Bovine intramuscular, subcutaneous, and perirenal stromal-vascular cells express similar glucocorticoid receptor isoforms, but exhibit different adipogenic capacity

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ABSTRACT: Understanding preadipocyte differentiation in economically important adipose depots will facilitate efforts to selectively increase intramuscular (i.m.) lipid accretion in cattle. The objectives of this study were to determine if glucocorticoid receptor (GR) expression differs among bovine stromal-vascular (S-V) cells derived from i.m., subcutaneous (s.c.), and perirenal (p.r.) adipose tissue, and to evaluate the effects of dexamethasone (DEX) on adipogenesis of these cell populations. Stromal-vascular cells isolated from i.m., s.c., and p.r. adipose tissues of 2 steers were propagated in culture and exposed to 0 or 250 nM DEX for 48 h. Cell lysates were subjected to GR immunoblot analysis, and immunoreactive protein bands of ~97, ~62, and ~48 kDa were detected and expressed relative to β-actin immunoreactivity. The abundance of each GR immunoreactive protein was similar among S-V cell populations (P > 0.50). Dexamethasone exposure decreased the abundance of the ~97 and ~62 kDa GR immunoreactive bands in S-V cells from the 3 depots (P < 0.001), but did not affect the expression of the ~48 kDa band (P = 0.96). Stromal-vascular cells isolated from 3 steers were grown in culture, and upon confluence, were exposed to 0, 25, or 2,500 nM DEX for 48 h. After an additional 10 d in differentiation media, differentiation was determined by glycerol-3-phosphate dehydrogenase (GPDH) specific activity and oil red O staining. The extent of differentiation differed by depot (p.r. > s.c. > i.m.; P < 0.05). Compared with control, 2,500 nM DEX increased GPDH activity in S-V cells from all depots (P < 0.05), and no interaction between depot and DEX concentration was observed (P = 0.99). We observed an adipose tissue depot by DEX concentration interaction (P = 0.03) for S-V cells with large (≥10 µm-diameter) lipid droplets. The percentage of p.r. S-V cells with large lipid droplets increased in response to DEX in a linear manner (P < 0.02), but only increased greater than control in s.c. cells exposed to 2,500 nM DEX (P = 0.002). Dexamethasone did not significantly increase the percentage of i.m. S-V cells with large lipid droplets (P > 0.27). Collectively, these data demonstrate differences in adipogenic activity among bovine i.m., s.c., and p.r. S-V cells, but indicate no relationship between adipogenic activity and glucocorticoid receptor abundance or function.

Key words: adipogenesis, bovine, dexamethasone, differentiation, glucocorticoid receptor, stromal-vascular cell

INTRODUCTION

Glucocorticoid induction of adipose tissue development has been shown to have anatomical specificity. Preferential accretion of abdominal adipose tissue is observed in humans treated with glucocorticoids (Rebuffé-Schifique et al., 1988; Fried et al., 1993; Boschmann, 2001). In swine, glucocorticoid stimulation of subcutaneous (s.c.) preadipocyte differentiation was greater in cells isolated from the shoulder than those from the ham (Ramsay et al., 1989). In humans and rats, the relative abundance of glucocorticoid receptors (GR) in adipose tissue is positively associated with the accumulation of adipose tissue in the various depots following...
glucocorticoid supplementation (Feldman and Loose, 1977; Brönnegard et al., 1990; Rebuffé-Scrive et al., 1990).

Preadipocytes are also considered a target for glucocorticoids, which are commonly added to the differentiation media for cultured stromal-vascular (S-V) cells and clonally derived preadipocytes (Sato et al., 1996; Takenouchi et al., 2004, Hirai et al., 2007). However, to our knowledge, no previous study has documented GR expression in bovine S-V cells. If differences in GR expression exist between bovine S-V cells from different adipose tissues, it could present a route to selectively alter adipose tissue accretion. Because intramuscular (i.m.) adipose tissue accretion is positively associated with beef palatability and is the main determinant of beef quality within a carcass maturity classification, any treatment that could selectively enhance i.m. adipose tissue development, or selectively reduce s.c. fat, would potentially benefit the beef industry.

