Deficient leukemia inhibitory factor signaling in muscle precursor cells from patients with type 2 diabetes

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Submitted 18 November 2011; accepted in final form 2 May 2012

Broholm C, Brandt C, Schultz NS, Nielsen AR, Pedersen BK, Scheele C. Deficient leukemia inhibitory factor signaling in muscle precursor cells from patients with type 2 diabetes. Am J Physiol Endocrinol Metab 303: E283–E292, 2012. First published May 29, 2012; doi:10.1152/ajpendo.00586.2011.—The cytokine leukemia-inhibitory factor (LIF) is expressed by skeletal muscle and induces proliferation of muscle precursor cells, an important feature of skeletal muscle maintenance and repair. We hypothesized that muscle precursor cells from patients with type 2 diabetes had a deficient response to LIF. The mRNA and protein expressions of LIF and its receptor (LIFR) were measured in skeletal muscle biopsies from healthy individuals and patients with type 2 diabetes by use of qPCR and Western blot. LIF signaling and response were studied following administration of recombinant LIF and siRNA knockdown of suppressor of cytokine signaling (SOCS)3 in myoblast cultures established from healthy individuals and patients with type 2 diabetes. Myoblast proliferation rate was assessed by bromodeoxyuridine incorporation. LIF and LIFR proteins were increased in both muscle tissue and cultured myoblasts from diabetic patients. Nonetheless, in the diabetic myoblasts, LIF-induced phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3 was impaired. The deficient response to LIF administration in the diabetic myoblasts was further emphasized by a lack of increase in LIF-stimulated cell proliferation and a decreased LIF-stimulated induction of the proliferation-promoting factors cyclin D1, JunB, and c-myc. SOCS3 protein was upregulated in diabetic myoblasts, and knockdown of SOCS3 rescued LIF-induced gene expression in diabetic myoblasts, whereas neither STAT1 or STAT3 signaling nor proliferation rate was affected. In conclusion, although LIF and LIFR proteins were increased in muscle tissue and myoblasts from diabetic patients, LIF signaling and LIF-stimulated cell proliferation were impaired in diabetic myoblasts, suggesting a novel mechanism by which muscle function is compromised in diabetes.

LIF signaling would be deficient in diabetic skeletal muscle. Previous studies have demonstrated that muscle precursor cells from patients with type 2 diabetes retain a “diabetic” phenotype in terms of impaired glucose transport (11), reduced lipid oxidation (12), and increased activity of inflammatory markers (14). Interestingly, a recent global comparison of mRNA and microRNA (miRNA) expression between skeletal muscle from patients with type 2 diabetes and healthy individuals suggested that alterations in the developmental program of the muscle precursor cells might be part of the muscular pathophysiology of type 2 diabetes (10). One of the proteins predicted to be targeted by the diabetes-differentially expressed miRNAs was the leukemia-inhibitory factor receptor (LIFR) (10). Interestingly, a recent study reported an increased LIFR expression in skeletal muscles of diabetic mice (37).

Leukemia inhibitory factor (LIF) regulates muscle precursor cells by inducing proliferation and inhibiting differentiation (1, 5, 33, 35). Moreover, LIF promotes muscle growth and regeneration in intact skeletal muscle (15, 34, 41). LIF signaling is initiated when LIF binds the specific LIFR in complex with the ubiquitously expressed gp130 receptor (13), which leads to phosphorylation and thereby activation of janus kinase (JAK)1 and signal transducer and activator of transcription (STAT)1 and STAT3 in cultured muscle cells (8, 35). LIF also induces the expression of suppressor of cytokine signaling (SOCS)3, which negatively regulates LIF signaling at the receptor level (8). LIF is produced by skeletal muscle and seems to regulate human myoblast proliferation in an autocrine or paracrine fashion (5).

On the basis of the idea of a dysfunctional regulation of muscle precursor cells in type 2 diabetes, we hypothesized that LIF signaling would be deficient in diabetic skeletal muscle. Accordingly, we examined LIF expression and signaling in skeletal muscle tissue and satellite cell-derived myoblasts from patients with type 2 diabetes.

