i.m. tissues from 3 steers. Data from triplicate (Exp. 1, 3, 4) or duplicate (Exp. 2) experiments were analyzed, and pooled cells from 2 wells of a 6-well plate were considered the experimental unit. In Exp. 4, each replicate consisted of cells from a different animal. In Exp. 1, 2, and 3, means were calculated using the fixed effects of serum lipids supplement, TRO, and treatment, respectively. In Exp. 4, to satisfy the conditions of normality and homogeneity of variance, GPDH data were ln-transformed. Least squares means were computed for the fixed effects of TRO, depot, and TRO × depot. Steer within depot and TRO × steer within depot were included as random effects and were defined as the experimental units for depot and TRO, respectively. For Exp. 1, 2, and 4, linear and quadratic regression models for dose responses were determined using the PROC REG procedure of SAS. In all experiments, when main effects were considered significant (P < 0.05), differences between means were investigated using Tukey’s multiple comparison test.

RESULTS AND DISCUSSION

Addition of serum lipids to serum-free differentiation media for 12 d had a quadratic effect (P < 0.01) on GPDH activity (Figure 1). Preadipocyte differentiation was not detected using control media without serum lipids. However, cells containing lipid droplets were
observed with the addition of 5 μL/mL or greater serum lipids supplement, and concentrations of 10 and 20 μL/mL serum lipids supplement (P < 0.01) increased GPDH activity compared with the control, but did not differ from each other (P = 0.37). Inclusion of 40 μL/mL serum lipids supplement showed no difference in GPDH activity (P = 0.99) compared with the control. Cell morphology and viability appeared to be negatively affected with addition of 40 μL/mL of serum lipids supplement (unpublished observations).

Previous studies using bovine (Wu et al., 2000) and ovine (Soret et al., 1999) stromal-vascular cells have shown that lipid supplementation enhances differentiation. Wu et al. (2000) demonstrated that addition of a lipid emulsion (containing phosphatidylcholine and triacylglycerols) to differentiation media stimulated differentiated stromal-vascular cells extracted from Japanese Black steers. Also, the inclusion of very low-, low-, or high-density lipoproteins, derived from human plasma, enhanced adipose conversion. Using the same serum lipids source used in our experiments, Soret et al. (1999) showed that 10 μL/mL of serum lipids supplement stimulated lipid accumulation in ovine stromal-vascular cells. Fatty acids, especially long chain PUFA, serve as substrates for esterification to triacylglycerol and phospholipids, or stimulate preadipocyte differentiation through ligand binding to PPAR, or both (Kliwer et al., 1997; Krey et al., 1997). Of note, linoleic acid has been found to increase lipid accumulation in human (Hutley et al., 2003) and porcine (Ding et al., 2003) stromal-vascular cells. Likewise, arachidonic acid has been found to enhance differentiation in the Ob1771 preadipocyte cell line (Massiera et al., 2003) and may exert its effects via its eicosanoid products (Krey et al., 1997), including the prostaglandins (Kliwer et al., 1997; Massiera et al., 2003). Expression of PPAR-γ protein has been documented in undifferentiated bovine (Torii et al., 1998) and porcine (Kim et al., 2000) stromal-vascular cells derived from adipose tissue. Ding et al. (2003) suggested that addition of PPAR-γ ligands, such as fatty acids (FA), stimulate differentiation in porcine stromal-vascular cells through ligand binding to PPAR-γ, as well as potentially serving as substrates for triacylglycerol synthesis. These mechanisms would explain the observed increase in differentiation of bovine s.c. preadipocytes due to addition of serum lipids.

