Dexamethasone Enhances Insulin-like Growth Factor-I Effects on Skeletal Muscle Cell Proliferation

Role of Specific Intracellular Signaling Pathways

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Abstract

IGF-I stimulation of cell proliferation and c-Fos expression in skeletal muscle cells is markedly enhanced by dexamethasone. The effect of dexamethasone is not mediated by changes in IGF-binding proteins, as evidenced by similar effects of dexamethasone on the actions of insulin, PDGF-BB, and the IGF-I analogue long R^3IGF-I. Dexamethasone also does not alter autocrine IGF-II secretion by muscle cells. To investigate the mechanism of the augmentation of IGF-I action, the effects of dexamethasone on intracellular IGF-I signaling pathways were determined. In dexamethasone-treated cells, the levels of IGF-I receptor tyrosine phosphorylation and receptor-associated phosphatidylinositol 3-kinase activity were increased. Dexamethasone-treated cells also showed increased and prolonged tyrosine phosphorylation of the Shc proteins. In contrast, dexamethasone decreased both tyrosine phosphorylation and expression of insulin receptor substrate 1 (IRS-1) and IRS-1–associated phosphatidylinositol 3-kinase activity. Thus, distinct signaling pathways activated by the IGF-I receptor in skeletal muscle cells are differentially regulated by dexamethasone. Potentiation of IGF-I action correlates with increased IGF-I receptor-associated phosphatidylinositol 3-kinase activity and tyrosine phosphorylation of Shc, but appears to be independent of activation of the IRS-1/phosphatidylinositol 3-kinase signaling pathway. (*J. Clin. Invest.* 1995. 96:1473–1483.) Key words: glucocorticoids • insulin-like growth factor-I receptor • insulin receptor substrate-1 • phosphatidylinositol 3-kinase • Shc

Introduction

IGF-I is a polypeptide hormone with a broad range of metabolic actions that include potent stimulation of cell growth and differentiation in multiple cell types (1). In human fibroblasts as well as in other cells, the growth-stimulatory effects of IGF-I have reportedly been markedly enhanced by glucocorticoids (2–4). Glucocorticoids have also been shown to enhance cell proliferation induced by other growth factors, including insulin, EGF, fibroblast growth factor, and PDGF, all of which act via cell surface receptors containing a ligand-activated tyrosine kinase (2, 3, 5–7). The mechanism by which glucocorticoids enhance the action of IGF-I and other growth factors has not been elucidated. The objective of this study was to investigate the effects of the glucocorticoid dexamethasone on mitogenic responses to IGF-I in cultured skeletal muscle cells and to define the mechanism of glucocorticoid-induced augmentation of IGF-I action.

The biologic effects of IGF-I are mediated by specific cell-surface receptors (8–10). IGF-I binding to the extracellular α subunits activates the tyrosine kinase intrinsic to the cytoplasmic portion of the IGF-I receptor, leading to autophosphorylation of specific tyrosine residues in the receptor β subunit (11, 12). One early molecular event that may link the receptor kinase to the biologic actions of IGF-I is tyrosine phosphorylation of insulin receptor substrate-1 (IRS- 1).[1] IRS-1 is a 135-kD cytoplasmic protein that migrates anomalously as a 175–185 kD tyrosine phosphoprotein on SDS–polyacrylamide gels (13–15). IRS-1 undergoes tyrosine phosphorylation in response to insulin and IGF-I stimulation of intact cells (16, 17) and represents a direct substrate for the activated insulin and IGF-I receptor kinases (18). Current evidence suggests that IRS-1 acts as a multisite "docking" protein by binding to downstream signal-transducing molecules. Phosphorylation of multiple tyrosine residues found in distinct IRS-1 amino acid motifs results in the association of IRS-1 with the src homology 2 (SH2) domains of other cytoplasmic signaling proteins, including phosphatidylinositol (PI) 3-kinase, Syp, GRB-2, and Nck (reviewed in reference 19). Two specific YMXM motifs mediate IRS-1 association with the 85-kD regulatory subunit of PI 3-kinase, leading to activation of the catalytic subunit of this enzyme (20, 21). Activated PI 3-kinase is then capable of phosphorylating the D-3 position of various phosphatidylinositols, forming PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3, which are believed to regulate certain aspects of cellular growth and metabolism (22). The IGF-I receptor kinase directly phosphorylates YMXM tyrosine residues in the IRS-1 protein and thus increases the amount of PI 3-kinase activity bound to IRS-1 (18, 23)

Pelicci et al. (24) identified a novel gene, *Shc,* which encodes three protein species of 46, 52, and 66 kD, respectively, termed $p46^{shc}$, $p52^{shc}$, and $p66^{shc}$. Two of the Shc proteins, $p46^{sch}$ and $p52^{sch}$, result from the presence of two translation-initiation sites on the same mRNA, whereas $p66^{sch}$ is likely to

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Received for publication 27 October 1994 and accepted in revised form 18 May 1995.

J. Clin. Invest.

0021-9738/95/09/1473/11 $2.00
Volume 96, September 1995, 1473–1483

1. *Abbreviations used in this paper:* αIGF-I R, anti–IGF-I receptor; αIRS-1, anti–IRS-1; DCBS, donor calf bovine serum; IRS-1, insulin receptor substrate-1; PI, phosphatidylinositol; αPY, anti-phosphotyrosine; SH2, src homology 2.

result from a distinct transcript (24). Evidence that the Shc proteins participate in insulin signaling has come from recent studies showing that all three protein species are rapidly phosphorylated in response to insulin stimulation in fibroblasts expressing elevated levels of human insulin receptors (25–27). Tyrosine phosphorylated Shc can then activate cellular signaling by binding the SH2 domain of the GRB-2 protein (25). GRB-2 forms a stable complex with Sos1, a guanine nucleotide releasing factor for $p21^{ras}$ (28), and subsequent activation of $p21^{ras}$ leads to the consecutive stimulation of Raf 1 kinase, MEK, and MAP kinase (reviewed in reference 29). Recent studies in NIH 3T3 cells overexpressing an Shc cDNA have demonstrated that the Shc proteins are also tyrosine phosphorylated in response to IGF-I and may be direct substrates for both the IGF-I and insulin receptor kinases (30). At present, Shc and IRS-1 represent the only known intracellular tyrosine phosphoproteins that are stimulated by IGF-I in mammalian cells and act as coupling molecules for specific downstream signaling pathways.

In this study, the effects of dexamethasone on IGF-I–induced mitogenesis were investigated in cultured skeletal muscle cells, because skeletal muscle represents an important site of action for the IGFs. Specific high-affinity IGF-I receptors are known to be expressed in skeletal muscle (31), and both IGF-I and IGF-II peptides are capable of stimulating muscle growth and differentiation (31, 32). In addition to IGF-I receptors, the presence of mRNA and peptide for both IGF-I and IGF-II has been documented in cloned muscle cell lines and normal fetal and adult muscle tissues (33), suggesting that a significant component of IGF-I action in muscle cells may be mediated through autocrine as well as paracrine mechanisms. Muscle cells also secrete IGF binding proteins, which may modify the biologic activity of the IGFs (34). The L6 skeletal muscle cells used in this study express abundant IGF-I receptors (31) and are markedly responsive to the mitogenic actions of IGF-I (31, 35). In addition, L6 cells express receptors for insulin and PDGF (31, 36), which makes it possible to study responses to multiple tyrosine kinase–associated growth factors.

To identify glucocorticoid-regulated steps that may explain the increased proliferative effects of IGF-I, we have investigated both extracellular and intracellular components of the mechanism of action of this growth factor. Here we report that dexamethasone markedly potentiates the mitogenic actions of IGF-I on L6 skeletal muscle cells and that this response is associated with increased activity of specific intracellular IGF-I signaling pathways.