MATERIALS AND METHODS

Type 2 diabetes cohort. Individuals with normal glucose tolerance and patients with type 2 diabetes were recruited by advertising in a local newspaper. Exclusion criteria were treatment with insulin, recent or ongoing infection, history of malignant disease, or treatment with anti-inflammatory drugs. With the use of a cross-sectional case-control design, participants (n = 47) were divided into two groups based on whether the participants had normal glucose tolerance or type 2 diabetes according to the results of an oral glucose tolerance test (Table 1). The WHO diagnostic criteria for type 2 diabetes were used. All participants were given both oral and written information about the experimental procedures before giving their written in-
formed consent. Before the experimental day, all participants underwent a clinical examination with blood samples. The study has previously been described in detail (28). Due to the lack of material, the group studied here is reduced compared with the group described previously (28). The study was approved by the Ethics Committee of Copenhagen and Frederiksberg Council, Denmark.

**Culture of human myoblasts.** Muscle precursor cells were isolated from five patients with type 2 diabetes and five healthy individuals (Table 2). We (14) have previously shown that these cells can be differentiated into myotubes in vitro and, as such, retain a “diabetic” phenotype, for example, in terms of insulin sensitivity, matching the donor phenotype. Muscle precursor cells were isolated from human skeletal muscle biopsies obtained from the vastus lateralis muscle using a biopsy needle with suction (4). Fat and connective tissue were removed, and the muscle tissue was minced into small pieces with sterile scissors. Subsequently, the muscle tissue was transferred to 5-ml sterile-filtered digestion solution [HAM-F10 medium containing 0.05% trypsin-EyDTA, 1 mg/ml collagenase IV, and 10 mg/ml fatty acid-free bovine serum albumin (BSA, Sigma)] and shaken for 5 min at 37°C. Digestion solution containing liberated muscle precursor cells was removed and added to 2 ml of fetal bovine serum (FBS) on ice to inactivate the enzymes. Additionally, 5 ml of digestion solution was added to the remaining tissue, and the procedure was repeated. Next, the mixture was spun at 800 × g for 7 min, and the supernatant was removed with suction. The pellet was washed in HAM-F10 medium, and the cell suspension (present in the pellet) was preplated in a 60-mm plate with growth medium (HAM-F10 supplied with 20% FBS and 1% penicillin-streptomycin) for 3 h. Finally, the cell suspension was transferred to a flask coated with Matrigel, and the muscle precursor cells were propagated.

Isolated muscle precursor cells were propagated in growth medium (HAM-F10 supplied with 20% FBS and 1% penicillin-streptomycin) and plated in six-well plates. Following seeding, the cells were grown until 70–80% confluence, and proliferating myoblasts were stimulated with recombinant human LIF (10 ng/ml, Sigma) dissolved in phosphate-buffered saline (PBS). Control cells were treated with PBS alone. Cell culture medium ingredients were from Invitrogen, and plates and flasks were from Nunc.

**Small interfering RNA transfection in myoblasts.** Transient transfections of human myoblasts were performed using pools of small interfering RNA (siRNA) oligos specifically targeting four different sites of the SOCS3 and LIFR mRNA (On Target Plus, Dharmacon) to minimize any off-target effects. Transfections were performed in proliferating myoblasts (at ~75% confluence) using 25 nM siRNA and Lipofectamine 2000 (Invitrogen) in antibiotic-free cell culture medium, and the cell suspension (present in the pellet) was preplated in a 60-mm plate with growth medium (HAM-F10 supplied with 20% FBS and 1% penicillin-streptomycin) for another 24 h. BrdU incorporation was measured using an ELISA reader according to the manufacturer’s protocol (cat. no. H11021, Roche).

**Caspase-3 activity assay.** Confluent myoblasts were given medium with reduced serum (HAM-F10 supplied with 10% FBS and 1% penicillin-streptomycin) together with LIF (10 ng/ml) for 24 h, after which cells were harvested. Caspase-3 activity was analyzed using a caspase-3/CPP32 colorimetric assay kit according to the manufacturer’s protocol (no. K106–25, BioVision).

