Multiple signaling pathways mediate LIF-induced skeletal muscle satellite cell proliferation

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Spangenberg, Espen E., and Frank W. Booth. Multiple signaling pathways mediate LIF-induced skeletal muscle satellite cell proliferation. Am J Physiol Cell Physiol 283: C204–C211, 2002.—There are many known growth factors/cytokines that induce skeletal muscle satellite cell proliferation. Currently, the signaling mechanisms in which these growth factors/cytokines activate satellite cell proliferation are not completely understood. Here, we sought to determine signaling mechanisms by which leukemia inhibitory factor (LIF) induces satellite cell proliferation in culture. First, we confirmed that LIF induces proliferation of C2C12 immortalized myoblasts and cultured primary rat satellite cells. In addition, we also found that this increase in proliferation can be inhibited by incubation of the cells in tyrphostin AG 490, a specific inhibitor of Janus-activated kinase (JAK) 2 activity. Furthermore, we also found that incubation of the cells at various time points with LIF (10 ng/ml) induces a significant, transient increase in JAK2 phosphorylation, signal transducers and activators of transcription (STAT3) phosphorylation, and STAT3 transcriptional activity. Increases in the STAT3-sensitive endogenous SOC3 protein followed these transient increases in STAT3 activation. In addition, AG 490 inhibited the increase in STAT3 phosphorylation. Finally, LIF did not change the phosphorylation status of extracellular signal-regulated protein kinase (ERK)1/2 or affect the phosphorylation status of Akt/protein kinase B. However, LY-294002, an inhibitor of phosphoinositide 3-kinase, blocked LIF-induced proliferation of satellite cells. These data suggest that LIF induces satellite cell proliferation by activation of the JAK2-STAT3 signaling pathway, suggesting that this may be an important pathway in muscle growth and/or hypertrophy.

Understanding the biochemical and molecular regulators of skeletal muscle size has important biological consequences. Various environmental factors such as physical inactivity associated with aging, bed rest, limb immobilization, spaceflight, and neuromuscular disease can contribute to reductions in muscle size (i.e., muscle atrophy). Therefore, determination of biological regulators that control muscle growth and/or hypertrophy is of the utmost importance.

Increases in satellite cell proliferation are necessary for full skeletal muscle growth or hypertrophy (15, 22, 30, 31). Satellite cells are quiescent myogenic precursor cells that are located between the basal lamina and the sarcolemma (36). During muscle growth/hypertrophy, there are increases in the concentrations of various autocrine/paracrine factors that are thought to enhance satellite cell proliferation and/or differentiation (15, 38). For example, insulin-like growth factor (IGF)-I (1) and satellite cell proliferation (34, 35) increase in mechanically overloaded muscles. Furthermore, transgenic expression or exogenous delivery of IGF-I can induce increases in muscle size with concurrent increases in satellite cell proliferation (8, 10). Given that multiple growth factors regulate satellite cell proliferation, it is likely that other growth factors will be found that regulate in vivo muscle growth and satellite cell proliferation. Therefore, we hypothesized that leukemia inhibitory factor (LIF) might be an important growth factor that would enhance satellite cell proliferation in culture.

During muscle hypertrophy or muscle regrowth induced by reloading, muscle damage can occur that consists of minor microdamage or large tears in the sarcolemma (38). This damage induces a large inflammatory response within skeletal muscle. However, what is currently unclear is the role(s) that this inflammatory response might play in muscle growth. Damage to muscle can induce the release of various cytokines from cells (i.e., neutrophils and macrophages) that are attracted to the injured tissue and is associated with the onset of the inflammatory response (38). The soleus and plantaris muscles exhibit increased LIF expression for up to 14 days after synergist ablation (32, 33). LIF expression is also elevated during myotoxin-induced skeletal muscle regeneration and in muscular dystrophy (19). All of these conditions are characterized by high levels of satellite cell proliferation (15). Furthermore, Austin et al. (5, 6) found that recombinant LIF can induce myoblast proliferation in culture and improve muscle regeneration in animals (4). Unfortunately, there are no data describing the biochem-
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Satellite cell isolation. Animals were anesthetized with a single intraperitoneal injection of a cocktail consisting of 75 mg/kg ketamine, 3 mg/kg xylazine, and 5 mg/kg acepromazine. Subsequently, all of the major hindlimb muscles except for the soleus muscle were removed and trimmed of excess fat and connective tissue. Satellite cells were then isolated according to the previously described methods (10) modified from Allen et al. (2) and were maintained in a 37°C humidified incubator (6% CO2). It was found that <10% of the total cells were nonmyogenic cells based on desmin and vimentin staining, which are isolation yields similar to those found by other investigations (2).