Increasing concentrations of TRO, previously shown to stimulate differentiation in stromal-vascular cells isolated from Japanese Black cattle (Ohyama et al., 1998; Torii et al., 1998), were added to our differentiation media in an attempt to increase adipogenesis of bovine s.c. preadipocytes. A quadratic increase in GPDH activity (P < 0.0001) was observed with increasing concentrations of TRO (Figure 2). There were no significant differences in GPDH activity among treatments of 20 μM or greater TRO. Addition of 20 to 60 μM TRO increased GPDH activity by 5-fold compared with control (P < 0.001). Similarly, Soret et al. (1999) reported a 1.9- to 2.5-fold increase in GPDH activity of stromal-vascular cells derived from 6- to 8-mo-old wether lambs when 100 nM rosiglitazone (another thiazolidinedione) was added to media containing 10 μL/mL serum lipids supplement. The concentrations of TRO found to stimulate differentiation in our experiment were within the range of TRO (1 to 100 μM) previously shown to enhance adipose conversion of perirenal (Ohyama et al., 1998) and i.m. (Torii et al., 1998) stromal-vascular cells isolated from Japanese Black cattle. Thiazolidinediones such as TRO, are known to be high affinity PPAR-γ ligands (Lehmann et al., 1995). Expression of PPAR-γ is primarily in adipose tissues of most species investigated (Chawla et al., 1994; Bräissant et al., 1996; Sundvold et al., 1997), including cattle (Torii et al., 1998), and has been implicated in the differentiation process through its activation by endogenous (Kliwer et al., 1997; Krey et al., 1997) and synthetic (Lehmann et al., 1995; Spiegelman, 1998) ligands. Ligand-bound PPAR-γ forms heterodimers with retinoid X receptors, which are then capable of binding to PPAR response elements in the promoters of adipogenic genes, thus stimulating differentiation (Kliwer et al., 1992). Inclusion of serum lipids in our differentiation media stimulated differentiation of bovine preadipocytes, and a PPAR-γ agonist induced additional increases in GPDH activity. This increase in differentiation was likely a result of TRO being a more potent activator of PPAR-γ than the FA in serum lipids. Enhanced ligand binding to PPAR-γ may increase adipogenic gene transcription, thus stimulating a greater number of cells to
differentiate or increase the adipogenic capacity of the differentiated cells, or both. Therefore, whereas TRO likely acted as the more potent PPAR-γ agonist, serum lipids may have contributed to differentiation as a source of FA for membrane phospholipid synthesis, a substrate for triacylglycerol synthesis, or both.

Octanoate (Aso et al., 1995; Sato et al., 1996; Wu et al., 2000) and acetate (Aso et al., 1995) have previously been incorporated into differentiation media of bovine stromal-vascular cells and preadipocytes, respectively. When used in combination with DEX and 1-methyl-3-isobutylxanthine, Sato et al. (1996) found that octanoate stimulated a 5-fold increase in GPDH activity using bovine i.m. stromal-vascular cells, whereas Wu et al. (2000) observed no significant response in omental cells. Han et al. (2002) suggested that octanoate may serve as a PPAR-γ ligand and possibly activate differentiation in the 3T3-L1 cell line. Acetate is considered the major precursor for FA synthesis in ruminant adipose tissues (Vernon, 1980), and Aso et al. (1995) showed increased acetate incorporation into bovine clonal preadipocytes during adipose accumulation following differentiation. The combination of 1 mM octanoate and 10 mM acetic acid with 0.25 μM DEX, and 280 nM insulin (TRT 1) resulted in the morphological differentiation of a small number of cells (Figure 3). However, these FA did not induce GPDH activity (Figure 4) in the bovine s.c. preadipocytes.

Lipid emulsions have also been shown to enhance stromal-vascular cell differentiation (Soret et al., 1999; Wu et al., 2000). Replacing octanoate and acetate with serum lipids was sufficient to generate GPDH activity (Figure 4), as well as visually increase the number of morphologically differentiated cells. In addition, a 1.7-fold increase in protein concentration was observed when serum lipids replaced octanoate and acetate, indicating that the supplement was more favorable for both cell viability and differentiation. The concentration of FA in the serum lipid supplement (0.19 mg/mL) used in this study was within the concentration range of octanoate (0.14 mg/mL) and acetate (0.60 mg/mL) provided to the cells in TRT 1. We did not determine if the short chain fatty acids inhibit adipogenesis or were simply insufficient to stimulate adipogenesis.

