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Epidermal growth factor receptor is required for estradiol-stimulated bovine satellite cell proliferation

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ABSTRACT

The objective of this study was to assess the role of the epidermal growth factor receptor (EGFR) in estradiol-17β (E_2)-stimulated proliferation of cultured bovine satellite cells (BSCs). Treatment of BSC cultures with AG1478 (a specific inhibitor of EGFR tyrosine kinase activity) suppresses E_2-stimulated BSC proliferation ($P < 0.05$). In addition, E_2-stimulated proliferation is completely suppressed ($P < 0.05$) in BSCs in which EGFR expression is silenced by treatment with EGFR small interfering RNA (siRNA). These results indicate that EGFR is required for E_2 to stimulate proliferation in BSC cultures. Both AG1478 treatment and EGFR silencing also suppress proliferation stimulated by LR3-IGF-1 (an IGF1 analogue that binds normally to the insulin-like growth factor receptor (IGFR)-1 but has little or no affinity for IGF binding proteins) in cultured BSCs ($P < 0.05$). Even though *EGFR* siRNA treatment has no effect on IGFR-1β mRNA expression in cultured BSCs, IGFR-1β protein level is substantially reduced in BSCs treated with *EGFR* siRNA. These data suggest that EGFR silencing results in post-transcriptional modifications that result in decreased IGFR-1β protein levels. Although it is clear that functional EGFR is necessary for E_2-stimulated proliferation of BSCs, the role of EGFR is not clear. Transactivation of EGFR may directly stimulate proliferation, or EGFR may function to maintain the level of IGFR-1β which is necessary for E_2-stimulated proliferation. It also is possible that the role of EGFR in E_2-stimulated BSC proliferation may involve both of these mechanisms.

1. Introduction

Estradiol (E_2) and combined E_2 and trenbolone acetate (TBA; a testosterone analogue) implants are routinely used to enhance muscle growth in feedlot steers [1,2]. In addition, E_2 replacement therapy reportedly enhances both muscle mass and strength in postmenopausal women [3–5]. Although the stimulatory effects of E_2 on muscle growth are well established, the mechanism(s) involved in these effects is not well understood.

In feedlot steers, E_2 implants stimulate muscle *IGF-1* mRNA expression [6] and when used in conjunction with TBA result in significantly increased muscle satellite cell number [7]. Although the effects of E_2 implants on alone on satellite cell number have not been assessed in vivo, E_2 treatment stimulates proliferation in cultured bovine satellite cells (BSCs) [8]. The ability of E_2 to stimulate satellite cell proliferation is particularly significant because satellite cells are the source of nuclei needed to support postnatal muscle fiber hypertrophy and are thus crucial in determining the rate and extent of muscle growth [9,10]. Consequently, in an effort to identify the mechanism(s) by which E_2 stimulates muscle growth, we have initially focused on elucidating the receptors involved in the E_2-stimulated increase in satellite cell proliferation.

In bovine skeletal muscle [6] and in BSCs cultured in the presence of 10% fetal bovine serum (FBS) [8,11], E_2 treatment increases *IGF-1* mRNA level. Estradiol stimulation of IGF-1 mRNA expression in BSC cultures is mediated

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through G protein-coupled estrogen receptor (GPER)-1 [8,11]. Because IGF-1 stimulates satellite cell proliferation [12], it is likely that this E_2-stimulated increase in IGF-1 expression is at least partially responsible for the increased satellite cell number observed in muscles of E_2-treated steers. However, we have established that under culture conditions in which E_2 treatment does not increase expression of either IGF-1 or IGF-1 receptor (IGFR-1), E_2 treatment still stimulates proliferation in cultured BSCs [8]. These data indicate that, in addition to increasing IGF-1 expression by binding to GPER-1, E_2 may stimulate rate of proliferation through interaction with other receptors. This hypothesis is confirmed by studies showing that silencing either estrogen receptor (ER)-α (ESR1) or IGFR-1 results in suppression of E_2-stimulated proliferation of cultured BSCs [13], indicating that both ESR1 and IGFR-1 are involved in the mechanism of E_2-stimulated satellite cell proliferation. Studies in breast cancer cells have shown that binding of E_2 to GPER-1 results in activation of matrix metalloproteinase (MMP2) and MMP9, resulting in proteolytic release of heparin-binding epidermal growth factor-like growth factor (hb-EGF) from the cell surface. Released hb-EGF binds to and activates the epidermal growth factor receptor (EGFR), resulting in increased proliferation [14–19]. Consequently, determine whether the EGFR receptor also is involved in E_2-stimulated proliferation of BSCs, we have examined the effects of inhibiting or silencing EGFR on E_2-stimulated proliferation of cultured BSCs.