Methods

Reagents. Culture medium and donor calf bovine serum were obtained from GIBCO BRL (Grand Island, NY). Recombinant human insulin and recombinant human IGF-I were gifts from Eli Lilly Co. (Indianapolis, IN). PDGF-BB was purchased from Genzyme Co. (Boston, MA). LongR^{3}IGF-I was obtained from GroPep Ltd. (Adelaide, Australia). Protein A-sepharose was obtained from Pierce Chemical Co. (Rockford, IL). Reagents for SDS-PAGE and the Bradford protein assay were purchased from Bio-Rad Laboratories (Richmond, CA). ^{125}I-labeled protein A was obtained from ICN Biomedicals Inc. (Irvine, CA). [^{3}H]-Thymidine, [α^{32}P]dCTP, and [γ-^{32}P]ATP were obtained from New England Nuclear Co. (Boston, MA). Dexamethasone and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Polyclonal anti-phosphotyrosine (αPY) antibody was prepared in rabbits by injection of phosphotyrosine polymerized by 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide with alanine, threonine, and keyhole limpet hemocyanin (KLH) as previously described (37). Non-immunoglobulin proteins were extracted with 6% octanoic acid precipitation, and αPY antibody was then purified by affinity chromatography on a phosphotyramine-sepharose column. Polyclonal anti-peptide antibodies were generated against two peptide sequences corresponding to regions of the IGF-I receptor (αIGF-I R) and IRS-1 (αIRS-1). These included the synthetic peptide ala-ser-phe-asp-glu-arg-gln-pro-tyr-ala-his, which contains the COOH-terminal amino acid sequence between residues 1308 and 1318 of the β subunit of the human IGF-I receptor reported by Ullrich et al. (9), and the peptide tyr-ala-ser-ile-asn-phe-gln-lys-gln-pro-glu-asp-arg-gln, corresponding to the last 14 amino acids in the COOH-terminal region of rat IRS-1 (15). These antibodies were prepared and purified on specific peptide affinity columns as previously described (38). Polyclonal anti-human Shc (αShc) antibodies were purchased from Transduction Laboratories (Lexington, KY). Polyclonal anti-myosin heavy chain antibodies were purchased from Sigma Chemical Co. Polyclonal anti-PDGF β receptor (αPDGF R) antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal antibodies to a COOH-terminal region of the rat insulin receptor β subunit (αIns R) were generously provided by Dr. C. R. Kahn (Boston, MA).

Cell culture. The line of L6 rat skeletal muscle cells has been described previously (39). Stock cells were stored frozen in liquid N_2, and a fresh vial of cells was thawed each 4 to 6 wk and maintained by passage in MEM supplemented with 10% donor calf bovine serum (DCBS), 2 mM glutamine, and nonessential amino acids in a 5% CO_2 atmosphere at 37°C. Cells were plated in MEM containing 10% DCBS in 35-mm wells for thymidine incorporation and proliferation experiments, or otherwise in 150-mm culture dishes. On the fourth day after plating, when the cells were 50–60% confluent, the medium was replaced with serum-free MEM containing 0.5% BSA, and cells were incubated for 72 h in the presence or absence of 1 μM dexamethasone (concentration confirmed by absorbance at 242 nm using a molar absorbance coefficient of $1.5 \times 10^4 M^{-1}\ cm^{-1}$). All experiments were carried out with undifferentiated myoblasts.

DNA synthesis. L6 myoblasts were plated at a density of 20×10^4 cells/well in 35-mm multiwell plates in MEM containing 10% DCBS and grown for 4 d to ~ 50% confluency. After removing the medium and washing with serum-free MEM, the monolayer was incubated for an additional 72 h in MEM containing 0.5% BSA in the absence or presence of 1 μM dexamethasone. During the last 16 h of this incubation, various concentrations of IGF-I or other hormones were added to the quiescent cells. The cells were then incubated for 1 h in fresh MEM containing 0.5% BSA, 25 mM Hepes (pH 7.6), and 1 mCi/ml [^{3}H]-thymidine. The medium was removed, and the monolayer was washed twice with ice-cold PBS and twice with ice-cold 10% trichloroacetic acid. The remaining cell material was solubilized in 2 ml 0.1 N NaOH at 37°C for 30 min, and the amount of [^{3}H] was quantitated by liquid scintillation counting. For each condition, experiments were carried out in triplicate.

Northern blotting. L6 cell monolayers were rinsed twice with ice-cold PBS and solubilized directly on the tissue culture plates with 4 M guanidinium isothiocyanate, containing 0.1 M Tris-HCl (pH 7.5), 0.66% *N*-lauryl-sarcosine, and 5% β-mercaptoethanol. Total cellular RNA was isolated by low-temperature 4 M guanidinium isothiocyanate-phenol-chloroform extraction, followed by cold ethanol precipitation (40), and quantitated by spectrophotometry at 260 nm. In all samples, intact ribosomal RNA bands were visualized after electrophoresis. Northern blots were performed after separation of RNA by electrophoresis through 1.2% agarose gels containing 2.2 M formaldehyde, overnight transfer to Genescreen Plus membranes (Stratagene, La Jolla, CA) by capillary action, and cross-linking of RNA to the membrane by UV irradiation. Prehybridization was performed at 42°C for 4 h in a solution containing 1 M NaCl, 1% SDS, 10% dextran sulfate, 20 mg/ml denatured salmon sperm DNA, and 50% formamide. Hybridization was for 16 h at 42°C after addition of the radiolabeled probe to the same solution. At the end of the hybridization period, the filters were washed twice for 20 min at 22°C in 2 × SSC with 1% SDS. This was followed by

one wash for 20 min at 50°C in 2× SSC with 0.1% SDS and two additional washes for 15 min at 60°C in 0.1 × SSC with 0.1% SDS. After autoradiography using Kodak XAR films (Eastern Kodak, Rochester, NY) with intensifying screens at −70°C for 12–48 h, scanning optical densitometry (Molecular Dynamics, Sunnyvale, CA) was performed to quantify the relative amounts of mRNA species.

The c-Fos probe was a 2.1-kb EcoRI–EcoRI restriction fragment of the coding sequence of the mouse *Fos* gene, and the c-Jun probe was a 2.1-kb PstI–EcoRI restriction fragment of the coding sequence of the human *Jun* gene (provided by Dr. B. Spiegelman, Boston, MA). After denaturation, the cDNA probes were labeled with [α^{32}P]dCTP to ~ 10^9 dpm/mg using a random hexamer priming kit (Multiprime; Amersham Corp. Arlington Heights, IL). Unincorporated label was separated with a Elutip-d column (Schleicher & Schuell, Inc., Keene, NH), and the specifically labeled DNA fragment was eluted using a high salt concentrated solution.

IGF-I and IGF-II radioimmunoassay. Media were collected after various periods of incubation with L6 cells in the absence or presence of dexamethasone, clarified by low speed centrifugation, and frozen at −70°C until assayed. For IGF-I and IGF-II determinations, binding proteins were removed by extraction with 0.8 M formic acid, 0.05% Tween-20, and 70% acetone according to the method of Bowsher et al. (41). A polyclonal antibody to IGF-I that recognizes rat IGF-I was used in the IGF-I RIA (42). For IGF-II determinations, a monoclonal antibody against human IGF-II that recognizes rat IGF-II with equal efficiency was used (Amano International Enzyme Co., Troy, VA). Assay sensitivities were 18 ng/ml for IGF-I and 3 ng/ml for IGF-II.

Phosphatidylinositol 3-kinase activity. After 72 h in serum-free medium without or with dexamethasone, cells were incubated in the absence or presence of IGF-I (100 nM) for the indicated times, washed once with ice-cold PBS containing 100 mM sodium orthovanadate, and twice with 20 mM Tris-HCl (pH 7.6) containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 mM sodium orthovanadate (buffer A). The cells were lysed in buffer A (1 ml/150-mm dish) containing 1% NP-40, 10% glycerol, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM EDTA, 2 mM PMSF, 4 mg/ml leupeptin, and 2 mM sodium orthovanadate (lysis buffer). Insoluble material was removed by centrifugation at 12,000 *g* for 10 min at 4°C, the protein concentration in the resulting supernatant was determined with the Bradford dye binding assay (43), and the final protein concentration was adjusted to 2 mg/ml with lysis buffer.