**Western blot analysis.** Muscle tissue was freeze-dried and dissected free of visual blood, fat, and connective tissue. Muscle lysate was prepared as described previously (29). Cells were lysed in ice-cold cell lysis buffer (20 mM Tris, 1 mM EDTA, 1% Triton, pH 7.4) with phosphatase inhibitor cocktails 1 and 2 (Sigma) and Complete mini protease inhibitor cocktail (Roche). Lysate proteins were separated by SDS-PAGE using 4–12% Bis-Tris gels (Invitrogen) and transferred by electrophoresis to polyvinylidene difluoride membranes (GE Healthcare). The membranes were blocked for 1 h at room temperature in either 5% skim milk, 1% fish skin gelatin, or 5% BSA and subsequently incubated overnight at 4°C with antibodies against human LIF (AF-250-NA, R&D), LIFR (sc6599, Santa Cruz), SOCS3 (no. 2923, Cell Signaling), phospho-STAT3 (Tyr705) (no. 9145, Cell Signaling), and STAT3 (no. 9139, Cell Signaling). Next day, the membranes were stained with reactive brown (Sigma). All membranes were stained with reactive brown (Sigma). The primer sequences are given in Table 3. The primers were synthesized by DNA Technology. The primer sets were mixed with siRNA mix and bromodeoxyuridine (BrdU). Control cultures were prepared simultaneously and treated the same way, but without addition of any oligonucleotide (control), or transfected with a scrambled oligonucleotide predesigned to not target any gene (Dharmacon; siRNA Scr).

Table 1. Clinical characteristics of participants in the cohort

<table>
<thead>
<tr>
<th></th>
<th>NGT</th>
<th>T2D</th>
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<tr>
<td>n</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>Sex (female/male)</td>
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<td>8/13</td>
</tr>
<tr>
<td>Age, yr</td>
<td>50.9 ± 9.4</td>
<td>55.7 ± 7.5</td>
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<tr>
<td>Current smoker</td>
<td>7</td>
<td>6</td>
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<tr>
<td>BMI, kg/m²</td>
<td>29.6 ± 7.8</td>
<td>31.5 ± 7.5</td>
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<tr>
<td>Fasting glucose, mM</td>
<td>5.1 ± 0.5</td>
<td>9.6 ± 5.1</td>
</tr>
<tr>
<td>Fasting insulin, pM</td>
<td>52.0 ± 46.4</td>
<td>111.9 ± 90.9†</td>
</tr>
<tr>
<td>HOMA2-IR</td>
<td>1.0 ± 0.9</td>
<td>3.0 ± 3.3*</td>
</tr>
<tr>
<td>Hemoglobin, A1C, %</td>
<td>5.5 ± 0.3</td>
<td>7.0 ± 1.8*</td>
</tr>
<tr>
<td>VO₂max, ml·kg⁻¹·min⁻¹</td>
<td>34.3 ± 18.7</td>
<td>24.7 ± 7.8*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. NGT, individuals with normal glucose tolerance; T2D, individuals with type 2 diabetes; BMI, body mass index; HOMA2-IR, homeostasis model assessment index for insulin resistance; VO₂max, maximal oxygen uptake. *P < 0.05, †P < 0.01, ‡P < 0.001.