Cell proliferation assay. Satellite cell proliferation was determined using a CyQUANT Assay Kit (Molecular Probes, Eugene, OR) according to the methods of Qu et al. (27) and the manufacturer’s directions. Briefly, the cells were counted with a hemacytometer and then seeded at ~4,000 cells/well on a 24-well plate in 500 µl of DMEM with 20% FBS and 1% chicken embryo extract (CEE). Concurrently, a standard curve was formulated in which the satellite cells were plated out in the increasing number from ~2,500 to ~25,000 cells/well. Each point on the standard curve was seeded in quadruplicate. The cells were allowed to adhere to the plate overnight. The following day, the incubation medium was removed and the cells were washed twice with phosphate-buffered saline (PBS). The plate(s) containing the standard curve was placed in a −80°C freezer. The wells of the proliferation plates were washed twice with PBS, and then 500 µl of either DMEM supplemented with 5% FBS, LIF with DMEM and 5% FBS, or IGF-I with DMEM and 5% FBS were added. After 24 h of incubation, the plates were removed, and medium and growth factors were refreshed. After a total of 48 h, the plates were removed and the wells were rinsed twice with sterile PBS. The plates were then frozen at −80°C.

To inhibit various signal pathways, the above experiment was conducted in the presence of various cell signaling inhibitors (i.e., AG 490, PD-098059, and LY-294002). After the cells were plated out, the inhibitors were added to DMEM supplemented with 5% FBS without LIF. After 3 h, the medium was replaced with identical medium, except it was supplemented with LIF and returned to the 37°C humidified incubator for 24 h. The cells were then subsequently removed, the specific medium was refreshed, and the plates were returned to the incubator for another 24 h. After a total of 51 h, the cells were removed and washed twice with sterile PBS and placed into a −80°C freezer. Each experimental condition was measured at least in quadruplicate and on at least two separate occasions.

To estimate proliferation, the plates containing the cells were removed from a −80°C freezer and allowed to defrost for 10 min. Cells were then incubated in the fluorescent dye and the cell lysis buffer from the CyQUANT Assay kit for 10 min. The fluorescent intensity was subsequently determined by an optical scanner (STORM 860; Molecular Dynamics) with excitation at 485 nm and emission at 535 nm. All images were subsequently stored on microcomputer for later analysis using ImageQuant software. The correlation of standard curves was $r^2 = 0.99$.

Western blots. To determine amounts of specific proteins and their phosphorylation status, cells were plated out at a density of ~1 × 10^6 cells/well and allowed to incubate for ~2 days until they reached ~75% confluency. The medium was aspirated, and cells were then washed twice with sterile PBS. The medium was replaced with LIF (10 ng/ml) and allowed to incubate until designated time points when the plates were removed, the medium was aspirated, and the cells were washed with ice-cold sterile PBS two times. Cells were lysed
and scraped in ice-cold cell lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 200 mM NaF, 20 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 200 mM phenylmethylsulfonyl fluoride, and 1 mM NaVO₄). Cell extracts were then rotated for 30 min at 4°C. The extract was then centrifuged at 13,000 rpm for 10 min. Supernatant protein was then quantified using the Bradford protein assay kit (Bio-Rad, Richmond, CA). Equal amounts of total protein were then resolved on SDS-PAGE gels and transferred to nitrocellulose membranes. All blots were then incubated with Ponceau S (Sigma) to ensure equal loading in all lanes (data not shown). The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween (TBS-T) and probed with the appropriate antibody overnight. The membranes were washed in TBS-T and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (1:5,000; Amersham Pharmacia Biotech, Piscataway, NJ). Finally, the HRP activity was detected using enhanced chemiluminescence reagent (ECL; NEN Life Science Products, Boston, MA). The membrane was exposed to autoradiographic film (Kodak-XAR5) with the exposure time adjusted to keep the integrated optical densities (IOD) within a linear and nonsaturated range. The IODs of the specific bands were then quantified using densitometry software (NIH Image). To determine the total protein expression, blots for each specific protein that was previously probed for the phosphorylation status were stripped in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) for 30 min at 55°C. The membrane was then reprobed with an antibody specific to the total specific protein as described above.

