Melengestrol acetate enhances adipogenic gene expression in cultured muscle-derived cells

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ABSTRACT: Melengestrol acetate (MGA) has been used in the United States for nearly 40 yr to enhance feedlot heifer performance, yet unequivocal studies have not been conducted to discover the mechanism of action. Our hypothesis was that MGA may induce various populations of muscle-derived cells (MDC) to the adipogenic pathway in both a bovine and murine cell culture model. To determine this, MDC were digested from the semimembranosus muscle tissue of six 14-mo-old crossbred steers. The addition of insulin, oleic acid, and ciglitizone (IOC) with cultured bovine MDC resulted in morphological differences compared with control cultures. Multilocular lipid droplets stained with Oil Red O were seen not only in single MDC, but also in fused myotubes. An increase \( P < 0.05 \) in relative PPAR\( \gamma \) messenger RNA (mRNA) levels was measured in MDC incubated with IOC. However, myogenin mRNA levels in MDC incubated with IOC were repressed \( P < 0.05 \) compared with nontreated MDC.

Cultures of MDC treated with 10 \( \mu M \) insulin, 10 \( \mu M \) oleic acid, 10 \( \mu M \) ciglitizone, 10 nM estradiol-17\( \beta \) (E\( \beta \)), and 10 nM MGA resulted in cultures with highly distributed lipid droplets not only in single cells, but also in the multinucleated myotubes. Relative C/EBP\( \beta \) and PPAR\( \gamma \) mRNA levels in total RNA isolated from MDC treated with MGA increased \( P < 0.05 \) compared with control cultures. Estradiol treatment had no effect \( P > 0.05 \) on these mRNA levels. The addition of both E\( \beta \) and MGA to MDC increased \( P < 0.05 \) C/EBP\( \beta \) mRNA levels and tended \( P = 0.06 \) to increase the PPAR\( \gamma \) mRNA level. There was no difference \( P > 0.10 \) in relative myogenin mRNA among the control, E\( \beta \), and MGA treatments. Relative C/EBP\( \beta \), PPAR\( \gamma \), and myogenin mRNA levels were investigated in murine C2C12, C3H 10T 1/2, and 3T3-L1 cells. Treatment of cultures with 10 nM MGA increased C/EBP\( \beta \) levels \( P < 0.05 \) in C2C12 myoblasts and tended \( P = 0.08 \) to increase C/EBP\( \beta \) levels in 3T3-L1 preadipocytes. These data indicate that populations of cells are present in postnatal skeletal muscle that, under the appropriate stimuli in a culture model, express adipogenic genes and accumulate lipids. In addition, the synthetic progestogen MGA appeared to upregulate the genes necessary for conversion to the adipogenic pathway.

Key words: melengestrol acetate, muscle-derived cell, transdifferentiation

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INTRODUCTION

The adipogenic transcriptional factors PPAR\( \gamma \) and C/EBP are highly involved not only in the differentiation of adipocytes (Umek et al., 1991), but also in the transdifferentiation of myoblasts to adipocytes (Hu et al., 1995). Increased expression of C/EBP restricted mitotic growth of preadipocytes and promoted differentiation of preadipocytes (Umek et al., 1991). A specific C/EBP antibody and nucleic acid hybridization analysis was conducted previously on liver, lung, adipose, intestine, and placenta tissues (Birkenmeier et al., 1989), but not on the muscle tissue. Several in vitro studies have indicated a role for PPAR\( \gamma \) and C/EBP\( \alpha \) in inducing adipocyte numbers in the bovine and porcine skeletal muscle (Torii et al., 1998; Pouloos and Hausman, 2006). These 2 adipogenic transcriptional factors increased under the transdifferentiation process of myoblasts when exposed to thiazolidinediones (TZD). However, these factors are not expressed when myoblasts are treated under optimal myogenic differentiation conditions (Hu et al., 1995; Kook et al., 2006; Singh et al., 2007). Therefore, PPAR\( \gamma \) and C/EBP can be used as specific markers for indicating myoblast transdifferentiation.

Thiazolidinediones and long-chain fatty acids (LCFA) are commonly used for activating differentiation of preadipocytes. Grimaldi et al. (1997) reported that treatment of C2C12 cells with TZD or LCFA reduced myogenic gene expression but induced adipogenic gene expression.