We hypothesized that bovine i.m., s.c., and perirenal (p.r.) S-V cells exhibit different levels of GR expression and different adipogenic responses to glucocorticoids. The objectives of this study were to compare GR expression among bovine i.m., s.c., and p.r. S-V cells, and to compare the effects of a glucocorticoid on the differentiation of these distinct cell populations.

**MATERIALS AND METHODS**

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

Isolation of the bovine S-V cells from i.m. and s.c. adipose depots of 3 Angus steers used in these studies was described previously by Grant et al. (2008a). Briefly, adipose tissue samples were collected from the carcass immediately after exsanguination. A rib section was taken dorsal to the 12th and 13th rib, and p.r. tissue obtained from each animal. Tissue samples were immediately placed in sterile, ice-cold PBS and transported to the laboratory. Unless otherwise stated, all reagents were of tissue culture grade and were purchased from Sigma (St. Louis, MO). Adipose tissues were isolated under sterile conditions and cut into approximately 2-mm³ sections. Aliquots of approximately 3 g were digested in 6 mL of Dulbecco’s modified Eagle’s medium (5.5 mM glucose; Invitrogen Corp., Carlsbad, CA) containing 2 mg/mL of collagenase and 2% BSA. After incubation, digested material was sequentially filtered through 1,000-, 500-, and 53-µm sterilized nylon mesh (Nitrex, Tetko, Briarcliff Manor, NY). Following isolation by centrifugation at 4°C for 10 min at 800 × g, cells were suspended in growth medium [Dulbecco’s modified Eagle’s medium (5.5 mM glucose) containing antibiotic-antimycotic (final concentration: 100 units/mL of penicillin G, 0.1 mg/mL of streptomycin sulfate, and 0.25 µg/mL of amphotericin B), 0.05 mg/mL of gentamicin, 33 µM biotin, 17 µM pantothenate, 200 µM ascorbate, 1,000 µM octanoate, and 10% fetal bovine serum (FBS)].

The resulting suspension of S-V cells was seeded at 0.05-g equivalent per cm² in 35 mm-diameter cell culture wells (Corning Inc., Corning, NY). Alternatively, cells were suspended in freezing medium (growth medium adjusted to contain 20% FBS and 10% dimethyl sulfoxide) and aliquoted into 1.8-mL cryogenic vials. Cells were placed at −20°C for 2 h, and overnight at −80°C, before storage in liquid N for later use.

**GR Expression**

Primary S-V cells from the i.m., s.c., and p.r. adipose tissue of 2 steers were propagated in culture and in their 4th passage, cells were seeded at a density of 3,600 cells/cm² in 35 mm-diameter cell culture wells. Cells were allowed to proliferate to confluence in growth medium, while incubated in a humidified atmosphere (37°C, 95% air and 5% CO₂). Growth medium was replaced every 2 d. After reaching confluence, cells were washed twice with PBS and the S-V cells were exposed to modified growth medium (1% FBS instead of 10% FBS) containing 0 or 250 nM dexamethasone (DEX) for 48 h. Identical procedures were followed in 4 individual trials.