Immunoprecipitation was carried out by incubation of the cell lysate overnight at 4°C with αIGF-I R or αIRS-1 antibodies, as indicated. Immunocomplexes were adsorbed with protein A-sepharose beads for 2 h at 4°C, and the pelleted beads were washed successively in PBS containing 1% NP-40 and 100 mM sodium orthovanadate (three times), 100 mM Tris-HCl (pH 7.6) containing 500 mM LiCl and 100 mM sodium orthovanadate (three times), and 10 mM Tris-HCl (pH 7.6) containing 100 mM NaCl, 1 mM EDTA, and 100 mM sodium orthovanadate (twice). For determination of PI 3-kinase activity in the immunoprecipitate, the sepharose beads were resuspended in 50 ml 10 mM Tris-HCl (pH 7.6) containing 100 mM NaCl, 1 mM EDTA, and 100 mM sodium orthovanadate, and then combined with 10 ml 100 mM $MgCl_2$ and 10 ml 2 mg/ml phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) sonicated in 10 mM Tris-HCl (pH 7.6) containing 1 mM EGTA. The PI 3-kinase reaction was started by the addition of 10 ml 10 mM ATP containing 30 mCi [γ-^{32}P]ATP. After 10 min at 22°C with constant vortexing, the reaction was stopped by the addition of 20 ml 8 N HCl and 160 ml chloroform/methanol (1:1, vol/vol). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate precoated with 1% potassium oxalate (Merck, Darmstadt, Germany). The plates were developed in chloroform/methanol/water/ammonia (60:47:11.3:2, vol/vol), dried, and visualized by autoradiography. The PI-3 product was identified by its comigration with a PI-4 standard and quantitated by scanning densitometry (Molecular Dynamics).

Immunoblotting. For identification and quantitation of specific proteins by immunoblotting, cell lysates were prepared and immunoprecipitations carried out as described above for the determination of PI 3-kinase activity. Immunoprecipitation was performed with αIGF-I R, αIns R, αPDGF R, αIRS-1, or αShc antibodies, as indicated. The pellets were boiled in Laemmli buffer with 100 mM DTT for 4 min, and the proteins were separated by SDS-PAGE and transferred onto nitrocellulose sheets (Schleicher & Schuell) using a transfer buffer containing 10 mM Tris, 192 mM glycine, 20% vol/vol methanol, and 0.02% SDS at 80 V for 2.5 h. To reduce nonspecific antibody binding, the sheets were soaked in 10 mM Tris (pH 7.8), 0.9% NaCl, and 0.01% sodium azide (rinse buffer) containing 5% BSA and 0.05% NP-40 for 2 h at 37°C. The sheets were then incubated with αPY, αIGF-I R, αIRS-1, or αShc antibodies as appropriate in rinse buffer containing 5% BSA for 10–16 h at 4°C. After washing twice with rinse buffer plus 0.05% NP-40, and once with rinse buffer plus 0.1% Tween-20, the sheets were incubated with ^{125}I-labeled protein A (1 μCi/ml) for 1 h at 22°C, washed again with rinse buffer as described above, air-dried, and subjected to autoradiography with Kodak X-Omat film in the presence of an intensifying screen at −80°C for 6–48 h. Specific protein bands on autoradiographic images were quantified by scanning optical densitometry (Molecular Dynamics), and the data were expressed using arbitrary units normalized to control values within each gel.

Statistical analysis. Data are presented as the mean±SE. Statistical analysis was performed by paired and unpaired Student's *t* tests as appropriate.

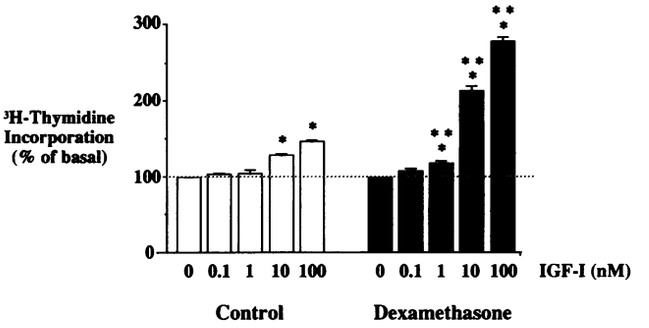

Figure 1. Effects of dexamethasone on IGF-I stimulation of DNA synthesis in undifferentiated L6 skeletal muscle cells. DNA synthesis was measured by determining the rate of [^{3}H]thymidine incorporation into DNA after cells were incubated in serum-free medium in the presence or absence of 1 μM dexamethasone for 72 h and stimulated with the indicated concentrations of human recombinant IGF-I for the last 16 h. Data are derived from five independent experiments. * $P < 0.05$ vs. 0 IGF-I, paired *t* test; ** $P < 0.05$ vs. controls, paired *t* test.

Results

DNA synthesis. IGF-I effects on DNA synthesis in undifferentiated L6 skeletal muscle cells were determined by measuring the rate of [^{3}H]thymidine incorporation into DNA. As shown in Fig. 1 (*left*), the addition of IGF-I to L6 cells grown in serum-free medium for 72 h resulted in a modest but significant dose-dependent increase in DNA synthesis. When cells were preincubated for 72 h in serum-free medium containing 1 μM dexamethasone (Fig. 1, *right*), the stimulation of DNA synthesis by IGF-I was markedly augmented. At a maximally effective IGF-I concentration (100 nM), pretreatment with dexamethasone resulted in an approximately four-fold increase in DNA synthesis compared with control cells. The effect of dexamethasone was not evident in the absence of IGF-I, and the augmentation of IGF-I–stimulated DNA synthesis was maximal after 48–72 h of incubation with dexamethasone (data not shown).

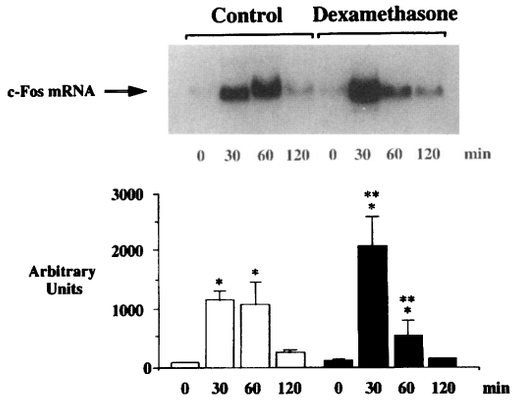

Figure 2. Effects of dexamethasone on IGF-I stimulation of c-Fos expression. Undifferentiated L6 cells were incubated in serum-free medium in the presence or absence of 1 μM dexamethasone for 72 h and then stimulated with 100 nM IGF-I for the indicated times. For each condition, total cell RNA was extracted and an equal amount (10 mg) analyzed by Northern blotting as described in Methods. A representative autoradiogram is shown on the top, and the results from quantitation of multiple experiments (n = 5) on the bottom. * $P < 0.05$ vs. 0 IGF-I; ** $P < 0.05$ vs. controls, paired t test.

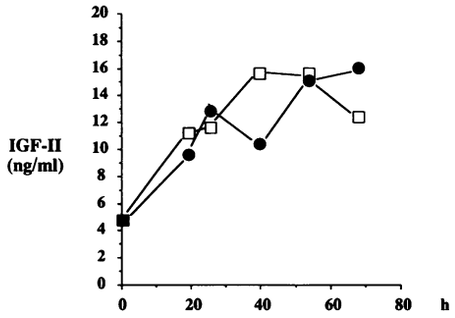

Figure 3. IGF-II concentrations in the medium after various periods of incubation of L6 cells in serum-free medium in the presence or absence of 1 μM dexamethasone. At the indicated times, IGF-II in the culture medium was determined by radioimmunoassay as described in Methods. The open squares indicate control cells, and the solid circles indicate dexamethasone-treated cells. Values represent the mean of independent determinations from two individual culture dishes.

These results are similar to the reported effects of glucocorticoids on IGF-I–stimulated DNA synthesis in human fibroblasts (2, 3).