The combination of TRO, DEX, serum lipids supplement, and insulin resulted in the greatest GPDH activity (TRT 3, Figure 4). Omission of DEX (TRT 4) tended to reduce GPDH activity (P = 0.06), whereas the removal of TRO (TRT 2) decreased GPDH activity by 68% (P < 0.001) and also reduced protein concentrations (P = 0.03). Wu et al. (1996) suggested that glucocorticoids are capable of increasing differentiation through the upregulation of CCAT/enhancer-binding proteins, which in turn, influence the expression of PPAR-γ in the 3T3-L1 cell line. Also, glucocorticoids may increase differentiation through the enhancement of arachidonic acid metabolism (Gaillard et al., 1991). Thus, the potential for synergism between DEX and TRO in enhancing differentiation exists. Soret et al. (1999) found the combination of DEX and a thiazolidinedione to be additive in stimulating the differentiation of stromal-vascular cells from 6- to 8-mo-old wether lambs. However, results using stromal-vascular cells from suckling lambs (Soret et al., 1999), and those from 5- to 7-d-old pigs (Tchoukalova et al., 2000) demonstrated that the combination of DEX and a thiazolidinedione did not stimulate differentiation above that of either compound when included individually. Soret et al. (1999) therefore suggested that the effects of these compounds on stromal-vascular cell differentiation are dependent on species, depot, and animal age. In addition, Soret et al. (1999) also found
Figure 4. Effect of adipogenic agents on glycerol-3-phosphate dehydrogenase (GPDH), activity and protein concentration of subcutaneous bovine clonally-derived preadipocytes. Components included (+) in or excluded (−) from the media were troglitazone (TRO; 40 μM), dexamethasone (DEX; 0.25 μM), serum lipids supplement (SL; Ex-Cyte, Serologicals Corp., Norcross, GA) (20 μL/mL), insulin (280 nM), octanoate (1 mM), and acetate (10 mM). Values are least squares means and SEM of 3 independent experiments. a–c, x–z Least squares means without a common superscript letter differ (P < 0.05).

that DEX and a thiazolidinedione increased protein concentrations individually, and in combination with serum lipids. Based on the reduced activity observed upon their removal from media, our study suggests that TRO was a more potent stimulator of differentiation than DEX.

Insulin appeared to enhance adipose differentiation, viability, or both of the s.c. preadipocytes, because a 74% reduction in GPDH activity (P < 0.001) was recorded upon omission of insulin from differentiation media (TRT 5, Figure 4). Insulin is known to stimulate adipogenesis of swine (Suryawan et al., 1997) and ovine (Adams et al., 1996) stromal-vascular cells and has been included in differentiation media of bovine stromal-vascular cells and preadipocytes (Aso et al., 1995; Torii et al., 1998; Wu et al., 2000). Adams et al. (1996) documented that exclusion of insulin from differentiation media prevented the adipogenesis of ovine stromal-vascular cells. Although the specific effects of insulin on preadipocyte differentiation have yet to be fully detailed, insulin has been shown to influence the expression of adipogenic genes. Insulin enhanced the transcription of genes such as fatty acid synthase (Moustaid et al., 1994; Paulauskis and Sul, 1988), glyceraldehyde-3-phosphate dehydrogenase (Nasrin et al., 1990), acyl-CoA synthetase, and stearoyl CoA desaturase-1 (Weiner et al., 1991), in studies using the 3T3-L1 cell line. Insulin also stimulates glucose uptake into 3T3-L1 adipocytes through increased translocation of the glucose transporter-4 protein to the plasma membrane (Calderhead et al., 1990). In addition, Stahl et al. (2002) showed that insulin increased cellular uptake of long chain FA through stimulating FA transport protein translocation from intracellular pools to the plasma membrane in 3T3-L1 adipocytes. Therefore, insulin may act as a modulator of cellular differentiation, as well as serve to increase substrate availability for adipogenesis in adipocytes. This suggests that the lack of insulin may have reduced glucose uptake or FA entry into the cells, which in turn reduced GPDH activity. The exclusion of insulin also caused a reduction in protein concentration (P < 0.001) compared with TRT 2, 3, or 4, and apparently reduced viability of these bovine preadipocytes. Ursø et al. (2001) demonstrated that apoptosis is inhibited in 3T3-L1 fibroblasts and adipocytes via the intracellular domains of both the insulin and IGF type I receptor.