Our objectives were to compare the effects of melengestrol acetate (MGA) on messenger RNA (mRNA)
levels for key adipogenic genes in both bovine primary culture and mouse immortalized cell line. Thus, we tried to determine whether they inhibit or stimulate the conversion of muscle cells to adipose tissue cells, respectively.

**MATERIALS AND METHODS**

All experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee.

**Bovine Muscle-Derived Cell Isolation**

Bovine muscle-derived cells (MDC) were isolated as described previously (Johnson et al., 1998). Muscle-derived cells were isolated from six 14-mo-old cross-bred steers. Using an aseptic technique, we collected approximately 500 g of semimembranosus muscle tissue and transported it to the cell culture laboratory. Cell isolation procedures were done in the sterile environment of a culture hood.

The muscle pieces were dissected from connective tissue, blood vessels, and adipose tissue and were then passed through a sterile meat grinder. The ground muscle was incubated with 0.1% pronase (Calbiochem, La Jolla, CA) in Earl's Balanced Salt Solution (Sigma, St. Louis, MO) for 1 h at 37°C, with mixing every 10 min. After incubation, the mixture was centrifuged at 1,500 × g for 4 min at room temperature. The resulting pellet was suspended in PBS (Invitrogen, Grand Island, NY; 140 mM NaCl, 1 mM KH₂PO₄, 3 mM KCl, 8 mM Na₂HPO₄) and the suspension was centrifuged at 500 × g for 10 min at room temperature. The supernatant was collected and centrifuged at 1,500 × g for 10 min at room temperature to pellet the mononucleated cells. The PBS wash and differential centrifugations were repeated 2 more times. The resulting mononucleated cell preparation was suspended in cold (4°C) Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen) and 10% (vol/vol) dimethylsulfoxide (Sigma). Cells were frozen in liquid nitrogen for use in future MDC studies.

**Bovine MDC Culture**

Cell culture plates (2 cm²) were precoated with 1:10 (vol/vol) diluted Matrigel (reduced growth factor form; BD Biosciences, Bedford, MA). After a 48-h incubation in DMEM containing 10% FBS at 37°C and 5% CO₂, the cultures were rinsed 3 times with serum-free DMEM, and DMEM containing 10% FBS was added. When cultures approached approximately 80% confluence, phenol red-free DMEM (Invitrogen, Grand Island, NY) containing 3% horse serum was added alone (control) or with insulin (10 μM), oleic acid (100 μM), and ciglitizone (10 μM; IOC; Sigma). Cultures that were induced to transdifferentiate were maintained with IOC and the indicated additions of estradiol-17β (E₂), trenbolone-17β (TBOH), or MGA for 168 h. The culture medium was replaced once after 72 h.

**Morphological Analysis**

Oil Red O and hematoxylin staining was conducted to confirm accumulated lipid droplets in the differentiated bovine MDC. After aspirating off the media, the cells were fixed with 10% neutral buffered formalin (30 mM NaH₂PO₄·H₂O, 54.6 mM NaHPO₄, 40% formalin) for 10 min at room temperature and then washed in flowing water. The fixed cells were treated with 100% propylene glycol for 2 min and washed in water. After washing, the cells were stained with 0.5% Oil Red O solution (2:3 vol/vol mixture of 0.5% Oil Red O in isopropl alcohol and distilled water) in darkness for 20 min and washed with 60% propylene glycol for 1 min. The cells were stained by Harris’ hematoxylin in darkness for 3 min and mounted in glycerol. Transdifferentiated bovine satellite cells were identified by the presence of Oil Red O-stained lipid droplets in the cytosol and myotubes.

**RNA Isolation and cDNA Synthesis**

At 168 h, total RNA was isolated using the tri-reagent (Sigma). The RNA was quantified by absorbance at 260 nm. Quality of the RNA was determined by agarose gel electrophoresis and by optical density measurements. Subsequently, 1 μg of total RNA was reverse transcribed to produce first-strand cDNA, using TaqMan reverse transcription reagents and MultiScribe (Applied Biosystems, Foster City, CA) according to the protocol provided by the manufacturer.