Luciferase assay. Primary rat satellite cells were plated at 3 x 10⁴ cells/well in a 24-well plate and cultured overnight in DMEM supplemented with 20% FBS and 1% CEE. Cells were washed twice with sterile PBS and then transfected in serum-free medium with a total of 1.0 μg of plasmid DNA containing the indicated plasmid reporter and indicated expression vector. The luciferase reporter plasmid (Clontech, Palo Alto, CA) used to measure STAT3 transcriptional activity contained four STAT3 binding sites upstream from a minimal SV40 promoter. The dominant negative STAT3 (DM-STAT3) (24) had been created by truncating the wild-type form of STAT3 after the Lys685 residue and leaving the SH2 domain intact (kindly donated by Dr. Alice Mui, Dept. of Surgery, University of British Columbia, Jack Bell Research Centre, Vancouver, BC, Canada). Transfection was accomplished using Lipofectamine and PLUS reagent (Life Technologies) according to the manufacturer’s directions. After 3 h, the medium was replaced with medium supplemented with LIF or LIF and AG 490. After 16 h, cells were lysed and assayed for luciferase activity with a luciferase assay kit (Clontech). Luciferase activity was normalized to total protein as previously described (39).

Statistics. All values are means ± SE. All differences between groups were determined using a two-tailed Student’s t-test. For all statistical tests, raw values were used and the 0.05 level was considered statistically significant.

RESULTS

LIF-induced C₂C₁₂ and primary rat satellite cell proliferation. After 48 h of incubation with 5% FBS alone, there was a 113% increase in the number of C₂C₁₂ cells (Fig. 1A) and a 103% increase in rat satellite cells (Fig. 1B) compared with the initial preplate number of cells. However, a 348% increase in C₂C₁₂ cell number (Fig. 1A) and a 190% increase in the number of rat satellite cells (Fig. 1B) occurred in the presence of 10 ng/ml LIF, compared with the preplate number of cells. Furthermore, data on IGF-I were included as positive control to demonstrate that the assay can detect increases in cell number, because it is well known that IGF-I induces myoblast and satellite cell proliferation (9–11). Optimal doses of LIF and IGF-I had similar increases in C₂C₁₂ myoblast proliferation. There were no indications of cell apoptosis or differentiation (data not shown). The LIF-induced (10 ng/ml) proliferation of rat satellite cells thus extends to the previous reports where LIF increased the proliferation of myoblasts (6).

LIF-induced proliferation of satellite cells was inhibited by pharmacological inhibition of the JAK2 pathway. For the first time, we have shown that AG 490, a JAK2 inhibitor (42), blocked LIF (10 ng/ml)-induced proliferation in both the C₂C₁₂ and rat satellite cells (Fig. 2). The MEK inhibitor PD-098059 and PI3K in-
inhibitor LY-294002 both blocked the LIF-induced proliferation of the C2C12 cells (Fig. 2A); however, only LY-294002 blocked LIF-induced proliferation of the rat satellite cells to the degree observed for FBS levels (Fig. 2B).

**LIF induced rapid phosphorylation of JAK2 and STAT3 in rat satellite cells.** LIF (10 ng/ml) induced a 138% increase in the phosphorylation of Tyr1007/1008 of JAK2 at 15 min of incubation (Fig. 3A). Also, LIF induced 132% and 112% increases in phosphorylation of Tyr705 STAT3 after 15 and 30 min of incubation, respectively (Fig. 3B). Both JAK2 and STAT3 phosphorylation returned to basal levels after 30 and 60 min, respectively, of LIF incubation. Also, C2C12 cells exhibited similar changes in phosphorylation of STAT3 after LIF incubation (data not shown).

**AG 490 inhibits LIF-induced STAT3 phosphorylation in rat satellite cells.** LIF (10 ng/ml) was unable to induce STAT3 phosphorylation at any time point when the rat satellite cells were incubated in the presence of AG 490 (Fig. 4). In addition, AG 490 did not appear to have any cytotoxic effects on our cells (data not shown), nor did AG 490 nonspecifically inhibit another signaling pathway (i.e., as evidenced by normal ERK1/2 phosphorylation).

