Effects of Testosterone, 17β-estradiol, and Progesterone on the Differentiation of Bovine Intramuscular Adipocytes

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ABSTRACT : The aim of this study was to investigate the effects of testosterone, 17β-estradiol, and progesterone on the differentiation of bovine intramuscular adipocytes (BIA). Stromal-vascular (SV) cells were obtained from *M. longissimus dorsi* of 20 months old Korean (Hanwoo) steers, and were cultured in DMEM containing 5% FBS. The proliferated BIA were induced to differentiate with 0.25 μM dexamethasone, 0.5 mM 1-methyl-3-isobutyl-xanthine and 10 μg/ml insulin. During differentiation, the cells were treated with testosterone, 17β-estradiol, and progesterone at concentrations of 10^{-10}, 10^{-9}, and 10^{-8} M, respectively, for 12 days. Regardless of its concentration, testosterone remarkably reduced lipid droplets in the cytosol of BIA. On the other hand, 17β-estradiol and progesterone increased the accumulation of lipid droplets in BIA. Testosterone significantly ($p<0.05$) decreased GPDH activities with a dose-dependent pattern. 17β-Estradiol treatment onto BIA during differentiation, however, increased GPDH activity showing the highest activity (11.3 nmol/mg protein/min) at 10^{-10} M. Treatment of BIA with progesterone also increased ($p<0.05$) GPDH activity with the highest activity (13.8 nmol/mg protein/min) at 10^{-9} M. In conclusion, the results in the current study suggest that testosterone inhibits differentiation of BIA by suppressing GPDH activity while 17β-estradiol and progesterone have adverse effects. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18. No. 11 : 1589-1593)***

Key Words : Testosterone, 17β-estradiol, Progesterone, Bovine Adipocyte, GPDH

INTRODUCTION

Castration is performed to produce high quality beef because steers have greater marbling score, tenderness, and overall flavor while they have higher backfat thickness and smaller *M. longissimus dorsi* than bulls (Bailey et al., 1966; Gregory et al., 1983). The major hormones affected by castration are steroid hormones including testosterone. Unfortunately, however, the exact role of steroid hormones in lipogenesis, especially in bovine species, is still poorly understood.

Fat development in beef cattle consists of adipogenesis and lipogenesis. Adipogenesis is a sequence of events influenced by a variety of hormones and nutritional signals during which adipose precursor cells proliferate. Insulin, insulin-like growth factor I (IGF-I), growth hormone, and glucocorticoids are important positive signals for adipocyte differentiation *in vivo* and *in vitro* (Ailhaud et al., 1994; Cornelius et al., 1994; Smas and Sul, 1995). Insulin-like growth factors (IGFs), transforming growth factor (TGF)-b, and epidermal growth factor (EGF) are involved in the growth and maintenance of muscle. Also, dehydroepiandrosterone-sulfate (DHEA-S) and cortisol are known to be related to the obesity and subcutaneous fat depth in pigs (Seong et al., 2003). 17β-Estradiol has been shown to regulate adipose tissue mass through effects on proliferation (Roncari and Van, 1978) and differentiation (Dieudonne et al., 2000).

Lipogenesis occurs when free fatty acids are incorporated into glycerol to store carbons in the form of triglycerides in adipocytes. Human studies suggest that lipogenesis is influenced by catecholamines, insulin, growth hormone, glucocorticoids, steroid hormones, thyroid hormones, and acylation-stimulating protein (Kissebah and krakower, 1994; Prins and O'Rahilly, 1997). Animal studies have shown 17β-estradiol to reduce lipogenesis (Harmosh and Hamosh, 1975; Kim and Kalkhoff, 1975) and increase lipolysis (Tomita et al., 1984; Valette et al., 1986).

The aim of this study was to investigate, under primary culture conditions, the direct effects of testosterone, 17β-estradiol, and progesterone on the differentiation of bovine adipocytes isolated from intramuscular fat depots by examining morphological changes and glycerol-3-phosphate dehydrogenase (GPDH) activity.