To assess whether treatment with dexamethasone and/or IGF-I altered the state of differentiation of L6 myoblasts, the amount of myosin heavy chain in the cell lysates was measured by immunoblotting with anti-myosin heavy chain antibodies. The induction of myosin heavy chain expression has been used as a marker of L6 cell differentiation because this protein increases severalfold when myoblasts fully differentiate into myotubes (44). In cells treated with dexamethasone for 72 h, myosin heavy chain content was decreased to 45.6±1.1% of control cells, $P < 0.05$. By contrast, IGF-I treatment for 16 h induced only a modest 20% decrease in myosin heavy chain content in control cells and had no effects on myosin heavy chain content in dexamethasone-treated cells. These results indicate that L6 myoblasts treated with dexamethasone were moderately less differentiated compared with control myoblasts.

c-Fos and c-Jun expression. The stimulation of proto-oncogene expression by a number of growth factors has been implicated as an intermediary event involved in the regulation of cell growth and proliferation. To investigate whether the effects of dexamethasone in potentiating IGF-I–stimulated cell proliferation were associated with a change in IGF-I stimulation of proto-oncogene expression, c-Fos and c-Jun steady-state mRNA levels were studied in undifferentiated L6 cells by Northern analysis. As shown in Fig. 2, treatment of L6 cells with 100 nM IGF-I markedly stimulated c-Fos mRNA expression. This response peaked after 30–60 min and then returned to nearly basal levels 120 min after addition of IGF-I. In cells treated with dexamethasone for 72 h, IGF-I stimulation induced a higher peak level of c-Fos mRNA at 30 min compared with control cells. The increase in c-Fos mRNA induced by IGF-I was more transient in dexamethasone-treated cells than in control cells, with c-Fos mRNA levels markedly decreasing after 60 min of stimulation with IGF-I. Thus, the augmentation of DNA synthesis in L6 myoblasts by dexamethasone was associated with a higher and earlier peak of c-Fos mRNA induction by IGF-I.

IGF-I also induced a 3.5-fold increase in the expression of c-Jun mRNA in undifferentiated L6 cells in the absence of dexamethasone. This response peaked after 30–60 min of stimulation and returned to near basal levels after 120 min. In contrast to the effect on c-Fos, however, pretreatment of cells with dexamethasone affected neither the magnitude nor the time course of c-Jun induction by IGF-I (data not shown).

Dexamethasone effects on IGF-II production and responses to other growth factors. Because the autocrine production of IGFs and IGF binding proteins may have important effects on muscle cell proliferation and/or differentiation, we considered the possibility that dexamethasone could augment IGF-I–stimulated mitogenesis in L6 cells via changes in the production of IGF-II and/or IGF binding proteins. To investigate the effect of dexamethasone on IGF-II production by L6 cells, monolayers were incubated in the presence or absence of dexamethasone (1 μM) for various times, and IGF-II concentration in the medium was determined by radioimmunoassay (Fig. 3). As previously reported by other investigators (45), IGF-II was produced by L6 cells and its concentration in the culture medium progressively increased during the 68 h of study. Treatment with dexamethasone did not alter IGF-II concentration in the culture medium at any time point. IGF-I was also measured in the culture medium under the same experimental conditions using a specific radioimmunoassay and found to be consistently below the detection limit of the assay (data not shown). These results rule out the possibility that the augmentation of IGF-I responsiveness by dexamethasone could occur through increased synthesis of IGF-II or IGF-I.

To determine whether dexamethasone increased IGF-I–stimulated cell growth by changing the levels or activities of IGF binding proteins, the effects of dexamethasone on DNA synthesis stimulated by IGF-I and the analogue longR^{3}IGF-I were compared. LongR^{3}IGF-I binds to the IGF-I receptor and stimulates biologic responses but has a very low affinity for IGF binding proteins (46). As shown in Fig. 4, dexamethasone increased the effects of IGF-I and longR^{3}IGF-I on L6 myoblast DNA synthesis to a similar extent. Dexamethasone also increased the stimulation of DNA synthesis by insulin and the BB form of platelet-derived growth factor (PDGF-BB) (Fig.

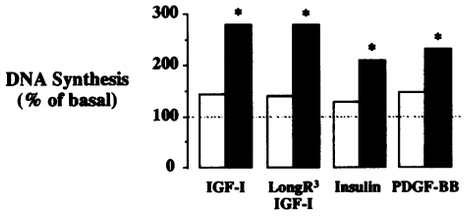

Figure 4. Effects of dexamethasone on growth factor stimulation of DNA synthesis in undifferentiated L6 skeletal muscle cells. Cells were incubated in serum-free medium in the presence or absence of 1 μM dexamethasone for 72 h. DNA synthesis stimulated by 10 nM IGF-I, 10 nM longR^{3}IGF-I, 10 nM insulin, or 10 ng/ml PDGF-BB was measured by determining the rates of [^{3}H]thymidine incorporation into DNA, as described in Methods. Open and cross-hatched bars represent growth factor–stimulated DNA synthesis in control and dexamethasone-treated cells, respectively, from three independent experiments. * $P < 0.05$ vs. control cells.

4). These latter two hormones act through their own specific cell surface tyrosine kinase receptors (31, 36) and do not interact with IGF binding proteins. These data indicate that the increase in IGF-I responsiveness in dexamethasone-treated cells cannot be explained by changes in the levels of IGF binding proteins. The potentiation of the mitogenic actions of insulin and PDGF-BB further suggests that the mechanism of action of dexamethasone is likely to involve changes in intracellular signaling steps that are shared by multiple growth factor receptors containing a tyrosine kinase domain.

Expression and tyrosine phosphorylation of IGF-I receptors and IRS-1. Autophosphorylation of IGF-I receptors on tyrosine residues and activation of the receptor-associated tyrosine kinase represent the initial postbinding events that mediate the intracellular actions of IGF-I. To investigate the influence of dexamethasone on these signaling steps, L6 cells were stimulated with a maximally effective dose of IGF-I (100 nM) and the pattern of tyrosine phosphoproteins was analyzed by immunoblotting with αPY antibody (Fig. 5). In both control and dexamethasone-treated L6 myoblasts, IGF-I stimulated the tyrosine phosphorylation of two major proteins of 105 kD and 175 kD. Sequential immunoprecipitation with an antibody specific to the IGF-I receptor β subunit, followed by blotting with αPY antibody, identified the 105-kD phosphoprotein as the IGF-I receptor β subunit. Immunoprecipitation with IRS-1 antibody identified the 175-kD phosphoprotein as the insulin receptor/IGF-I receptor substrate IRS-1.

To determine the effects of dexamethasone on IGF-I receptors, L6 myoblasts were incubated in the presence or absence of 1 μM dexamethasone for 72 h, stimulated for various time periods with 100 nM IGF-I (0–180 min), and the cell lysates immunoblotted with phosphotyrosine antibody. The results from densitometric analysis of multiple experiments are shown in Fig. 6 *(left)*. The total amount of basal and stimulated IGF-I receptor tyrosine phosphorylation was increased modestly but significantly in dexamethasone-treated cells. IGF-I receptor content determined by immunoblotting with αIGF-I R antibody was increased in dexamethasone-treated cells by ~ 70% (Fig. 6, *right*).

To determine the effects of dexamethasone on the cellular content of insulin and PDGF β receptors, total cell lysates from control and dexamethasone-treated L6 myoblasts were subjected to immunoprecipitation and immunoblotting with αIns

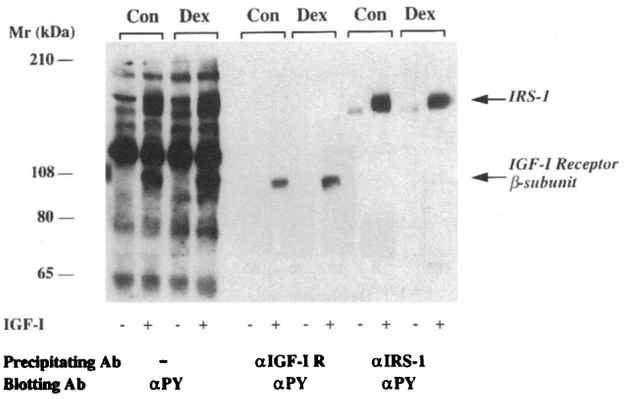

Figure 5. Identity of IGF-I–stimulated tyrosine phosphoproteins in undifferentiated L6 skeletal muscle cells. Cells were incubated in the presence or absence of 1 μM dexamethasone for 72 h and then stimulated with 100 nM IGF-I for 10 min or left unstimulated. Total cell lysates from control (*Con*) and dexamethasone-treated (*Dex*) L6 cells were obtained and either left untreated or immunoprecipitated with an antibody specific to the COOH-terminal region of the IGF-I receptor β subunit (αIGF-I R) or an antibody specific to the COOH-terminal region of rat IRS-1 (αIRS-1). Total cell lysates, αIGF-I R, and αIRS-1 immunoprecipitates then were resolved by SDS-PAGE on 7% acrylamide gels and blotted with phosphotyrosine antibody (αPY) and ^{125}I-labeled protein A, as described in Methods.