**LIF does not induce ERK1/2 or Akt phosphorylation in rat satellite cells.** There were no significant changes in the phosphorylation of ERK1/2 or Akt at any time point in the presence of LIF (10 ng/ml) (Fig. 5). Also,
C2C12 cells did not demonstrate changes in phosphorylation of ERK1/2 or Akt (data not shown).

LIF increases transcriptional activity of STAT3 in rat satellite cells. LIF (10 ng/ml) induced an increase in STAT3-activated luciferase activity by 118% in primary rat satellite cells compared with the same STAT3-luciferase construct without LIF (Fig. 6A). In addition, AG 490 (STAT3 + AG) inhibited the LIF-induced activation of the STAT3 reporter construct, whereas the DM-STAT3 inhibited any basal and LIF activation of the reporter construct.

There was a significant increase in the expression of endogenous protein SOCS-3 after 5 and 7 h of LIF incubation ($P < 0.05$) (Fig. 6B). The SOCS-3 gene is transcriptionally sensitive to STAT3 activation (16).

DISCUSSION

This study shows for the first time that the induction of satellite cell proliferation by 10 ng/ml LIF utilizes the JAK2-STAT3 signaling pathway. Tyrophostin AG 490, an inhibitor of JAK2 activity (42), blocked the LIF-induced increase in satellite cell proliferation. This was further confirmed in that LIF induced an
increase in the levels of tyrosine phosphorylation at residues 1007/1008 of JAK2, which others (28) have shown is necessary to elevate the kinase activity of JAK2. LIF also increased Tyr705 phosphorylation of STAT3, which is known to be a downstream target of JAK2. Phosphorylation of residue 705 is an absolute requirement for increasing transcriptional activity of multiple genes where STAT3 acts as a transcription factor (12). Therefore, we found an association between the increased phosphorylation status of JAK2 and STAT3 in the presence of LIF. In addition, AG 490 not only inhibited the proliferation of satellite cells in the presence of LIF but also blocked the LIF-induced increase in STAT3 phosphorylation and transcriptional activity, showing that increases in JAK2 activity do indeed activate STAT3 in rat satellite cells. These findings strongly suggest that JAK2 inhibition, or another pathway activated and/or inhibited by AG 490, may be involved in controlling LIF-induced satellite cell proliferation. Previous studies have shown that AG 490 has JAK2 inhibitory activity (21) and has been widely used to assess the functional role of this enzyme in various scientific contexts (for review, see Ref. 42). Furthermore, we did not find any alteration in ERK1/2 phosphorylation in the presence of AG 490 and LIF, again confirming that AG 490 specifically inhibits JAK2 kinase activity. In summary, these data suggest that LIF induces increased proliferation of rat satellite cells and that activation of the JAK2 and STAT3 signaling pathways at least partially contributes to the increased satellite cell proliferation.

One of the known features of the JAK-STAT pathway is its rapid activation postreceptor stimulation (16). The data presented here show that LIF increased phosphorylation of JAK2 and STAT3 by 15 min in the culture conditions employed. STATs, which are normally localized in the cytoplasm, are activated when phosphorylated on the tyrosine residue located around residue 700 (28). Tyrosine phosphorylation of STAT3 induces either STAT3 proteins to form homodimers or a STAT3 and another isoform of STAT to form a heterodimer with their reciprocal phosphotyrosine-SH2 domain interactions (12). The active STAT dimer then rapidly translocates to the nucleus by the importin α/β localization pathway (28). Binding of STAT3 to specific DNA-binding elements results in increased transcriptional activity of various gene targets. Our data show that LIF enhanced the transcriptional activity of a STAT3-driven promoter driving the luciferase reporter gene in transfected satellite cells. STAT3 has been shown to upregulate transcriptional control of many genes, including those that encode many proteins that are known to control cell cycle (i.e., cyclin D), cellular growth (i.e., c-Myc and SOCS-3), and apoptosis (i.e., Bcl-xL and Bcl-2) (23). All of these proteins are known to be operative in skeletal muscle satellite cells, and, therefore, it is very possible that LIF-induced STAT3 activation may play a significant role in some aspect of early signal transduction in skeletal muscle satellite cell proliferation.