Removal of the serum lipids supplement from TRT 3 (Figure 4) resulted in substantial cell detachment from the plates 2 d after exposure to differentiation media (unpublished observations). The serum lipids supplement appeared to contain components that aided in
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Figure 5. Effect of troglitazone supplementation on glycerol-3-phosphate dehydrogenase (GPDH) activity. The effect was linear ($P < 0.01$). Values are least squares means and SEM for 2 independent experiments using subcutaneous and intramuscular bovine stromal-vascular cells from 3 steers. $^{a,b}$Least squares means without a common superscript letter differ ($P < 0.05$). Because there were no depot effects or depot $\times$ treatment interactions ($P > 0.30$), the data were pooled. Inset shows the results for the subcutaneous (SC) and intramuscular (IM) bovine stromal-vascular cells; the axis labels are the same.

maintaining cell viability, attachment, or both during differentiation. The latter appears to be dependent upon the presence of DEX, TRO, or both, because medium containing no serum lipids supplement was capable of supporting cell viability in the absence of DEX and TRO (Figure 1).

To examine if the adipogenic effect of TRO was depot-specific, we exposed stromal-vascular cells derived from both i.m. and s.c. adipose tissues to increasing concentrations of TRO. Increasing concentrations of TRO caused a dose-dependent linear increase in GPDH activity in both depots ($P < 0.01$), and no depot effects ($P = 0.32$) or depot $\times$ treatment interactions ($P = 0.69$) were detected. Pooled data are shown in Figure 5 and results for each depot are shown in the Figure 5 inset. Addition of 40 or 60 $\mu$M TRO increased GPDH activity 2.9- and 3.4-fold, respectively, compared with control ($P < 0.02$). The conditions established with clonally derived s.c. preadipocytes were suitable for differentiation of s.c. and i.m. stromal-vascular cells. Additionally, clonally derived preadipocytes and heterogeneous stromal-vascular cells responded similarly to TRO (Figures 2 and 5). Despite the lack of a depot effect on GPDH, bovine s.c. stromal-vascular cells appear to accumulate more lipid than bovine i.m. stromal-vascular cells. This observation warrants further investigation and refutes our hypothesis that an exogenous PPAR-$\gamma$ agonist would enhance the differentiation of bovine i.m. and s.c. preadipocytes, with the relative response being greater in i.m. than s.c. cells.

Previous studies using stromal-vascular cells isolated from 6- to 8-mo-old wether lambs (Soret et al., 1999)
and 4- to 5-yr-old Holstein cows (Wu et al., 2000) have shown a greater relative increase in GPDH activity in omental compared with s.c. cells with addition of a PPAR-γ agonist to differentiation media. The authors suggested that omental cells might be limited in their ability to produce natural PPAR-γ ligands compared with s.c. cells. In contrast, studies using human stromal-vascular cells have shown that thiazolidinedione addition stimulated differentiation in s.c. cells, whereas omental cells were less responsive to treatment (Adams et al., 1997; Sewter et al., 2002; Tchkonia et al., 2002). Adams et al. (1997) observed no differences in PPAR-γ protein abundance between depots and offered no definitive explanation for the depot-specific responses to thiazolidinedione. However, more current studies showed that PPAR-γ messenger RNA (Sewter et al., 2002; Tchkonia et al., 2002) and protein expression (Tchkonia et al., 2002) were greater in human s.c. stromal-vascular cells than omental and, thus, may account for depot differences in adipogenesis. When taken together, the aforementioned studies suggest that regional variation in adipogenesis exists in stromal-vascular cells isolated from different depots. Although bovine i.m. and s.c. stromal-vascular cells have been shown to respond to lipid supplements (Sato et al., 1996; Wu et al., 2000) and thiazolidinedione (Torii et al., 1998), this study represents the first direct depot comparison of bovine i.m. and s.c. preadipocytes to a PPAR-γ ligand.

This study established that bovine clonally-derived subcutaneous preadipocytes were capable of increased differentiation in response to serum lipids, DEX, and TRO. As well, the relative increase in differentiation of both s.c. and i.m. preadipocytes was similar when exposed to TRO. This suggests that PPAR-γ is likely involved in the differentiation of both depots. The use of clonally derived preadipocytes enabled the optimization of differentiation conditions, which allowed for the first direct comparison between bovine i.m. and s.c. preadipocytes. This culture system will permit future investigations on the cellular mechanisms involved in bovine adipose tissue development, and elucidation of depot differences in preadipocyte differentiation.

**LITERATURE CITED**