2. Materials and methods

2.1. BSC isolation

Satellite cell isolation was done as described previously [20–22]. Steers were sacrificed by bolting, followed by exsanguination. With the use of sterile techniques, approximately 500 g of the semimembranosus muscle was dissected out and transported to the cell culture laboratory. Subsequent procedures were conducted in a sterile field under a tissue culture hood. After removal of connective tissue the muscle was passed through a sterile meat grinder. The ground muscle was incubated with 0.1% Pronase in Earle balanced salt solution for 1 h at 37°C with frequent mixing. After incubation the mixture was centrifuged at 1,500× *g* for 4 min, the pellet was suspended in PBS (140 m*M* NaCl, 1 m*M* KH_2PO_4, 3 m*M* KCl, 8 m*M* Na_2HPO_4, pH 7.4), and the suspension was centrifuged at 500× *g* for 10 min. The resultant supernatant was centrifuged at 1,500× *g* for 10 min to pellet the mononucleated cells. The PBS wash and differential centrifugation were repeated 2 more times. The resulting mononucleated cell preparation was suspended in cold (4°C) Dulbecco modified Eagle medium (DMEM) that contained containing 10% FBS and 10% (vol/vol) dimethylsulfoxide (DMSO) and frozen. Cells were stored frozen in liquid nitrogen for use in future studies. Clonal analysis of satellite cell cultures established from these preparations showed that between 80% and 90% of the cells were able to establish colonies that contained fusion, indicating that between 80% and 90% of the cells in the preparations were myogenic. Phenol red was not present in any of the culture media used in this study.

2.2. ^{3}H-Thymidine incorporation assays

Incorporation of ^{3}H-thymidine was measured as previously described [7,8,23]. Before plating of cells, individual wells were treated with 125 μL/cm^2 DMEM that contained 0.95 mg/mL reduced growth factor Matrigel (Becton Dickson & Co, Franklin Lakes, NJ, USA). Cells were plated in DMEM that contained 10% FBS at a density empirically established so that cultures were approximately 50% confluent when proliferation rate was determined. For assays that assessed the effect of the EGFR tyrosine kinase inhibitor, AG1478, plating media was removed at 72 h and cultures were fed with test media that consisted of DMEM plus 1% IGF binding protein (IGFBP)-3–free swine serum (SSS) (SSS was prepared by passing sera obtained from 6-wk-old male pigs castrated within 1 wk of birth through an IGFBP-3 immunoaffinity column [23]) or DMEM/1% SSS plus appropriate additions of LR3-IGF-1/mL (GroPep Bioreagents Pty Ltd, Thebarton, SA, Australia; catalog BU100) (an IGF-1 analogue that binds normally to the IGFR-1 but has little or no affinity for IGFBPs [24]), hb-EGF (R&D Systems, Minneapolis, MN, USA; catalog 259-HE), E_2 (Steraloids, Inc, Newport, RI, USA; catalog E0950-000), or the EGFR tyrosine kinase inhibitor AG1478 [25] (EMD Bioscience/EMD Millipore USA, Billerica, MA, USA; catalog121767) [26]. Estradiol was initially dissolved in 95% ethanol, and all cultures including the control contained a final concentration of 0.1% ethanol. AG1478 was dissolved in cell culture-grade DMSO, and in experiments that used AG1478 all cultures including the control contained a final concentration of 0.1% DMSO and 0.1% ethanol. The concentrations of ethanol and DMSO added to the cultures did not affect proliferation rate. In experiments that assessed the effect of AG1478, cultures were preincubated for 30 min with AG1478 before addition of LR3-IGF-1, hb-EGF, or E_2 to the culture. For knockdown studies cultures were treated with control or EGFR small interfering RNA (siRNA) as described in Section 2.3 before addition of test media that consisted of DMEM/1% SSS or DMEM/1% SSS containing indicated levels of E_2, LR3-IGF-1, hb-EGF, or FBS at 96 h. At 93 h for AG1478 studies and 117 h for EGFR-silencing studies, ^{3}H-thymidine was added to the media on the cells at a concentration of 1 mCi/mL and incubated for 3 h. After the ^{3}H-thymidine incubation (96 h for AG1478 studies and 120 h for EGFR knockdown studies), test media was removed, and cells were washed 3 times with cold DMEM. Cells were fixed with 1 mL of cold 5% trichloroacetic acid (TCA) overnight at 4°C. Unincorporated ^{3}H-thymidine was removed when 5% TCA was removed, and cells were washed twice with cold 5% TCA. Incorporation of ^{3}H-thymidine into cellular DNA was determined by dissolving the cell material in 0.5 *M* sodium hydroxide for 30 min. Cell material was then placed in scintillation vials and counted in a scintillation counter. All experiments contained triplicate measurements.

2.3. siRNA knockdown of EGFR

Silencing of EGFR expression in BSC cultures was achieved with custom-designed siRNA specific for bovine *EGFR*