R and αPDGF R antibodies, respectively. In dexamethasone-treated cells, insulin receptor content was significantly increased compared with control cells (161.9±9.7% of control cells, $P < 0.05$). By contrast, although the mean PDGF β receptor content was slightly higher in dexamethasone-treated than in control cells, this difference did not approach statistical significance (132.6±25.0% of control cells, $P = 0.24$).

The effects of dexamethasone on IGF-I stimulation of IRS-1 tyrosine phosphorylation are shown in Fig. 7 *(left)*. Maximal IRS-1 tyrosine phosphorylation was significantly decreased in dexamethasone-treated compared with control cells 10 min after stimulation with IGF-I. Dexamethasone resulted in a more marked 65% decrease in the total cell content of IRS-1. Thus,

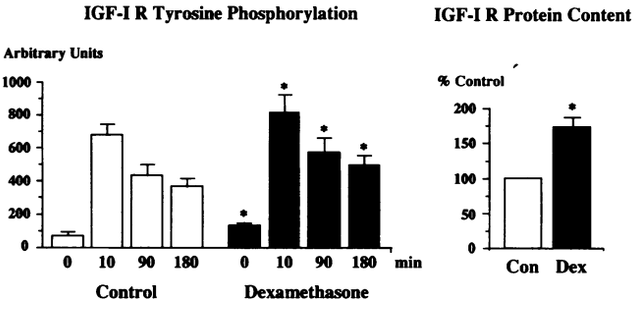

Figure 6. Tyrosine phosphorylation and total content of IGF-I receptors (IGF-I R) in control (*open bars*) and dexamethasone-treated (*cross-hatched bars*) L6 myoblasts. Cell monolayers were incubated in the presence or absence of 1 μM dexamethasone for 72 h and then stimulated with 100 nM IGF-I for the indicated times. Tyrosine phosphorylation and total content of IGF-I receptors were determined by immunoblotting with αPY and αIGF-I R antibodies, respectively, as described in Methods. Data represent five independent experiments. * $P < 0.05$ vs. control cells, paired t test.

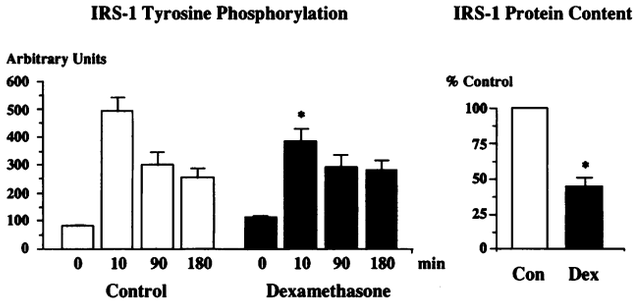

Figure 7. Tyrosine phosphorylation and total content of IRS-1 in control (*open bars*) and dexamethasone-treated (*cross-hatched bars*) L6 myoblasts. Cells were incubated in the presence or absence of 1 μM dexamethasone for 72 h and then stimulated with 100 nM IGF-I for the indicated times. Tyrosine phosphorylation and total content of IRS-1 were determined by immunoblotting with αPY and αIRS-1 antibodies, respectively, as described in Methods. Data represent five independent experiments. * $P < 0.05$ vs. control cells, paired t test.

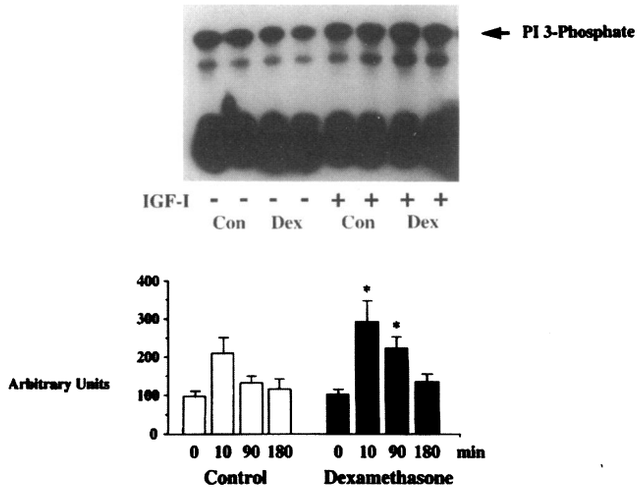

Figure 8. Effects of dexamethasone on PI 3-kinase activity associated with IGF-I receptors. After incubation in the presence or absence of 1 μM dexamethasone for 72 h, intact L6 cells were stimulated with 100 nM IGF-I. PI 3-kinase activity was measured in anti-IGF-I receptor antibody immunoprecipitates by in vitro assay with [^{32}P]ATP and PI, followed by analysis of phosphorylated lipids by thin layer chromatography, as described in Methods. For IGF-I receptor-associated PI 3-kinase activity, TLC plates were subjected to autoradiography for 24–36 h. The autoradiogram in the upper part of the figure shows a representative experiment performed with IGF-I stimulation of intact cells for 10 min. The bar graph in the lower part shows the quantitation of multiple experiments performed with IGF-I stimulation (100 nM) for different periods of time. Open bars and solid bars represent control and dexamethasone-treated cells, respectively, from three independent experiments. * $P < 0.05$ vs. control cells, paired t test.

the effects of dexamethasone on the expression and tyrosine phosphorylation of IRS-1 were distinct from the effects on IGF-I receptors.

IGF-I receptor- and IRS-1–associated PI 3-kinase activity. The effects of IGF-I on cultured cells include the activation of PI 3-kinase after association of phosphorylated IRS-1 with the p85 subunit of this enzyme (15, 18, 23). IGF-I also increases the amount of PI 3-kinase activity recoverable in αIGF-I R immunoprecipitates, although this receptor-associated activity is thought to represent a minor fraction compared with IRS-1–associated PI 3-kinase activity (47). Because dexamethasone treatment of L6 cells resulted in opposite effects on tyrosine phosphorylation and expression of IRS-1 and IGF-I receptors, we investigated its effects on both IRS-1 and IGF-I receptor-associated PI 3-kinase activity. For this purpose, NP-40 lysates prepared from L6 cells stimulated with 100 nM IGF-I for various times were subjected to immunoprecipitation with either αIRS-1 or αIGF-I R antibodies. The immunoprecipitates were then reconstituted and assayed for PI 3-kinase activity with PI substrate in the presence of [^{32}P]ATP. Previously published work using HPLC analysis of the deacylated lipid products has shown that the lipids produced in PI 3-kinase assays of αIRS-1 and αIGF-I R immunoprecipitates display a HPLC elution profile identical to that of authentic deacylated PI 3-monophosphate (15, 47).

Stimulation of L6 cells with 100 nM IGF-I increased IGF-I receptor-associated PI 3-kinase activity by approximately two-fold (Fig. 8). Autocrine secretion of IGF-II from L6 myoblasts may account, at least in part, for the observed amount of basal receptor-associated PI 3-kinase activity. In the basal state, dexamethasone-treated cells had a similar amount of PI 3-kinase activity associated with IGF-I receptors in comparison with control cells. However, the amount of IGF-I–stimulated PI 3-kinase activity that co-immunoprecipitated with IGF-I receptors was increased in dexamethasone-treated cells compared with control cells.

The amount of PI 3-kinase activity present in αIRS-1 immunoprecipitates was several-fold greater than in αIGF-I R immunoprecipitates, which is in agreement with previously reported studies (47). In control cells, IGF-I stimulation induced a rapid and marked (42-fold) increase in the amount of PI 3-kinase activity associated with IRS-1 (Fig. 9). In cells treated with 1 μM dexamethasone for 72 h, there was no difference in the basal level of PI 3-kinase activity associated with IRS-1, but IGF-1 stimulation induced only a 23-fold increase in IRS-1–associated PI 3-kinase activity. Thus, in comparison with control cells, dexamethasone reduced by 45% the levels of IGF-I–stimulated PI 3-kinase activity associated with IRS-1.