Table 2. Clinical characteristics of muscle precursor cell donors

<table>
<thead>
<tr>
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<tr>
<td>n</td>
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<td>5</td>
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<tr>
<td>Age, yr</td>
<td>64.8 ± 4.8</td>
<td>57.7 ± 9.9</td>
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<tr>
<td>BMI, kg/m²</td>
<td>25.1 ± 3.1</td>
<td>32.4 ± 2.7†</td>
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<tr>
<td>Fasting glucose, mM</td>
<td>5.5 ± 0.3</td>
<td>8.9 ± 2.6*</td>
</tr>
<tr>
<td>OGTT, mM</td>
<td>5.5 ± 3.1</td>
<td>14.0 ± 4.8†</td>
</tr>
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</table>

Data are presented as mean ± SD. Ctrl, healthy controls; DM, patients with type 2 diabetes; OGTT, glucose value at 2 h after oral glucose tolerance test. *P < 0.05, †P < 0.01.
cDNA and SYBR Green PCR Master Mix (Applied Biosystems) in a total reaction volume of 10 μl. 18S was amplified using predeveloped assay reagents (Applied Biosystems) and mixed with cDNA and TaqMan Universal Master Mix (Applied Biosystems) in a total reaction volume of 10 μl. Detection of mRNA levels was performed in triplicates using an ABI PRISM 7900 sequence detector (Applied Biosystems). To adjust for variations in the cDNA synthesis, each gene was normalized to that of 18S ribosomal RNA using the comparative \(2^{-\Delta\Delta CT}\) method (23).

Statistical analysis. All analyses were performed using SAS software version 9.1. If data were not normally distributed, a logarithmic transformation was performed. For comparisons between two groups, Student’s \(t\)-test was used. For evaluation of LIF-induced signaling over time in control cells and diabetic cells, two-way ANOVA for repeated measures was performed. The residuals obtained from the ANOVA models were evaluated, and the model was accepted only if the residuals were normally distributed. Normally distributed data are presented as means ± SE, and log-transformed data are presented as geometric means ± SE. A level of \(P < 0.05\) was accepted as statistically significant.

RESULTS

LIF and LIFR protein levels were increased in diabetic skeletal muscle and myoblasts. The clinical characteristics of the 47 individuals in this study are presented in Table 1. Muscular LIF

Table 3. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>Human LIF</td>
<td>5'-gtccaggttgtgaggggaac-3'</td>
<td>5'-tgccatgccctctttatc-3'</td>
</tr>
<tr>
<td>Human LIFR</td>
<td>5'-tcacacccccatgcacag-3'</td>
<td>5'-gtatgcagttggtgcacacag-3'</td>
</tr>
<tr>
<td>Human JunB</td>
<td>5'-gctgctttaggtttgga-3'</td>
<td>5'-aatccagctacggagatctg-3'</td>
</tr>
<tr>
<td>Human cyclin D1</td>
<td>5'-gacctctcatctctga-3'</td>
<td>5'-gaagatcgtggcacttg-3'</td>
</tr>
<tr>
<td>Human SOCS3</td>
<td>5'-agacctcagttcggacca-3'</td>
<td>5'-taggcctcttgtacag-3'</td>
</tr>
<tr>
<td>Human myoD</td>
<td>5'-cactacagcggcactec-3'</td>
<td>5'-gtctctgagactctc-3'</td>
</tr>
<tr>
<td>Human myogenin</td>
<td>5'-gctgctttaggtttgga-3'</td>
<td>5'-gtctctgagactctc-3'</td>
</tr>
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</table>

LIF, leukemia-inhibitory factor; LIFR, LIF receptor.