**Real-Time Quantitative PCR from Cultured Bovine MDC, C2C12, C3H 10T 1/2, and 3T3-L1 Cells**

Real-time quantitative PCR was used to measure the quantity of C/EBPβ, PPARγ, stearoyl-CoA desaturase (SCD), and myogenin gene expression relative to the quantity of 18S ribosomal RNA (rRNA) in total RNA isolated from cultured bovine MDC and cells of mouse origin (see below). Measurement of the relative quantity of cDNA was performed using TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of the appropriate forward and reverse primers, and TaqMan detection probe, Assays-on-Demand Gene Expression Products (Applied Biosystems), and 1 μM cDNA mixture. The forward and reverse primers used for C/EBPβ, PPARγ, SCD, and, myogenin are shown in Table 1. Commercially available eukaryotic 18S rRNA and Assays-on-Demand primer and probes were used as an endogenous control (Applied Biosystems; GeneBank accession number X03205) and for gene expression of the mouse-origin cell line. The ABI Prism 7000 detection system and thermal cycling variables (Applied Biosystems) recommended by the manufacturer (50 cycles of
Table 1. Forward and reverse primers for real-time PCR for adipogenic gene messenger RNA

<table>
<thead>
<tr>
<th>Marker gene: gene no.</th>
<th>Primer</th>
</tr>
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<tbody>
<tr>
<td>C/EBPβ: NM_176788</td>
<td>Forward: 5'-CCA GAA GGA GGT GGA GCA ACT G-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TCG GCC AGC GTC TTT AAC-3'</td>
</tr>
<tr>
<td>PPARγ: NM_181024</td>
<td>Forward: 5'-ATC TGC TGC AAG CCT TGG A-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TGAGT ACG GCC TGT GCA AAG A-3'</td>
</tr>
<tr>
<td>Stearoyl-CoA desaturase: AB 075020</td>
<td>Forward: 5'-TGC CCA CCA CAA GTT TTC AG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCC AAC CCA CGT GAG AGA AG-3'</td>
</tr>
<tr>
<td>Myogenin: BC 118336</td>
<td>Forward: 5'-AGG TGA ATG AAG CCT TCG A-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCA GCC GCT CTA TGT ACT GGA T-3'</td>
</tr>
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Results

Bovine MDC

The cultures in these experiments were treated with insulin, oleic acid, and IOC to induce transdifferentiation in bovine MDC cultures. Addition of IOC to MDC produced morphological differences compared with the control (Figure 1A and 1B). Multilocular lipid droplets stained with Oil Red O were located not only in single bovine MDC, but also in fused multinucleated myotubes. Real-time reverse transcription-PCR analysis was used to analyze adipogenic and myogenic gene expression during MDC differentiation. Relative PPARγ mRNA levels in total RNA isolated from MDC incubated with IOC were increased (P < 0.05) compared with nontreated MDC. However, mRNA levels of myogenin (a transcription factor inducing the myogenic process) were repressed (P < 0.05) in the MDC incubated with IOC compared with nontreated MDC (Figure 1C).

Muscle-derived cell cultures maintained with IOC were used as a positive control (Figure 2A). Treating cultures with 10 nM E2 (Figure 2B) and 20 nM TBOH (Figure 2C) induced (morphologically) a small distribution of lipid droplets in MDC as compared with the control cultures. However, cultures treated with 10 nM MGA had a larger distribution of lipid droplets, not only in a single cell, but also in the multinucleated myotubes relative to the control cultures (Figure 2D). Relative C/EBPβ and PPARγ mRNA levels in total RNA isolated from MDC treated with MGA were increased (P < 0.05) compared with the control culture (Figure 3). Although no difference was observed compared with the control, the addition of both E2 and MGA to MDC increased the C/EBPβ mRNA level (P < 0.05) and tended (P = 0.06) to increase the PPARγ mRNA level (Figure 3). There was no difference in relative myogenin mRNA levels in the control, E2, and MGA treatments (P > 0.10). The SCD gene did not differ among treatments (P > 0.10). This may be because the SCD gene was typically expressed in the late phase of adipogenic differentiation in MDC. Time course differences may cause variation in SCD gene expression because in vitro studies usually take a shorter time to perform than in vivo ones.

Statistical Analysis

Data were analyzed as a completely randomized design using the MIXED model (SAS Inst. Inc., Cary, NC). Differences between the control and treatments were determined using the LSD procedure. Unless otherwise noted, means were considered different at P < 0.05, and trends were considered at P < 0.10.