MATERIALS AND METHODS

Chemicals

Hank's Balanced Salt Solution (HBSS) treated with 100 U/ml penicillin, 100 μg/ml streptomycin, fungizone and amphotericin B solution uesed to transport adipose tissue from slaughter to laboratory. Dubelcco's modified Eagle's medium (DMEM; without phenol red), insulin, dexamethasone, 1-methyl-3-isobutyl-xanthine (MIX), NADH, testosterone, 17β-estradiol, progesterone, penicillin-streptomycin, and amphotericin B from Sigma (St. Louis,

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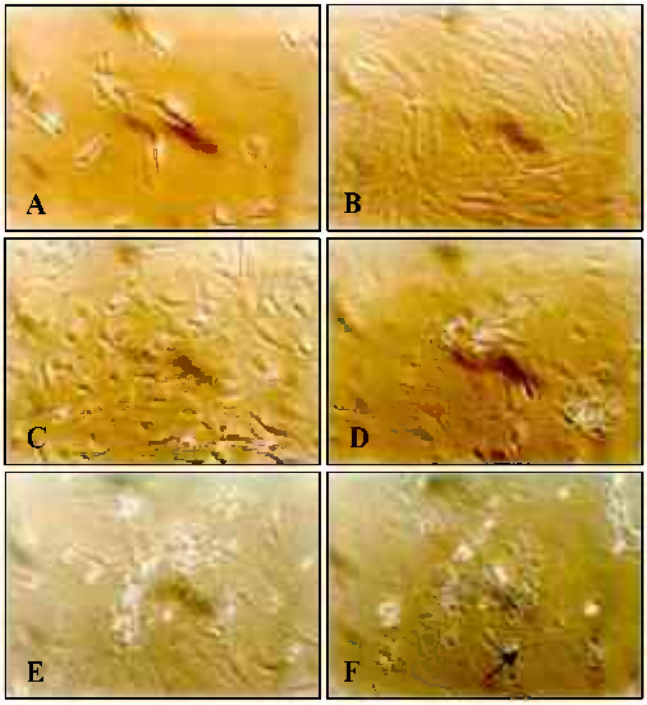

Figure 1. Morphological changes of bovine intramuscular adipocytes (BIA) during proliferation (A and B) and differentiation (C through F). Stromal-vascular (SV) cells were obtained by collagenase digestion of fat tissues taken from M. longissimus dorsi of 20 month old Korean (Hanwoo) steers, and were seeded at a density of 1×10^4 cells/ml. The cells were cultured in DMEM containing 5% FBS, penicillin-streptomycin (penicillin G sodium 10,000 unit/ml and streptomycin sulfate 10,000 μg/ml), and amphotericin B (250 ng/ml) in a humidified atmosphere with 5% CO_2 and 95% air. After reaching confluence, cells were treated with 0.25 μM dexamethasone, 0.5 mM MIX, and 10 μg/ml insulin to induce differentiation. After 48 h later, cells were cultured in DMEM containing 5% FBS and 10 μg/ml insulin only. A: day 1 of proliferation, B: day 6 of proliferation, C: day 1 of differentiation, D: day 4 of differentiation, E: day 8 of differentiation, F: day 12 of differentiation. Magnification, 400×.

MO, USA). Fetal bovine serum (FBS; charcoal/dextran treated) from HyClone (Logan, UT, USA) were used.

Preparation of bovine intramuscular adipocytes

Bovine intramuscular adipocytes (BIA) used for all subsequent assays were obtained from 20 months old Hanwoo steers. Approximately 100 g of *M. longissimus dorsi* was taken from the 13th rib area and was immediately placed in 40°C HBSS. In the laboratory hood, fatty pads in *M. longissimus dorsi* were dissected with scissors, and collagenase digestion was performed for an hour. After centrifugation, the suspension was filtered through a 250 μm nylon mesh in order to remove undigested tissues and other debris. Stromal-vascular (SV) cells in the pellets after centrifugation (1,500 rpm, 5 min) were suspended and washed with DMEM with 5% FBS and the cells were counted and seeded.

Cell culture

SV (Stromal-vascular) cells were seeded into 6-well tissue culture plates (Corning Glass Work, Corning, NY, USA) at a density of 1×10^4 cells/ml. The cells were cultured in DMEM containing 5% FBS, penicillin-streptomycin (penicillin G sodium 10,000 unit/ml and streptomycin sulfate 10,000 μg/ml), and amphotericin B (250 ng/ml) in a humidified atmosphere with 5% CO_2 and 95% air. After reaching confluence, cells were treated with 0.25 μM dexamethasone, 0.5 mM MIX, and 10 μg/ml insulin to induce differentiation. After 48 h later, cells were cultured in DMEM containing 5% FBS and 10 μg/ml insulin only. Testosterone, 17β-estradiol, and progesterone were supplemented into differentiation media to reach final concentrations of 10^{-10}, 10^{-5}, and 10^{-8} M, respectively, for 12 days. None treated BIA was used as a control group. The media were changed in 48 h intervals.