Although the activation of the JAK2-STAT3 pathway is rapid whereas the downstream result (i.e., increased proliferation) requires 48 h, the importance of this early activation cannot be discounted because activation of the proliferative response does not seem to occur without it. For example, multiple studies have documented that the JAK2-STAT3 is transient in activation, so it is clear within the literature that this is a trademark of this pathway (42). Furthermore, studies that have measured the physiological “end point” of

Fig. 6. A: LIF (10 ng/ml) induces increased luciferase activity of a reporter construct that contains 4 copies of a high-affinity STAT3 binding site. Luciferase activity is normalized to the protein content of the cell lysates. TA refers the plasmid containing no STAT3 binding sites in the vector. AG indicates AG 490 (10 μM). DM-STAT contains dominant negative STAT3. *Statistically different from all conditions (P < 0.05). B: increased endogenous suppressors of cytokine signaling (SOCS)-3 protein expression in satellite cells after LIF (10 ng/ml) incubation. LIF-induced increased SOCS-3 expression is transcriptionally regulated through activation of the transcription factor STAT3 (16). All units are expressed as the IOD of the band for total protein expression. A representative blot is shown beneath the bar graph. *Statistically different from control condition (P < 0.05).
LIF incubation (i.e., increase proliferation) in muscle cells have not determined the mechanisms that regulate this end point. Therefore, it is possible that certain signaling proteins (i.e., JAK2-STAT3) act as early response mechanisms to increase cellular proliferation. Recently, Wu et al. (41) proposed a model indicating that gene transcription can be short lived, with the resulting phenotype long lived. In other words, it is possible that the half-lives of mRNA and the protein products of the genes regulated by STAT3 are long compared with the amount of time that STAT3 is inducing transcription of the gene. For example, we noted an increase in SOCS-3, an endogenous protein product of STAT3-activated transcription, by 5–7 h after satellite cells were exposed to LIF and after the transient spike in JAK2 and STAT3 phosphorylation. Clearly, it is likely that other signaling pathways contribute to the maintenance of the increased proliferation signal. For example, we found that LY-294002 did inhibit the LIF-induced proliferation, so it is possible that PI3K is acting to potentiate the initial signal.

Neither ERK1/2 nor Akt phosphorylation was significantly altered by LIF administration to cultured satellite cells. Also, LIF-induced enhancement of rat satellite cell proliferation was not abated by application of the specific MEK inhibitor PD-098059 to prevent ERK's activation. However, the PI3K inhibitor LY-294002 did prevent LIF's induction of rat satellite cell proliferation. Apparently basal/permissive levels of PI3K activity are necessary for LIF to signal a stimulation of rat satellite cell proliferation through JAK2. Therefore, while JAK2 stimulation is required for LIF's stimulation of rat satellite cell proliferation, basal activities of PI3K are also required. It is well documented in other cell types that JAKs have extensive cross talk with other signaling pathways, including PI3K (25, 28).

Whether LIF's stimulation of satellite proliferation plays a regulatory role in the hypertrophy of skeletal muscle remains to be tested. However, LIF has been shown to promote cardiac muscle hypertrophy (20), neural regeneration (17), and maintenance of embryonic stem cell pluripotency (13, 29). All of these results suggest that the physiological role of LIF is complex and warrants further investigation. Furthermore, LIF is known to be elevated in skeletal muscle during regeneration, which is characterized by satellite cell proliferation (19). Satellite cell proliferation begins to occur ~3 days after increased loading (34, 35), and further LIF expression in skeletal muscle increases 2 days after increased loading (32). This associative evidence suggests that the time points at which satellite cell proliferation and LIF expression are elevated in the animal correlate well, and our observations in cultured satellite cells suggest that LIF could play a role in satellite cell activation during muscle hypertrophy. In addition, exogenous infusion of recombinant LIF (2–20 ng/day) was sufficient to improve the recovery of regenerating muscle (7, 18, 40). These amounts of LIF are within the range of LIF (10 ng) that we found to have a significant effect on satellite cell proliferation, and our data may describe some of the biochemical mechanisms that may activate these satellite cells. The current data, combined with other studies (15, 38), suggest that many different growth factors, such as IGF-I, hepatocyte growth factor, or LIF, can induce proliferation of cultured satellite cells through different signaling pathways, and they suggest a potential complex regulatory interaction of regulatory factors in satellite cell proliferation during the enlargement of skeletal muscle.

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