Fig. 1. Leukemia-inhibitory factor (LIF) and LIF receptor (LIFR) levels in muscle tissue from normal glucose-tolerant individuals (NGT) and type 2 diabetes (T2D) individuals. Muscle biopsies were obtained from vastus lateralis muscle from NGT (n = 26) and T2D (n = 21). A: LIF mRNA expression; geometric mean is shown. B: LIF protein, expressed as means ± SE. C: LIFR mRNA expression; geometric mean is shown. D: LIFR protein, expressed as means ± SE. AU, arbitrary units; RB, reactive brown membrane stain. *\(P < 0.05\), ***\(P < 0.001\).
mRNA levels were upregulated in patients with type 2 diabetes compared with individuals with normal glucose tolerance (1.09 ± 0.18 vs. 0.61 ± 0.12, \( P < 0.05 \); Fig. 1A). In accordance, LIF protein was increased in muscle biopsies from patients with type 2 diabetes compared with individuals with normal glucose tolerance (1.18 ± 0.14 vs. 0.87 ± 0.08, \( P < 0.05 \); Fig. 1B). LIFR mRNA expression was not different in the two subject groups (Fig. 1C). However, in line with the prediction analysis based on diabetes-differentially expressed miRNA performed by Gallagher et al. (10), LIFR protein was increased in muscle biopsies from patients with type 2 diabetes compared with individuals with normal glucose tolerance (1.18 ± 0.14 vs. 0.87 ± 0.08, \( P < 0.05 \); Fig. 1D). Although LIF and LIFR mRNA levels were not different between myoblasts derived from healthy individuals (control myoblasts) and patients with type 2 diabetes (DM myoblasts; Fig. 2, A and C, and Table 2), both LIF protein (\( P < 0.01 \); Fig. 2B) and LIFR protein (\( P < 0.05 \); Fig. 2D) were also upregulated in DM myoblasts. Control and DM myoblasts had the same levels of myogenin and MyoD mRNA (data not shown), indicating that the two groups were at the same myogenic stage.

**LIF signaling was decreased in DM myoblasts.** The above results raised the question whether downstream LIF signaling would be increased or decreased in DM myoblasts. Human recombinant LIF increased the ratio of pSTAT1(\( \text{Y}^701 \))/STAT1 \( (P < 0.001) \) in control and DM myoblasts, however, the ratio of LIF-induced pSTAT1(\( \text{Y}^701 \))/STAT1 was decreased in DM myoblasts compared with control myoblasts \( (P < 0.05) \); Fig. 3A). Interestingly, the basal expression of STAT1 was increased in DM myoblasts (Control myoblasts 0.57 ± 0.13 vs. DM myoblasts 1.92 ± 0.30, \( P < 0.05 \) and could explain the decreased LIF-stimulated pSTAT1(\( \text{Y}^701 \))/STAT1 ratio in DM myoblasts. LIF also increased the ratio of pSTAT3(\( \text{Y}^705 \))/STAT3 \( (P < 0.001) \) in control and DM myoblasts. However, the ratio of pSTAT3(\( \text{Y}^705 \))/STAT3 was reduced in DM myoblasts compared with control myoblasts \( (P < 0.05) \); Fig. 3B). There was no difference in the total amount of STAT3 protein between the two groups. The negative regulator of LIF signaling, SOCS3, was unaffected by LIF stimulation at the investigated time points but was substantially increased in DM myoblasts \( (P < 0.05) \); Fig. 3C).

To further investigate whether reduction of SOCS3 would rescue LIF-induced phosphorylation of STAT1 and STAT3 in DM myoblasts, SOCS3 was knocked down using siRNA. Validation of SOCS3 knockdown was performed using Western blot and qPCR. SOCS3 was significantly downregulated at
mRNA levels in SOCS3 siRNA-treated samples compared with control-treated samples in both control myoblasts \((P < 0.05)\) and DM myoblasts \((P < 0.05; \text{Fig. } 4A)\), whereas at the protein level SOCS3 was significantly downregulated in DM myoblasts \((P < 0.05)\) and also tended to be downregulated in control myoblasts \((P = 0.086; \text{Fig. } 4B)\). As the reduction of SOCS3 protein in DM myoblasts was greater than in control myoblasts, the levels of SOCS3 protein were comparable in control and DM myoblasts after SOCS3 knockdown in both groups.

LIF in combination with SOCS3 knockdown increased the ratio of pSTAT1/STAT1 tended to be increased in DM myoblasts when scrambled control was compared with SOCS3 knockdown (without LIF), and the ratio did not increase further with LIF treatment (Fig. 4C). LIF in combination with SOCS3 knockdown also increased the ratio of pSTAT3/STAT3 in myoblasts after SOCS3 knockdown \((P < 0.001)\), but the ratio remained markedly higher in control myoblasts than in DM myoblasts despite a comparable SOCS3 protein level in the two transfected groups \((P < 0.01; \text{Fig. } 4D)\). Thus, SOCS3 knockdown could not rescue LIF signaling through either STAT1 or STAT3, suggesting that additional control mechanisms are involved and altered in DM myoblasts.