Morphology and glycerol-3-phosphate dehydrogenase (GPDH) activity

Differentiated BIA were identified by the presence of lipid droplets in cytoplasm under inverted microscope.

To analyze GPDH activities, at 12 days of differentiation, cells were washed with DMEM and lysed with homogenizing buffer containing 0.25M sucrose, 1 M $Na_2EDTA{\cdot}2H_2O$, 5 mM Tris-base, and 1 mM dithiothreitol (pH 7.4). The lysates were centrifuged at 12,500 rpm for 10 min at 4°C. The reaction mixture contained 100 mM triethanolamine-EDTA premix, 0.1 mM β-mercaptoethanol, 0.176 mM NADH, and 0.8 mM dihydroacetone phosphate. One unit of activity was expressed as the amount of enzyme causing the oxidation of 1 μmol NADH per min.

Statistical analysis

Differences between control and steroid hormone treated groups in GPDH activity were analyzed using the general linear model (GLM) of SAS (copy right 1999-2000 SAS institute Inc. Cary, NC, USA). The significances were tested with statistical probabilities at the level of $p<0.05$.

RESULTS

A representative general changes in morphology of BIA during proliferation and differentiation was shown in Figure 1. After twenty-four hours of seeding, BIA attached on the surface of culture flasks forming a fibroblast-like shape (Figure 1A). Once BIA attached onto the bottom of flask, they proliferate rapidly and reached confluence after 4 to 6 days (Figure 1B). When BIA was treated with differentiation media, it looked like they shrank for the first

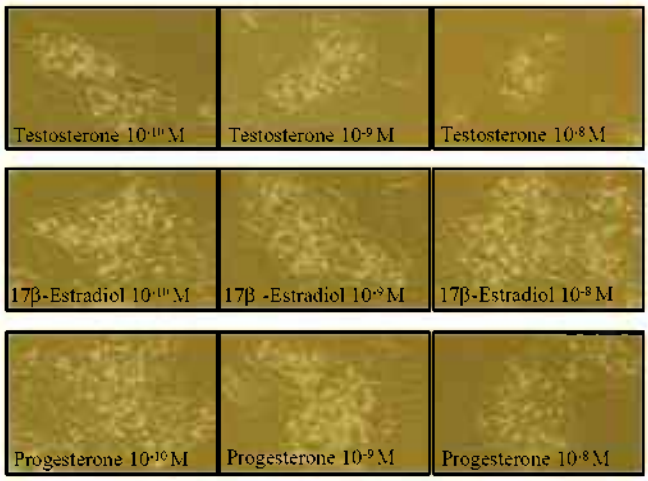

Figure 2. Morphological changes of bovine intramuscular adipocytes (BIA) treated with steroid hormones, testosterone, 17β-estradiol, and progesterone, at concentrations of 10^{-10}, 10^{-9}, and 10^{-8} M, respectively, for 12 days. For detailed culture conditions, refer to Figure 1. Whitish spots shown in each photograph represent lipid droplets in the cytosol of differentiated BIA. Magnification: 200×.

24 h (Figure 1C), but this type of morphological change was considered as a preliminary stage of the active synthesis of triglycerides (Figure 1D). At around 10 days after differentiation induction, it is easy to confirm the existence of round-shaped lipid droplets in cells all over the culture flask (Figure 1E, F). These serial changes in morphology during proliferation and differentiation of BIA made us to do subsequent steroid hormone experiments.

Regardless of its concentration, testosterone treatments onto BIA for 12 days during differentiation, significantly reduced lipid droplets in the cytosol comparing to 17β-estradiol and/or progesterone treated cells (Figure 2). The decrease in differentiation by testosterone treatments showed dose-dependent pattern, the higher testosterone concentration, the fewer the number of differentiated BIA. On the other hand, 17β-estradiol and progesterone showed strong lipogenic activities by expressing the more differentiated BIA in the same area of culture flasks than testosterone treated BIA. As the concentration of 17β-estradiol in the media increased, the degree of differentiation was enhanced showing adverse dose-dependent effects compared to testosterone. Treatment of BIA with progesterone resulted in similar changes in morphology as treat of 17β-estradiol with the highest degree of differentiation at concentration of 10^{-10} M.

Above mentioned morphological changes of BIA caused by steroid hormone treatments were also confirmed by staining of the lipid droplets with Oil-Red O (data not shown).