Murine-Origin Cells

Insulin, oleic acid, and cigitizone were added to C2C12 mouse-origin myoblasts to determine their effects on the transdifferentiation process, as measured by morphological and genetic differences. Addition of
IOC to cultured C2C12 cells resulted in morphological differences compared with control cultures (Figure 4A and 4B). Multilocular lipid droplets stained with Oil Red O were located not only in single C2C12 cells, but also in fused multinucleated myotubes. Real-time reverse transcription-PCR analysis was used to ana-
lyze adipogenic and myogenic processes during C2C12 cell differentiation. Relative PPARγ mRNA levels in total RNA isolated from C2C12 cells incubated with IOC did not differ ($P = 0.19$) from nontreated C2C12 myoblasts. However, relative myogenin mRNA levels in total RNA isolated from C2C12 myoblasts incubated with IOC were decreased ($P < 0.05$) compared with nontreated C2C12 myoblasts (Figure 4C). The mesenchymal precursor cells, C3H 10T 1/2, followed a pattern similar to that of mouse-origin myoblasts. Insulin, oleic acid, and ciglitizone addition to C3H 10T 1/2 mesenchymal precursor cells increased ($P < 0.05$) relative PPARγ mRNA levels, as measured by real-time PCR (Figure 5A). However, there was no difference in C/EBPβ, PPARγ, and myogenin mRNA levels in 3T3-L1 mouse preadipocytes during the final differentiation ($P > 0.10$; Figure 5B).

The C2C12 myoblast cells maintained with IOC were used as a positive control (Figure 6A). Treating cultures with 10 nM MGA increased ($P < 0.05$) relative C/EBPβ mRNA levels in total RNA isolated from C2C12 myoblasts (Figure 6C). Melengestrol acetate-treated 3T3-L1 cells tended to increase relative C/EBPβ mRNA levels compared with the control; however, there was no effect of MGA treatment in the 10T 1/2 mesenchymal precursor cells (Figure 6B and 6C).

![Figure 3](image3.png)

**Figure 3.** Relative C/EBPβ, PPARγ, stearoyl-CoA desaturase (SCD), and myogenin messenger RNA (mRNA) levels in total RNA isolated from transdifferentiation-inducing bovine muscle-derived cell (MDC) cultures with IOC (insulin [10 μM], oleic acid [100 μM], and ciglitizone [Sigma, St. Louis, MO; 10 μM]), 10 nM estradiol-17β (E2), 10 nM melengestrol acetate (MGA), and 10 nM MGA + 10 nM E2 (M + E). Cultures were established as described in the culture methods, and at 48 h, the culture received 10% fetal bovine serum (Invitrogen, Grand Island, NY) and Dulbecco’s modified Eagle’s medium (Invitrogen) containing IOC with E2, MGA, and E2 + MGA, and without treatment. After a change of medium at 72 h, total RNA was isolated and relative mRNA was determined in 168-h cultures. Bars are means ± SE relative to the control. *Means differ from the control ($P < 0.05$). **Means differ from the control ($P = 0.06$). Values are the means of 6 culture dishes delivered from 6 animals.

![Figure 4](image4.png)