**LIF-induced cell proliferation was impaired in DM myoblasts.** We have recently shown that in myocytes LIF stimulates expression of cyclin D1, JunB, and c-myc \((5)\), which are indicative of myoblast proliferation and have STAT3 binding sites in their promoters \((6, 9, 19, 25, 27, 40)\). We repeated this
finding as cyclin D1, JunB, and c-myc (borderline) were induced by LIF in a time-dependent fashion in myoblasts; cyclin D1 mRNA \((P < 0.05; \text{Fig. } 5A)\), JunB mRNA \((P < 0.01; \text{Fig. } 5B)\), and c-myc mRNA \((P = 0.08; \text{Fig. } 5C)\). However, the LIF-induced expression of the three factors was blocked in DM myoblasts, with a significant effect of group on cyclin D1 mRNA \((P < 0.05; \text{Fig. } 5A)\), JunB mRNA \((P < 0.05; \text{Fig. } 5B)\), and c-myc mRNA \((P < 0.001; \text{Fig. } 5C)\). Strikingly, SOCS3 knockdown fully rescued LIF-induced expression of cyclin D1, JunB, and c-myc in DM myoblasts (there was no statistical difference between LIF signaling in control myoblasts and DM myoblasts after SOCS3 knockdown; Fig. 5, A–C). LIF-induced gene expression in SOCS3 siRNA-treated control myoblasts was statistically comparable to untreated control myoblasts. The mRNA expressions of cyclin D1, JunB, and c-myc were not different between control myoblasts and DM myoblasts under basal conditions (data not shown). LIF increased cell proliferation, estimated by BrdU incorporation, in control myoblasts with and without SOCS3 knockdown \((P < 0.05; \text{Fig. } 6A)\). Nonetheless, LIF did not induce cell proliferation in DM myoblasts, and we
were unable to demonstrate any rescue effect of SOCS3 knockdown in DM myoblasts on LIF-induced cell proliferation (Fig. 6A). Further supporting a deficient LIF signaling in DM myoblasts, we found that LIFR knockdown only decreased proliferation in control myoblasts ($P < 0.05$), whereas proliferation of DM myoblasts was unaffected (Fig. 6B). This was despite a more significant knockdown of LIFR in the DM myoblasts at both mRNA level ($P < 0.01$ for DM myoblasts and $P < 0.05$ for control myoblasts; Fig. 6C) and protein level, where LIFR was only significantly knocked down in DM myoblasts ($P < 0.05$; Fig. 6D). LIF has been shown to affect myoblast number by decreasing apoptosis (17). We found that LIF decreased caspase-3 activity in control myoblasts ($P < 0.05$), but the LIF-stimulated decrease in caspase-3 activity was not significant in DM myoblasts (Fig. 7).

**DISCUSSION**

We demonstrate an increased protein expression of LIF and LIFR in muscle tissue and cultured myoblasts from diabetic patients. However, following LIF administration, DM myoblasts demonstrated a deficient LIF signaling in terms of reduced activation of STAT3 and reduced induction of STAT3-dependent markers of proliferation, including c-myc, JunB, and cyclin D1, as well as a lack of induction of BrdU incorporation during proliferation. This suggests that LIF resistance in muscle precursor cells might be part of the muscular pathophysiology of type 2 diabetes.