**Immunoblot Analysis**

After 48 h of exposure to 0 or 250 nM DEX, S-V cell monolayers were washed twice with ice-cold PBS and subsequently solubilized by the addition of hot (95°C) electrophoresis sample buffer [62 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol]. Cell lysates from 2 wells per treatment were pooled and immediately stored at −20°C. Protein concentrations were determined using the bicinchoninic acid assay (Pierce Biotechnology Inc., Rockford, IL; Sapan et al., 1999). Before electrophoresis, samples were diluted to equal protein concentrations (0.33 µg/µL) by the addition of electrophoresis sample buffer supplemented with 3-mercaptoethanol (5%) and bromophenol blue (0.01%). Samples were then boiled for 3 min and 20 µg of protein per sample were subjected to SDS-PAGE using 7.5% (37.5:1 acrylamide/bis acrylamide; Bio-Rad Laboratories, Hercules, CA) separating mini-gels, with 4% (37.5:1 acrylamide/bis acrylamide) stacking gels. After PAGE, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) at 4°C for 2 h at 100 V in a buffer containing 25 mM Tris, 193 mM glycine, and 15% methanol using a Bio Rad Mini-Trans-Blot electrophoretic transfer cell. Lanes with the molecular weight standards were cut from the membrane and stained with amido black. The remaining membranes were then cut just above the 45 kDa band to allow for separate β-actin and GR antibody labeling. Nonspecific antibody binding was prevented by incubating the membranes for 1 h in blocking solution [Tris buffered...
saline (198.2 mM Tris, 1.3 M NaCl, 26.8 mM KCl; pH 7.2) containing 0.1% Tween-20 (Bio-Rad Laboratories) and 5% nonfat dry milk. The membranes were then incubated overnight at 4°C in blocking solution containing 1 μg/mL of a polyclonal (rabbit) anti-GR antibody (PA1–511A, Affinity BioReagents Inc., Golden, CO). After the primary antibody incubation, membranes were washed 3 times with blocking solution and incubated for 1 h in blocking solution containing 1:1,000 (vol/vol) of an alkaline phosphatase conjugated goat anti-rabbit IgG antibody (A3683, Sigma). Membranes were washed 3 times with blocking solution and 3 times with TBS (0.1% Tween-20), and immunoreactive bands were detected upon the addition of 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium (Bio-Rad Laboratories). Utilizing a similar procedure, the appropriate membrane sections were incubated for 1 h in blocking solution containing 0.16 μg/mL of a monoclonal anti-β-actin antibody (ab6276, Ab Cam Inc., Cambridge, UK) and subsequently incubated for 1 h in blocking solution containing 1:1,000 (vol/vol) of an alkaline phosphatase conjugated goat anti-mouse IgG antibody (A3562, Sigma). Images of immunoblots were acquired with a Fluor-S MultiImager (Bio-Rad Laboratories) (A3562, Sigma). Abundance of the ~97 kDa isoform by 37 ± 9%, and reduced ~66 kDa isoform expression by 73 ± 24% in S-V cells from all depots (P < 0.001). However, the level of expression of the ~48 kDa immunoreactive band was not affected by DEX treatment (P = 0.96).

Supplementation of differentiation medium with DEX increased GPDH activity in S-V cells from all depots (P < 0.001), and there was no interaction (P = 0.99) between DEX concentration and depot of origin of the S-V cells (Figure 2). Addition of 2,500 nM DEX did not significantly increase GPDH activity over 25

**S-V Cell Differentiation**

Stromal-vascular cells from the i.m., s.c., and p.r. adipose tissue of 3 steers were seeded at a density of 4,600 cells/cm² in 35-mm-diameter cell culture wells. These second passage cells were allowed to proliferate to confluence in growth medium. Growth medium was replaced every 2 d. After reaching confluence, plates were washed twice with PBS and differentiation media were applied. Differentiation medium consisted of growth medium supplemented with 280 nM bovine insulin and 5 μL/mL of bovine serum lipids (Ex-Cyte, Serologicals Corp., Norcross, GA). Stromal-vascular cells were exposed to 0, 25, or 2,500 nM DEX for 48 h. Each treatment was applied to 2 wells of a 6-well plate, in 2 replicate plates for each of the 3 steers. After 48 h, fresh differentiation medium was provided every 2 d for 12 d. Cell differentiation was quantified biochemically by measuring glyceraldehyde-3-phosphate dehydrogenase (GPDH) enzyme activity using the procedure described by Grant et al. (2008a). Protein concentrations of soluble cell lysates were determined by bicinchoninic acid assay (Pierce Biotechnology). Intra- and interassay CV and assay sensitivity were 6.2%, <7.4%, and 20 μL/mL, respectively.

**Morphological Differentiation**

Cell differentiation was morphologically assessed by observation of cells containing lipid droplets stained by oil red O with cell nuclei counter-stained using giemsa as described by Grant et al. (2008a). Cells were visualized within 8 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). Five fields of view at 200× magnification, selected a priori, were photographed for each treatment replicate. Total cells were counted, and the percentage of oil red O positive cells and differentiated cells was determined. Differentiated cells were defined as having one or more lipid droplet(s) ≥ 10 μm in diameter.