Treatment of BIA with testosterone significantly ($p<0.05$) decreased GPDH, an index enzyme for adipocyte

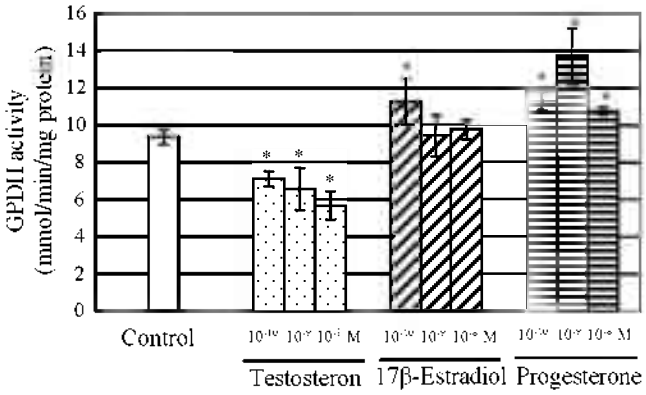

Figure 3. Glycerol-3-phosphate dehydrogenase (GPDH) activity in bovine intramuscular adipocytes (BIA) treated with steroid hormones, testosterone, 17β-estradiol and progesterone, for 12 days during differentiation. For detailed culture conditions, refer to Figure 1. Values are mean±SE (n = 5). * $p<0.05$.

differentiation, activity (Figure 3). 17β-Estradiol treatment onto BIA during differentiation, on the other hand, increased GPDH activity ($p<0.05$) showing the highest activity (11.3 nmol/mg protein/min) at 10^{-10} M. Treatment of BIA with progesterone also increased ($p<0.05$) GPDH activity with the highest activity (13.8 nmol/mg protein/min) at 10^{-9} M.

DISCUSSION

Using rat preadipocytes in primary culture and chronically exposed to steroid hormones, Dieudonne et al. (2000) reported that androgens elicit an antiadipogenic effect, whereas estrogens behave as proadipogenic hormones. They also suggested that these effects could be related to changes in insulin-like growth factor I receptor (IGF-I R) and PPAR γ2 expression. Testosterone-treated brown adipose tissue (BAT) showed fewer and smaller lipid droplets than control cells and a dose-dependent inhibition of uncoupling protein I mRNA expression while progesterone and 17β-estradiol-treated cells showed more and larger lipid droplets (Rodriguez et al., 2002). Male and female sex hormones have direct and opposite effects on the adrenergic receptor balance and lipolytic activity in BAT (Monjo et al., 2003).

The mechanisms by which testosterone regulates body composition are poorly understood. Testosterone and dihydrotestosterone regulate lineage determination in mesenchymal pluripotent cells by promoting their commitment to the myogenic lineage and inhibiting their differentiation into the adipogenic lineage through an androgen receptor-mediated pathway (Singh et al., 2003). From the study with castrated and castrated treated with testosterone rats, Xu et al. (1993) suggested that the testosterone-induced increase in lipolytic response to

catecholamines in rat white adipocytes is mediated through several events including an increased β-adrenergic receptor density, probably an increased adenylate cyclase activity and an increased protein kinase A/hormone sensitive lipase activity at the postreceptor level with apparent absence of effect on the expression of G-proteins. In 3T3-L1 adipocytes, testosterone reduced adiponectin, an adipose-specific secretory protein, secretion into the culture media and castration-induced increase in plasma adiponectin was associated with a significant improvement of insulin sensitivity (Nishizawa et al., 2002).

17β-Estradiol has been shown to regulate adipose tissue mass by increasing adipocyte number through effects on proliferation (Roncari and Van, 1978) and differentiation (Dieudonne et al., 2000). Using human subcutaneous abdominal adipocytes, Palin et al. (2003) demonstrated that the highest concentration of 17β-estradiol (10^{-7} mol/L) significantly reduced lipoprotein lipase (LPL) expression relative to control, while the lower concentrations significantly increased LPL expression relative to control.

Lacasa et al. (2001) reported that progesterone, like insulin, controls adipocyte determination and differentiation 1/sterol regulatory element-binding protein 1c gene expression which provides a potential mechanism for the lipogenic actions of progesterone on adipose tissue. In ovariectomized and adreanlectomized rats, 17-estradiol plus progesterone tended to increase lipoprotein lipase in the parametrial but not retroperitoneal fat depot, but no effects were found of estrogen or progesterone alone (Rebuffe-Scrive, 1987).

In conclusion, the results in the current study suggest that testosterone inhibits differentiation of BIA by suppressing GPDH activity while 17β-estradiol and progesterone have adverse effects.

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