This study combines LIF and LIFR expression analyses of skeletal muscle tissue from a well-characterized human cohort ($n = 47$) with mechanistic studies of LIF signaling in human muscle precursor cells. Our muscle tissue data were in line with the results obtained when culturing isolated muscle precursor cells, hereby showing that a deficiency in muscular LIF response is established already in the muscle precursor cells in patients with type 2 diabetes. Given the well-described role of LIF during muscle precursor cell proliferation (1, 5, 33, 35), this further emphasizes the potential importance of LIF-signaling deficiency in diabetes. Our replications in the cell experiments represent primary cells from separate individuals rather than repeated experiments using the same cell line. By using this approach, we account not just for technical variation but also for biological variation in our sample set. In line with previous studies investigating muscle precursor cells from patients with type 2 diabetes (11, 12, 14), this methodological design allowed us to conclude that the in vivo phenotype is retained in the muscle precursor cells. Whereas previous studies of myoblasts derived from insulin-resistant and type 2 diabetic individuals have focused on pathways involved in the metabolic response (11, 12, 18, 36), we aimed to investigate a pathway involved in regulating muscle precursor cells. Thus, this is the first study to investigate the proliferative status of muscle precursor cells derived from patients with type 2 diabetes.

A weakness of this study is the difference in BMI between the human donors of the muscle precursor cells; the control muscle precursor cell donors had a BMI of 25.1 ± 3.1, whereas the type 2 diabetes muscle precursor cell donors had a significantly higher BMI of 32.4 ± 2.7 (Table 2), raising the question whether the differences in LIF response between the subject groups are related to obesity rather than type 2 diabetes. However, we show that both LIF and LIFR upregulation in human muscle tissue was clearly

Fig. 5. Responsiveness of cyclin D1, JunB, and c-myc to LIF stimulation in Ctrl and DM myoblasts. Myoblasts were isolated from Ctrl and DM individuals. SOCS3 knockdown was performed in Ctrl myoblasts (Ctrl + SOCS3 KD) and DM myoblasts (DM + SOCS3 KD) 48 h before LIF stimulations. Proliferating myoblasts were stimulated with LIF for 3 and 6 h. A: cyclin D1 mRNA expression. B: JunB mRNA expression. C: c-myc mRNA expression. Time effect was evaluated by 2-way ANOVA including all groups in the model. Group effect was evaluated by comparing groups in pairs by 2-way ANOVA. Data are expressed as means ± SE; $n = 5$ in each group.
occurring in patients with type 2 diabetes and not in obese individuals with normal glucose tolerance, indicating that obesity is not enough to cause LIF and LIFR up regulation.

We demonstrate a resistance to LIF signaling in muscle precursor cells derived from patients with type 2 diabetes. As LIF is important in both muscle hypertrophy (34) and regeneration (2, 20, 41), this finding implies that the regulation of muscle adaptation and regeneration in patients with type 2 diabetes might be disturbed. This idea is in line with the recent findings from a study considering global miRNA expression in the skeletal muscle of patients with type 2 diabetes identifying 62 differentially expressed miRNAs and predicting developmental processes as one of the main differentially targeted mechanisms (10). Interestingly, our current study also provides further laboratory verification of the prediction model developed in that global miRNA study as LIFR, among other targets, was predicted to be upregulated at the protein level (10). Furthermore, the observation that LIFR protein was upregulated in skeletal muscle of diabetic individuals is in accord with a recent study reporting increased muscular LIFR production in a mouse model of diabetes (37). This suggests that overproduction of LIFR in skeletal muscle may be an important event in the muscular pathophysiology of diabetes leading to altered cytokine signaling in skeletal muscle.

LIF and LIFR expression profiles in muscle tissue are retained in myoblasts in vitro, suggesting that the diabetic environment modulates the epigenetic regulation of LIF or LIFR promoters in the quiescent muscle precursor cells. Indeed, alterations in the microenvironment caused by both
metabolic diseases like type 2 diabetes and the nutritional composition can, over time, induce epigenetic modifications (3, 21, 22, 30, 38). Previous studies have shown that the phenotype is retained in isolated muscle precursor cells established from patients with type 2 diabetes in terms of reduced glucose transport (11) and increased inflammatory state (14). This indicates that diabetes induces epigenetic alterations in several genes. The LIF promoter was recently found to be demethylated in breast cancer cells, resulting in increased LIF production (32). However, whether LIF and LIFR genes are subjected to epigenetic regulation in diabetic skeletal muscle has not been investigated. Nonetheless, this study shows that the muscle precursor cells, in addition to mature muscle fibers, are “permanently” affected by type 2 diabetes.