**Statistical Analysis**

Data were analyzed using the Mixed Model procedure (PROC MIXED, SAS Institute Inc., Cary, NC). For the GPDH and immunoblot data, pooled cells from 2 wells of a 6-well plate were considered the experimental unit, whereas for morphological differentiation experimental units were individual wells. For the immunoblot data, means were calculated using the fixed effects of DEX, depot, and DEX × depot, with steer included as a random variable. To satisfy the conditions of normality and homogeneity of variance, GPDH data were log transformed. For GPDH and morphological differentiation data, least square means were calculated using the fixed effects of DEX, depot, and DEX × depot, with steer and steer × replication included as random variables. When the main effects were significant (P < 0.05), mean differences were analyzed utilizing Tukey’s multiple comparisons. Correlation analysis was performed utilizing the CORR procedure of SAS where the variables analyzed were GPDH activity, percentage of differentiated cells, and percentage of oil red O positive cells.

**RESULTS**

The immunoblot analysis of protein isolates from i.m., s.c., and p.r. bovine S-V cells revealed 3 GR immunoreactive bands of ~97, ~66, and ~48 kDa (Figure 1). No differences in the abundance of these immunoreactive bands across the 3 depots were found (P > 0.50). The relative expression of the ~97 and ~48 kDa bands was approximately 2-fold more abundant than the ~66 kDa band in these cells. Dexamethasone exposure reduced the level of the ~97 kDa isoform by 37 ± 9%, and reduced ~66 kDa isoform expression by 73 ± 24% in S-V cells from all depots (P < 0.001). However, the level of expression of the ~48 kDa immunoreactive band was not affected by DEX treatment (P = 0.96).
nM DEX levels \( (P = 0.45) \). Intrinsic differences existed in the propensity of the S-V cells from different depots to undergo adipogenic differentiation. Independent of treatment, p.r. S-V cells were the most adipogenic, s.c. S-V cells were intermediate, and i.m. S-V cells were the least adipogenic \( (P < 0.001) \).

The percentage of p.r. cells with large lipid droplets \((\geq 10 \, \mu m\)-diameter\) increased in response to DEX in a linear manner \( (P < 0.02) \), but only increased above control in s.c. cells exposed to 2,500 nM DEX \( (P = 0.002) \); Figure 3). However, there was no increase in the percentage of i.m. cells with large lipid droplets upon DEX exposure \( (P > 0.27) \). These observations reflect an adipose tissue depot \( \times \) DEX concentration interaction \( (P = 0.03) \). The percentage of cells with only small lipid droplets \(< 10 \, \mu m\)-diameter\) averaged 21.3% and
was not influenced by depot \((P = 0.18)\) or DEX exposure \((P = 0.84; \text{Figure 4})\). Photomicrographs representing average fields of view are shown in Figure 5.

Correlation analysis revealed a linear relationship \((r = 0.95, P < 0.001)\) between the percentage of morphologically differentiated cells (cells with at least 1 lipid droplet \(\geq 10\ \mu\text{M diameter}\)) from all depots and GPDH activity in the 3 depots (Figure 6). Independently, the correlation between the percentage of morphological differentiated cells and GPDH activity was i.m. S-V cells \((r = 0.97, P < 0.001)\); s.c. S-V cells \((r = 0.90, P < 0.001)\); and p.r. S-V cells \((r = 0.98, P < 0.001)\). The percentage of cells containing lipid droplets only smaller than 10 \(\mu\text{M}\) in diameter was not correlated \((P = 0.19)\) with GPDH activity in s.c. S-V cells, was negatively correlated with GPDH activity in i.m. S-V cells \((r = -0.54, P = 0.02)\), and tended to be negatively correlated with GPDH activity in p.r. S-V cells \((r = -0.44, P = 0.07)\).