**Figure 4.** Mouse-origin myoblast C2C12 cells stained with Oil Red O and hematoxylin. Control (Cont; A). Insulin (10 μM), oleic acid (100 μM), and ciglitizone (Sigma, St. Louis, MO; 10 μM; IOC; B). Relative PPARγ and myogenin messenger RNA (mRNA) levels in total RNA isolated from transdifferentiation-inducing bovine muscle-derived cell cultures (C). Bars are means ± SE relative to the control obtained from triplicate cultures. *Means differ from the control ($P < 0.05$). Color version available in the online PDF.
Melengestrol acetate, an orally active synthetic progestin, has been fed to enhance heifer performance in the United States for nearly 40 yr. The endocrine mechanism of MGA in heifers is via inhibition of the preovulatory surge of LH (Imwalle et al., 2002). Several heifer studies have indicated that treatment with MGA along with E2 and trenbolone acetate reduces feed efficiency and ribeye area but increases carcass fatness and backfat thickness (Hutcheson et al. 1993; Mader and Lechtenberg, 2000; Macken et al., 2003). Treatment with MGA reduces bovine satellite cell proliferation, and this may explain reductions in carcass muscling (Sissom et al., 2006). These data also indicated that MGA enhances IGF concentrations in both mouse and bovine satellite cells. Several implant studies have demonstrated that anabolic implants not only enhance proliferation of bovine satellite cells (Johnson et al., 1998), but also induce the expression of IGF-I in bovine satellite cells (Kamanga-Sollo et al., 2004). However, it has been reported that trenbolone acetate and E2 implants have a negative impact on marbling score (Roebber et al., 2000) and fatty acid composition (Duckett et al., 1999). A study by Smith et al. (2007) confirmed that anabolic implants affected not only fat thickness, but also marbling scores. Interestingly, the numbers of intramuscular adipocytes per gram in implanted cattle were greater than those in nonimplanted cattle. These data indicated that anabolic implants affected the proliferation of multipotent precursor cells in the LM (sixth to ninth rib) area compared with nonimplanted cattle. Our data indicated that cells treated with MGA, alone or combination with E2, had similar or greater mRNA levels of adipogenic transcription factors. The MGA treatment may attenuate E2 effects on mRNA levels of key adipogenic factors in the bovine MDC model. In vitro transdifferentiation of MDC was induced with 3% horse serum combined with IOC. Long-chain fatty acids and TZD have been used as key regulators for adipose differentiation or lipid homeostasis (Grimaldi et al., 1997; Kliewer et al., 1997). Thiazolidinediones are well defined as specific ligands that bind to the nuclear transcriptional factor PPARγ. Grimaldi et al. (1997) reported that TZD- and LCFA-treated myoblasts had less formation of multinucleated myotubes and less expression of myogenic genes in the mouse cell model. Treatment with unsaturated LCFA, such as linoleic, linolenic, or arachidonic acid, has been reported to promote lipid accumulation processes in the mouse myoblast (Grimaldi et al., 1997). However, few studies have shown the functional activation of oleic acid, even though oleic acid constitutes the largest percentage of fatty acids in the bovine adipose tissue (Chung et al., 2006). The previous report also indicated that the addition of MUFA was significantly positively correlated not only with intramuscular fat content, but also with melting point in high-marbling cattle. Thus, we hypothesized that oleic acid may be a critical factor in enhancing the adipogenic pathway in bovine MDC.
Investigation of a suitable transdifferentiation mixture for bovine MDC showed the oleic acid-containing mixture to be more effective in the accumulation of lipid droplets in bovine myoblasts (data not shown). We also demonstrated the adipogenic and myogenic transcription factors to be highly involved in transdifferentiation from myoblast to adipoblast. In the current study, relative myogenin, C/EBP, PPAR, and SCD mRNA levels were used to determine myogenic or adipogenic development of MDC. The relative expression level of myogenin, used as a muscle-specific marker, was low in MDC and C2C12 myoblast cultures, but was not different in 10T 1/2 cells. These current findings are similar to results of the mouse myoblast transdifferentiation study by Grimaldi et al. (1997). Those authors reported that LCFA and TZD inhibited not only the formation of multinucleated myotubes, but also the expression of myogenic genes. However, LCFA and TZD enhanced the expression of adipogenic markers such as adipocyte lipid-binding protein and fatty acid transporter. The transcriptional factor C/EBPβ was expressed at an early stage of adipogenic differentiation (Wu et al., 1995). The adipocyte-specific transcriptional factor PPARγ is highly expressed during differentiation of preadipocytes (Tontonoz et al., 1994) and is highly involved in the transdifferentiation process of MDC (Grimaldi et al., 1997).

Recently, research has demonstrated that LCFA may specifically affect the cell-surface protein GPR40 (Briscoe et al., 2003). In addition, GPR41 or GPR43 was specifically affected by short-chain fatty acids such as acetate or propionate, and may act as cell-surface signaling ligands (Brown et al., 2003; Hong et al., 2005). Thus, fatty acids, such as oleic acid, can regulate signaling cascades at the cell-surface level, and this cascade controls the adipogenic or myogenic pathways of the myoblast in the muscle.

Our research indicates that cells are present in postnatal skeletal muscle that, under the appropriate stimuli in a culture model, will differentiate into adipocytes.
as determined by lipid accumulation. These data lead us to believe we could possibly “turn on” this process in the finishing animal during the feeding period, thereby enhancing the marbling process. In addition, anabolic steroids such as E2 and TBOH appeared to attenuate this conversion. However, the synthetic progestin MGA appeared to upregulate genes necessary for the transdifferentiation of MDC to adipocytes. Interestingly, anecdotal industry reports suggest that feeding MGA to finishing heifers may improve marbling scores. However, mouse-origin immortalized cell lines responded differentially to MGA, E2, and TBOH. One possible reason is the different fatty acid composition between the different species. Bovine fat has increased MUFA relative to fat in other species. This may cause each cell type to have a different sensitivity to different fatty acids. More research is needed to determine if these changes observed in vitro will occur in the finishing animal.

LITERATURE CITED