The finding that both LIF and LIFR proteins were increased in DM myoblasts could suggest that downstream LIF signaling was overactivated. Surprisingly, LIF activation of STAT1 and STAT3 was impaired, suggesting that DM myoblasts are resistant to LIF signaling. In accord with the finding that SOCS3 mRNA levels are increased in skeletal muscle of patients with type 2 diabetes in vivo (31), we observed an increase in SOCS3 protein expression in DM myoblasts. SOCS3 inhibits LIF signaling and cell proliferation induced by the JAK1-STAT1-STAT3 pathway (8). Thus, our finding that SOCS3 was increased in DM myoblasts could possibly explain the decreased LIF signaling through this pathway. Nonetheless, SOCS3 siRNA knockdown in DM myoblasts did not rescue LIF-induced STAT1 and STAT3 signaling, suggesting that other factors are responsible for the inhibitory effects on proximal LIF signaling in DM myoblasts.

A reduced proliferative capacity has been demonstrated in smooth muscle cells isolated from patients with type 2 diabetes (24). In this study, we show that LIF-induced cell proliferation is impaired in myoblasts from patients with type 2 diabetes. This was supported by the finding that cyclin D1, JunB, and c-myc, which promote cell proliferation in several cell types, including myoblasts (6, 27, 40), were unresponsive to LIF activation in DM myoblasts. Strikingly, SOCS3 knockdown fully rescued LIF-induced expression of cyclin D1, JunB, and c-myc in DM myoblasts, suggesting that SOCS3 inhibits cell cycle-promoting genes in DM myoblasts. Nonetheless, SOCS3 knockdown did not restore LIF-stimulated myoblast proliferation, signifying that factors in addition to SOCS3 are responsible for the impaired LIF signaling in DM myoblasts. Knockdown of the LIFR only significantly reduced proliferation of control myoblasts, not DM myoblasts, further validating that LIF-regulated cell proliferation is disturbed in DM myoblasts. Interestingly, recent studies in C2C12 cells have suggested that LIF increases myoblast cell number by decreasing apoptosis (16, 17). In this study, we confirmed that, in healthy human myoblasts, LIF reduces apoptosis, estimated by caspase-3 activation. This function of LIF was impaired in DM myoblasts, suggesting that diabetes affects different LIF signaling pathways. Thus, in human myoblasts LIF increases myoblast number by increasing mitosis and decreasing apoptosis, and in DM myoblasts both of these functions of LIF are impaired.

In conclusion, LIF and LIFR proteins were upregulated in both muscle biopsies and myoblasts from individuals with type 2 diabetes. Nonetheless, LIF signaling through STAT1 and STAT3 was abrogated, and LIF failed to induce proliferation of myoblasts from diabetic subjects. Parts of the resistance to LIF signaling could be ascribed to increased levels of SOCS3 in diabetic myoblasts. By investigating LIF signaling in myoblasts from diabetic individuals, we have identified a novel mechanism whereby skeletal muscle precursor cell regulation is altered in diabetic muscle. This may have severe consequences for muscle regeneration and hypertrophy in patients with diabetes. Whether other pathways involved in muscle precursor cell regulation are altered in diabetic muscle remains to be investigated in future studies.

GRANTS

The Centre of Inflammation and Metabolism is supported by a grant from the Danish National Research Foundation (no. 02-512-55). The study was further supported by the Danish Medical Research Council for Independent Research: Medical Sciences and the Commission of the European Communities (contract no. LSHM-CT-2004-005272). The Copenhagen Muscle Research Centre is supported by grants from the Capital Region of Denmark and the University of Copenhagen.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

C. Broholm, B.K.P., and C.S. designed study; C. Broholm, C. Brandt, N.S.S., and A.R.N. researched and interpreted data; C. Broholm prepared figures and tables; C. Broholm, and C.S. drafted article; C. Broholm, C. Brandt, N.S.S., A.R.N., and C.S. revised manuscript and approved final version.

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