**DISCUSSION**

To our knowledge, this is the first study that documents the expression of GR in cultured bovine S-V cells. We hypothesized that bovine S-V cells from different anatomical depots would express different levels of GR and that GR abundance would be positively associated with responsiveness to DEX. Immunoblot analysis of protein homogenates resulted in distinct GR immunoreactive bands of \(\sim 97, \sim 66, \text{and } \sim 48\ \text{kDa}\). In contrast to our hypothesis, GR abundance was similar among S-V cells isolated from different bovine adipose tissues. Although only one GR gene has been identified, multiple promoters, alternative splicing, alternative translation initiation, and posttranslational modifications result in production of various isoforms that fractionate into several bands upon electrophoresis (Brönnegård et al., 1995; Yudt and Cidlowski, 2002). The \(\sim 97\ \text{kDa}\) isoform is the best characterized GR\(\alpha\) isoform and presumably the primary mediator of glucocorticoid action.

We found that upon DEX exposure the \(\sim 97\ \text{kDa}\) GR immunoreactive band was downregulated, which is similar to previous results in human adipocytes (Brönnegård et al., 1995). Upon glucocorticoid binding, GR\(\alpha\) itself can inhibit GR\(\alpha\) expression by repressing the transcription of the GR gene (Nobukuni et al., 1995) and by reducing GR\(\alpha\) messenger RNA stability or translatability (Bamberger et al., 1996). The ability of DEX to downregulate the \(\sim 97\ \text{kDa}\) GR immunoreactive band detected in our study suggests that cultured bovine S-V cells express a functional GR\(\alpha\).

The function of the \(\sim 66\ \text{kDa}\) putative GR isoform is uncertain. Although the abundance of the \(\sim 66\ \text{kDa}\) immunoreactive band was not different among S-V cells isolated from different adipose depots, its abundance was also decreased by DEX exposure, suggesting a possible role in glucocorticoid signaling.

The specific identity of the \(\sim 48\ \text{kDa}\) putative isoform is not known. However, this immunoreactive band may be functionally equivalent to a splice variant named GR\(\rho\), which has been identified in myeloma tumor cells (Yudt and Cidlowski, 2002). In some cells, the level of expression of this protein may represent 10 to 50% of the total GR proteins (de Lange et al., 2001). We found the relative expression of the \(\sim 48\ \text{kDa}\) immunoreactive band was 40% of the total GR immunoreactive bands in bovine S-V cells. The GR\(\rho\) isoform is shorter than the GR\(\alpha\) (676 vs. 777 AA) and lacks a ligand binding domain (Yudt and Cidlowski, 2002), but may be involved in the upregulation of GR\(\alpha\) mediated gene expression (de Lange et al., 2001).

The responsiveness to glucocorticoids has been reported to be closely related to the abundance of GR in many tissues (Rousseau et al., 1972; Bamberger et al., 1996). Likewise, bovine S-V cells from i.m., s.c., and p.r. depots had similar GR abundance, and DEX increased GPDH activity by approximately 3 fold compared with control cells from all depots. Additionally, DEX numerically increased the percentage of cells with large lipid droplets by several fold in all depots. Because both GR abundance and responsiveness to DEX are similar among S-V cells from different anatomical depots, this implies that differences in adipogenic capacity among these cell populations may be GR independent. Alternatively, the decreased adipogenic capacity of i.m. S-V cells may suggest that cell cultures isolated from i.m. adipose tissue may contain a decreased proportion of adipogenic cells. This seems unlikely because clonal analysis of cells isolated from i.m. and s.c. adipose tissue and cultured under similar conditions as cells in this study revealed no difference in the percentages of colonies that were adipogenic, although the proportion of cells accumulating lipid within colonies were greater in s.c. cells (Grant et al., 2008b).
Figure 5. Effect of dexamethasone (DEX) concentration on morphological differentiation. Bovine stromal-vascular cells isolated from intramuscular (i.m.; a, b, c), subcutaneous (s.c.; d, e, f), and perirenal (p.r.; g, h, i) adipose tissue of 3 steers were grown to confluence and subsequently exposed to 0 (a, d, g), 25 (b, e, h), or 2,500 (c, f, i) nM DEX for 48 h and differentiation media for 10 additional days. Photomicrographs were taken 12 d after addition of treatments. Lipid droplets in cells were stained with oil red O and cell nuclei were counterstained with giemsa. Photomicrographs shown represent average fields of view. Bar = 100 µM (panel i).
Almost all mammalian cells accumulate minuscule lipid droplets that mainly serve as cholesterol ester reservoirs used in the synthesis and maintenance of membranes (Murphy and Vance, 1999; Wolins et al., 2005). Conversely, energy storage is primarily found in adipocytes that package triacylglycerides in large (10 to 100 µm in diameter) lipid droplets (Wolins et al., 2005). Other cell types have a limited capacity to store triacylglycerides and seldom accumulate large lipid droplets (Wolins et al., 2005). We used the presence of at least 1 lipid droplet ≥10 µm-diameter as a morphological indicator of preadipocyte differentiation. The percentage of cells with a lipid droplet ≥10 µm diameter was highly correlated with GPDH activity among the S-V cells of the 3 depots, which validates our criteria for determining adipogenic differentiation. In fact, the percentage of cells with only lipid droplets <10 µm diameter was not correlated with GPDH activity in s.c. S-V cells and was negatively correlated with GPDH activity in p.r. and i.m. S-V cells.