The observed findings of decreased IRS-1–associated PI 3-kinase activity and increased IGF-I receptor-associated PI 3-kinase activity correlate with the decrease in both the expression and tyrosine phosphorylation of IRS-1 and with the increase in the expression and tyrosine phosphorylation of IGF-I receptors, respectively. Because IRS-1 tyrosine phosphorylation and IRS-1–associated PI 3-kinase activity are decreased by dexamethasone under conditions where IGF-I–stimulated mitogenesis is augmented, the IRS-1/PI 3-kinase pathway is unlikely to be involved in the potentiation of IGF-I action by glucocorticoids.

Shc tyrosine phosphorylation. The decrease in IRS-1 phosphorylation and associated PI 3-kinase activity together with the increase in IGF-I receptor tyrosine phosphorylation suggest that the effects of dexamethasone on IGF-I–stimulated mitogenesis could be mediated by signaling events initiated by the IGF-I receptor but distinct from IRS-1 signaling pathways. The three Shc proteins (46, 52, and 66 kD), which are rapidly tyrosine phosphorylated in response to insulin and growth factor stimulation of intact cells (25–27), represent an alternative intracellular pathway that could be involved in glucocorticoid action. Tyrosine phosphorylation of proteins in this molecular

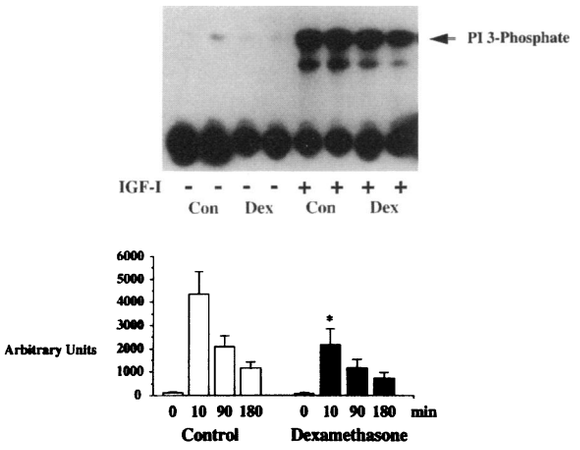

Figure 9. Effects of dexamethasone on PI 3-kinase activity associated with IRS-1. L6 cells were incubated in the presence or absence of 1 μM dexamethasone for 72 h and then stimulated with 100 nM IGF-I. PI 3-kinase activity was measured in anti-IRS-1 immunoprecipitates by in vitro assay with [^{32}P]ATP and PI, followed by analysis of phosphorylated lipids by TLC, as described in Methods. For IRS-1-associated PI 3-kinase activity, TLC plates were subjected to autoradiography for 3–6 h. The autoradiogram in the upper part of the figure shows a representative experiment performed with IGF-I stimulation of intact cells for 10 min. The bar graph in the lower part of the figure shows the quantitation of four independent experiments with 100 nM IGF-I stimulation for different periods of time. Open bars and cross-hatched bars represent control and dexamethasone-treated cells, respectively. * $P < 0.05$ vs. control cells, paired t test.

weight range in total cell lysates of IGF-I–stimulated L6 myoblasts may not have been observed by immunoblotting with αPY antibody (see Fig. 5) because of the limited sensitivity of this technique.

To investigate tyrosine phosphorylation of the Shc proteins, L6 myoblasts were treated with 100 nM IGF-I for various times, and cell lysates were subjected to immunoprecipitation with polyclonal Shc antibody and subsequent immunoblotting with phosphotyrosine antibody. As shown in Fig. 10 *(left)*, IGF-I markedly stimulated tyrosine phosphorylation of p52^{shc} and, to a lesser extent, of p66^{shc} and p46^{shc}. Tyrosine phosphorylation reached a maximum 5 min after addition of IGF-I and then

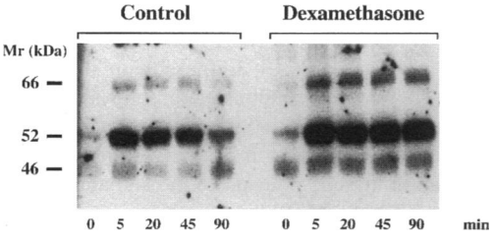

Figure 10. Effect of IGF-I on tyrosine phosphorylation of the Shc proteins in control and dexamethasone-treated L6 myoblasts. Cell monolayers were incubated in the presence or absence of 1 μM dexamethasone for 72 h and then stimulated with 100 nM human recombinant IGF-I for the indicated times. Total cell lysates were subjected to immunoprecipitation with Shc antibody, and the resulting immunoprecipitates were resolved by SDS-PAGE on 10% acrylamide gels, transferred to nitrocellulose, and blotted with the αPY antibody and ^{125}I-labeled protein A, as described in Methods.

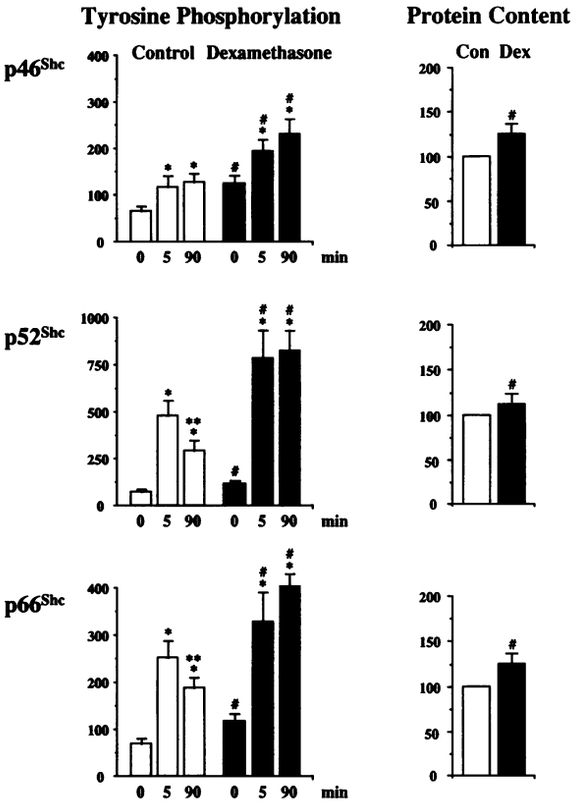

Figure 11. Effects of dexamethasone on tyrosine phosphorylation and content of p46^{shc}, p52^{shc}, and p66^{shc} in L6 myoblasts. To determine the levels of tyrosine phosphorylation of the Shc proteins, multiple experiments performed according to the procedure described in Fig. 10 were performed and the gels analyzed by scanning densitometry. Data on Shc tyrosine phosphorylation are presented on the left and expressed as arbitrary units. The total cell content of the Shc proteins was determined by a similar procedure except that immunoblotting was performed with Shc antibody. Data on Shc protein content are presented on the right and expressed as a percentage of control cells. Open and cross-hatched bars represent control and dexamethasone-treated cells, respectively, from five independent experiments. * $P < 0.05$ vs. 0 min IGF-I; # $P < 0.05$ vs. control cells; and ** $P < 0.05$ vs. 5-min IGF-I; paired t test.

slowly decreased after 90 min. A similar time course of tyrosine phosphorylation of the Shc proteins has been observed in 3T3 NIH fibroblasts overexpressing insulin receptors (26). When cells were treated with dexamethasone for 72 h, the level of IGF-I–stimulated tyrosine phosphorylation of all three Shc proteins was significantly higher than control cells. This difference was evident after 5 min of stimulation with 100 nM IGF-I and became more pronounced at later time points (Fig. 10, *right*). Quantitation of multiple experiments (Fig. 11, *left*) indicated that in control cells, IGF-I stimulation for 5 min increased the levels of tyrosine phosphorylation of p46^{shc}, p52^{shc}, and p66^{shc} by 1.7-fold, 6-fold, and threefold, respectively. These effects were augmented by approximately 80% in cells treated with dexamethasone, and there was also an increased level of basal tyrosine phosphorylation of all three Shc proteins in dexamethasone-treated cells. In addition, while tyrosine phosphorylation of p52^{shc} and p66^{shc} decreased significantly in control cells after 90 min of stimulation with IGF-I, a similar decrease was not

observed in cells treated with dexamethasone in which tyrosine phosphorylation of $p52^{shc}$ and $p66^{shc}$ persisted at high levels (Fig. 11, *left*). Although dexamethasone induced a modest increase in protein content of all Shc proteins (by ~ 15–25%; Fig. 11, *right*), this did not account for the increase in Shc tyrosine phosphorylation. The effect of dexamethasone on Shc tyrosine phosphorylation correlates with and may be mechanistically related to the increase in IGF-I–stimulated mitogenesis induced by dexamethasone.