Glucocorticoid influence on preadipocyte differentiation has been well documented in various species (Ramsay et al., 1989; Xu and Björntorp, 1990) and cell lines (Gaillard et al., 1991; Smas et al., 1999). In our study, bovine p.r. S-V cells were responsive to DEX and exhibited the greatest propensity to differentiate. Interestingly, porcine p.r. preadipocytes have been reported to be unresponsive to glucocorticoid exposure (Ramsay et al., 1989), whereas s.c. S-V cells exhibited dose-dependent increases in GPDH activity in response to hydrocortisone (Ramsay et al., 1989; Suryawan et al., 1997). In muscle- and adipose-S-V cultures from neonatal pigs, DEX addition produced similar magnitudes of increase in preadipocytes and adipocytes (Hausman and Poulos, 2004). Similar to our results in the bovine, adipogenesis was much less in porcine i.m. compared with s.c. adipose-S-V cells (Hausman and Poulos, 2004).

Dexamethasone promoted similar percentage increases in adipogenic enzyme activity in i.m., s.c., and p.r. bovine S-V cells. This indicated that adipogenic differences among these cells are likely controlled by factors unrelated to GR expression or activation. Peroxisome proliferator-activated receptor γ2 is considered the master regulator of adipogenesis (Schoonjans et al., 1996; Knouff and Auwerx, 2004). Therefore, differences in adipogenic capacity between bovine S-V cells could be related to differences in PPARγ2 expression. However, the activity of PPARγ2 is regulated by the availability of lipophilic molecules that could be different between S-V cells populations. Consequently, adipogenic differences among bovine S-V cells isolated from distinct adipose depots may result from intrinsic differences in the endogenous activation of PPARγ2 among distinct bovine S-V cell populations.

Figure 6. Relationship between the glycerol-3-phosphate dehydrogenase (GPDH) specific activity and percentage of cells with a lipid droplet ≥10 µm (r = 0.95; P < 0.001) in stromal-vascular cells isolated from intramuscular (i.m.), subcutaneous (s.c.), and perirenal (p.r.) adipose tissues.

We found that cultured S-V cells isolated from bovine i.m. adipose tissue have a limited ability to accumulate lipid when compared with s.c. and p.r. S-V cells. Because GR is equally expressed in i.m., s.c., and p.r. S-V cells, and DEX increased adipogenic enzymatic activity in equal proportions in these cell populations, the observed differences in adipogenic capacity among these cell types appears to be unrelated to GR function. Additional research with the objective of comparing expression of other proteins known to be involved...
in the regulation of S-V cell differentiation may help elucidate differences in adipogenic capacity among S-V cell populations and will facilitate efforts to selectively increase i.m. lipid accretion in cattle.

LITERATURE CITED