Discussion

Glucocorticoids have been shown to potentiate the mitogenic actions of IGF-I in multiple cultured cell types (2–4), but the mechanism of this response is unknown. In the present study, the glucocorticoid dexamethasone was shown to augment the stimulatory effects of IGF-I on DNA synthesis and expression of the c-Fos proto-oncogene in undifferentiated L6 skeletal muscle cells. The mechanism of these effects of dexamethasone was investigated by examining elements of the IGF-I signaling pathway.

IGFs and IGF binding proteins secreted by muscle cells have important roles in the control of muscle cell growth and differentiation. Autocrine IGF-II secretion appears to be a major determinant of the differentiation of cultured muscle cells from myoblasts to myotubes in low-serum medium (45). In addition, increasing IGF-II secretion into the culture medium by muscle cells during differentiation induces a 60% decrease in the expression of IGF-I receptors (48). Although dexamethasone thus could influence the degree of muscle cell differentiation and the responsiveness of muscle cells to IGF-I by inducing changes in IGF-II synthesis, IGF-II levels in the medium were unaffected by dexamethasone in this study. IGF-I receptor expression is increased by dexamethasone in skeletal muscle cells (49), but this appears to be independent of changes in IGF-II.

Glucocorticoids have been reported to regulate the levels of IGF binding proteins in experimental animals and human subjects. For example, dexamethasone stimulates transcription of the IGFBP-1 gene and synthesis of the IGFBP-1 protein in rat hepatoma cells (50). Although dexamethasone may also affect IGF binding protein synthesis in L6 cells, changes in binding proteins cannot explain the effects of dexamethasone on DNA synthesis and cell proliferation. When human recombinant IGF-I and the analogue $longR^3$IGF-I were compared, a similar augmentation of hormone action by dexamethasone was observed, even though $longR^3$IGF-I has a much lower affinity for binding proteins. Dexamethasone also increased the growth stimulatory effects of insulin and PDGF-BB, two other hormones that do not interact with IGF binding proteins.

Having demonstrated that the effects of dexamethasone on the responsiveness to IGF-I in L6 myoblasts involve steps proximal to the induction of the proto-oncogene c-Fos and distal to the interaction of IGF-I with binding proteins, we investigated the initial steps in intracellular signaling by IGF-I. Treatment of L6 myoblasts with dexamethasone significantly increased the level of tyrosine phosphorylation and total cell content of IGF-I receptors. The increased level of tyrosine phosphorylation likely reflects an increased number of cell surface receptors that bind and are activated by IGF-I. Similar to these findings, dexamethasone has been shown to enhance IGF-I binding in porcine granulosa cells by increasing the number of cell surface receptors (4). The increased level of receptor tyrosine phosphorylation in dexamethasone-treated cells raises the possibility that higher tyrosine kinase activity at the cell surface could result in increased tyrosine phosphorylation of IGF-I receptor substrates. In contrast with the increased tyrosine phosphorylation and content of IGF-I receptors, however, dexamethasone decreased tyrosine phosphorylation and content of the IGF-I receptor substrate IRS-1. Previous studies in 3T3-L1 adipocytes have demonstrated a similar effect of glucocorticoids on the content of IRS-1 that appears to be mediated primarily by inhibition of the transcription of IRS-1 mRNA (51). The oppositely directed effects of dexamethasone on receptor and IRS-1 tyrosine phosphorylation correlate with the levels of expression of these two proteins in L6 myoblasts. In previous studies (38), we have observed discoordinate changes of insulin receptor and IRS-1 tyrosine phosphorylation in the skeletal muscle of glucocorticoid-treated rats compared with controls, indicating that the levels of tyrosine phosphorylation of the receptor and the substrate IRS-1 can also be independently regulated in vivo.

Dexamethasone significantly increased IGF-I receptor-associated PI 3-kinase activity, whereas IRS-1–associated PI 3-kinase activity was decreased by the glucocorticoid. The oppositely directed effects of dexamethasone on receptor and IRS-1–associated PI 3-kinase activity are directly correlated with and appear to reflect the levels of expression and tyrosine phosphorylation of these two proteins in L6 cells. In particular, IGF-I receptor expression and tyrosine phosphorylation were increased by 70 and 25%, respectively, and IGF-I receptor-associated PI 3-kinase activity was increased by 40% in dexamethasone-treated L6 myoblasts. PI 3-kinase association with the IGF-I receptor could occur via the formation of IGF-I receptor · IRS-1 · PI 3-kinase ternary complexes, as demonstrated for the insulin receptor (52). However, PI 3-kinase may also directly associate with the IGF-I receptor through the YAHM motif at the COOH-terminus, which conforms to the required YXXM structure and has been shown to directly bind PI 3-kinase in the case of the insulin receptor (53). A direct interaction between partially purified insulin receptors and fusion proteins containing the NH_2- or COOH-terminal SH2 domains of PI 3-kinase has been demonstrated in vitro, and a tyrosyl phosphopeptide derived from the insulin receptor COOH-terminus containing the Y^{1322}THM sequence has been shown to increase PI 3-kinase catalytic activity (53). Whereas a significant amount of PI 3-kinase activity was found to be associated with the IGF-I receptor in L6 myoblasts, we were unable to detect phosphorylated IRS-1 associated with the IGF-I receptor by phosphotyrosine antibody blotting of αIGF-I R immunoprecipitates (see Fig. 5). However, these results do not allow us to conclude that the association of PI 3-kinase with the IGF-I receptor was direct because the amount of receptor-associated IRS-1 may be too small to be detected by standard immunoprecipitation/immunoblotting methods. It has been suggested that PI 3-kinase localization may be relevant to the signaling capability of the enzyme because PI 3-kinase activation appears to involve its redistribution within the cell (54). Although quantitatively small relative to the entire cellular PI 3-kinase, it is thus possible that a separate pool of IGF-I receptor-associated PI 3-kinase could have important and distinct cellular functions.

Several observations suggest a key if not essential role for IRS-1 in the control of cell proliferation by insulin and IGF-I. Expression of IRS-1 in the hematopoietic 32D cell line, which lacks endogenous IRS-1, allowed these cells to respond to insulin with DNA synthesis (55). By contrast, inhibition of IRS-1

expression by an antisense IRS-1 RNA resulted in a 30–50% decrease in insulin-stimulated IRS-1 tyrosine phosphorylation and in a coordinate decrease in IRS-1–associated PI 3-kinase activity in CHO cells (56). This was associated with a marked inhibition of insulin-stimulated DNA synthesis and decreased transcriptional activation of the c-Fos promoter, indicating that a relatively modest reduction of IRS-1 function is sufficient to cause a substantial impairment of insulin action. In an additional study, microinjection of αIRS-1 antibody in Rat-1 fibroblasts overexpressing insulin receptors completely inhibited insulin and also IGF-I stimulation of DNA synthesis (57). In spite of the demonstrated relationship between IRS-1 and hormone-stimulated DNA synthesis, however, the decreased levels of expression and tyrosine phosphorylation of IRS-1 and IRS-1–associated PI 3-kinase activity in L6 myoblasts treated with dexamethasone provide strong evidence against the involvement of IRS-1 in the mechanism of the augmented responsiveness to IGF-I. The decrease in IRS-1 tyrosine phosphorylation and associated signaling apparently is not marked enough to inhibit mitogenesis and/or induction of c-Fos expression in response to IGF-I, and it appears that other signaling mechanisms mediate the actions of dexamethasone in skeletal muscle cells.

IGF-I, insulin, and PDGF-BB act via distinct cell surface receptors that have intrinsic hormone-stimulated tyrosine kinase activity, and the effects of all three of these hormones on DNA synthesis are augmented by dexamethasone in L6 myoblasts. In previous studies, glucocorticoids have been shown to potentiate the mitogenic actions of other growth factors that also act via membrane receptors with tyrosine kinase activity, including EGF and FGF (5–7). Increased receptor expression alone cannot account for the increased mitogenic responses to multiple hormones. In the current study, while IGF-I and insulin receptors were increased by dexamethasone, PDGF β receptors were not. It is thus probable that the effects of dexamethasone on the responsiveness to IGF-I in L6 myoblasts involve intracellular signaling mechanisms that are common to multiple tyrosine kinase receptors. Phosphorylation of the Shc proteins represents an additional IGF-I stimulated signaling event that is alternative to the IRS-1/PI 3-kinase pathway. The Shc proteins also may transduce mitogenic signals (24) and therefore represent potential intracellular targets subject to regulation by glucocorticoids. Whereas IRS-1 is not involved in signaling by other growth factors, the Shc proteins appear to represent an important component of mitogenic signaling by multiple tyrosine kinase growth factor receptors, including insulin, IGF-I, EGF, and PDGF (24, 28, 30).

Treatment of L6 myoblasts with IGF-I resulted in easily detectable stimulation of tyrosine phosphorylation of all three Shc proteins. This response is of particular interest, because L6 myoblasts express a physiologic number of IGF-I receptors that is similar to IGF-I receptor abundance in fetal muscle tissue (31, 58). It has been shown previously that tyrosine phosphorylation of Shc can be induced by insulin in NIH-3T3 or CHO fibroblasts overexpressing insulin receptors but not in untransfected fibroblasts (25–27), and that IGF-I induces Shc tyrosine phosphorylation only in NIH-3T3 fibroblasts overexpressing Shc (30). In PC-12 pheochromocytoma cells, both EGF and nerve growth factor can stimulate Shc tyrosine phosphorylation, whereas insulin has no detectable effect on this response under conditions where insulin-stimulated tyrosine phosphorylation of insulin receptors and IRS-1 and activation of PI 3-kinase in IRS-1 immunoprecipitates occur (59). Taken together, these results suggest that IGF-I stimulation of the Shc pathway may be cell context specific and thus play a physiologically relevant role in L6 myoblasts. In dexamethasone-treated L6 cells, IGF-I stimulation of tyrosine phosphorylation of all three Shc proteins was increased. The level of Shc tyrosine phosphorylation was elevated at early time points and remained increased for at least 90 min after IGF-I stimulation.

Several observations suggest that tyrosine phosphorylation of the Shc proteins may be involved in the control of cell proliferation and/or transformation. In cells transformed by the oncogenic tyrosine kinases v-src, v-fps, and erbB-2, all three Shc proteins are "constitutively" tyrosine phosphorylated with a relatively high stoichiometry of tyrosine phosphorylation (60, 61). On the other hand, microinjection of antibodies to the Shc proteins into Rat1 fibroblasts overexpressing human insulin receptors inhibits DNA synthesis induced by insulin, IGF-I, and EGF, indicating that Shc is an important component of a mitogenic signal transduction pathway that is shared by these hormones (62). The increase in the maximal amount of Shc tyrosine phosphorylation and the persistence of the Shc proteins in the phosphorylated state after IGF-I stimulation of L6 myoblasts treated with dexamethasone may thus represent a mechanism leading to the increased mitogenic response. Although it is possible that dexamethasone may increase IGF-I stimulation of DNA synthesis and c-Fos expression by distinct mechanisms, the presence of a higher amount and extent of tyrosine phosphorylation of the Shc proteins may be sufficient to determine all of the increased responsiveness to IGF-I. This conclusion is consistent with the recent observation that cells transfected with a Shc cDNA and expressing increased amounts of the Shc proteins are able to advance into the early phases of the cell cycle and are more sensitive to the growth-promoting effects of insulin (30). Whether an increased level of Shc tyrosine phosphorylation potentiates IGF-I–stimulated mitogenesis in L6 cells by increased activity of the GRB-2/p21^{ras} pathway (25, 28) or other Shc-dependent pathways remains to be determined. The increase in Shc tyrosine phosphorylation observed in L6 myoblasts treated with dexamethasone may also contribute to the reduced state of differentiation of these cells. This possibility is substantiated by the observation that expression of the N-ras oncogene in C2 myoblasts, which results in permanent activation of the p21^{ras} pathway, causes inhibition of cell differentiation (63).

The basis for the increased and prolonged tyrosine phosphorylation of Shc observed in L6 myoblasts treated with dexamethasone is unclear. Although total cell content of the Shc proteins was increased by dexamethasone, the magnitude of this change was modest compared with the increase in tyrosine phosphorylation and does not explain the prolonged state of Shc phosphorylation. The amino acid sequence NPXY has been shown to be a consensus binding site for Shc (64). Interestingly, NPXY sequences are present in the juxtamembrane region of both the insulin and IGF-I receptors, and the tyrosine residue in these motifs (Tyr-960 and Tyr-950 in the human insulin and IGF-I receptors, respectively) has been shown to be required for interaction with IRS-1 (65). Under conditions where IRS-1 content is markedly reduced and Shc content significantly increased (as in L6 cells treated with dexamethasone), it is possible that preferential interaction of the IGF-I receptor with Shc could occur and that this could result in greater tyrosine phosphorylation of the Shc proteins. The increase in Shc tyro-

sine phosphorylation by ~ 80% directly correlates with the increase in expression and tyrosine phosphorylation of IGF-I receptors (by 70 and 25%, respectively). Therefore, an increased amount of phosphorylated receptors may also contribute to the augmentation of Shc phosphorylation in dexamethasone-treated L6 myoblasts.

Differential regulation of the IRS-1/PI 3-kinase and Shc signaling pathways by dexamethasone appears to affect two noninteracting signaling systems. Although the Shc proteins contain an SH2 domain, a direct interaction between the SH2 domain of the Shc proteins and tyrosine phosphorylated IRS-1 does not appear to occur (25). Similarly, tyrosine phosphorylated Shc proteins do not interact with the SH2 domains of the p85 subunit of PI 3-kinase, probably because they lack the specific YXXM/YMXM consensus sequences that are required for the association of tyrosine phosphorylated proteins with the SH2 domains of p85 (66). Thus, PI 3-kinase activity was not detected in anti-Shc immunoprecipitates (61), and overexpression of Shc proteins in fibroblasts does not modify basal PI 3-kinase activity or activation induced by insulin and IGF-I (30).

Although it is apparent that both the IRS-1/PI 3-kinase and the Shc signaling pathways may participate in the regulation of cell proliferation in response to insulin and IGF-I, the relative contribution of each pathway to this process has not yet been elucidated. In addition, the role of specific signaling systems in mammalian cells expressing a physiologic number of receptors has not been investigated. The results of our study show, for the first time, that the glucocorticoid dexamethasone, an important regulator of mitogenic responses in multiple cell types, can specifically augment the activity of the Shc signaling pathway by increasing the expression and tyrosine phosphorylation of the Shc proteins. In the same cells, dexamethasone inhibits a second distinct signaling pathway that involves tyrosine phosphorylation of IRS-1 and activation of PI 3-kinase. Further investigation of glucocorticoid regulation of the distinct signaling pathways activated by IGF-I receptors may contribute to our understanding of the physiologic relevance of the multiple intracellular signal transduction systems in skeletal muscle cells and other cell types.

Acknowledgments

We acknowledge Dr. R. R. Bowsher for his valuable assistance in performing the IGF-I and IGF-II radioimmunoassays, and Velta Ramolins and Karen Pickhardt for excellent technical support.

This work was supported in part by grants from the Juvenile Diabetes Foundation and the National Institutes of Health (DK43038, DK/GM 48503, Trauma Center grant GM36428, and Diabetes and Endocrinology Research Center grant DK36836). F. Giorgino was supported by postdoctoral fellowships from the Juvenile Diabetes Foundation International and the Mary K. Iacocca Foundation.

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