Synergistic stimulation of myogenesis by glucocorticoid and IGF-I signaling

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Pansters NA, Langen RC, Wouters EF, Schols AM. Synergistic stimulation of myogenesis by glucocorticoid and IGF-I signaling. J Appl Physiol 114: 1329–1339, 2013. First published August 30, 2012; doi:10.1152/japplphysiol.00503.2012.—Muscle wasting is associated with poor prognosis in chronic obstructive pulmonary disease (COPD). Exercise stimulates muscle recovery, but its efficacy is variable, depending on the clinical condition and medical treatment. Systemic glucocorticoids, commonly administered in high doses during acute disease exacerbations or as maintenance treatment in end-stage disease, are known to contribute to muscle wasting. As muscle mass recovery involves insulin-like growth factor (IGF)-I signaling, which can be stimulated by anabolic steroids, the impact of glucocorticoids and the effect of simultaneous IGF-I stimulation by anabolic steroids on muscle recovery and growth were investigated. The effects of, and interactions between, glucocorticoid and IGF-I signaling on skeletal muscle growth were assessed in differentiating C2C12 myocytes. As proof of principle, we performed a post hoc analysis stratifying patients by glucocorticoid use of a clinical trial investigating the efficacy of anabolic steroid supplementation on muscle recovery in muscle-wasted patients with COPD. Glucocorticoids strongly impaired protein synthesis signaling, myotube formation, and muscle-specific protein expression. In contrast, in the presence of glucocorticoids, IGF-I synergistically stimulated myotube fusion and myofibrillar protein expression, which corresponded with restored protein synthesis signaling by IGF-I and increased transcriptional activation of muscle-specific genes by glucocorticoids. In COPD patients on maintenance glucocorticoid treatment, the clinical trial also revealed an enhanced effect of anabolic steroids on muscle mass and respiratory muscle strength. In conclusion, synergistic effects of anabolic steroids and glucocorticoids on muscle recovery may be caused by relief of the glucocorticoid-imposed blockade on protein synthesis signaling, allowing effective translation of glucocorticoid-induced accumulation of muscle-specific gene transcripts.

protein synthesis; glucocorticoid receptor; anabolic steroid; muscle atrophy; muscle regeneration

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is a chronic disabling disease characterized by progressive, irreversible airflow obstruction, skeletal muscle wasting, and weakness. It is well established that muscle wasting negatively impacts physical performance and mortality, independent of the severity of airflow obstruction (38). Therefore, muscle mass maintenance or recovery after exacerbation-induced catabolic stress is considered an important therapeutic target. Effective and feasible single or multimodal therapeutic approaches focusing on muscle recovery are currently limited, and further development requires elucidation of the mechanisms governing muscle plasticity during muscle atrophy and recovery.

COPD-related factors that may contribute to muscle atrophy include disuse, inflammation, and systemic glucocorticoids (GC) (12, 28, 35, 55). GCs are frequently applied during acute exacerbations and sometimes also as maintenance treatment during end-stage disease. The efficacy of GC as maintenance medication is controversial (19), and Schols et al. (40) even reported a dose-dependent increased mortality risk in severe disease. In addition, proposed catabolic effects of GC could impair beneficial effects of pulmonary rehabilitation (11). Nevertheless, ill-manageable, frequently exacerbating patients often receive long-term, low-dose GC.

Besides increased proteolysis, GC-induced muscle atrophy involves decreased protein synthesis signaling. This relies on reduced activity of mammalian target of rapamycin (mTOR) complex-I (mTORC1) and decreased phosphorylation of its downstream targets p70-S6K1 and 4E-BP1, which stimulate translation capacity and suppress translation initiation, respectively (14, 17, 27, 34, 37, 58). Conversely, anabolic steroids, like nandrolone decanoate (ND), prevent muscle atrophy and stimulate muscle growth and hypertrophy, partly by increasing muscle insulin-like growth factor (IGF)-I production and signaling in a para/autocrine fashion (18, 23, 41, 44, 50). In contrast to GC, IGF-I signaling reduces proteolysis and increases protein synthesis, including mTORC1 signaling (17, 45).

Muscle regeneration and myogenesis involve activation, proliferation, and subsequent differentiation of satellite cells into myoblasts that fuse with existing or form new myofibers (7, 9, 53). This also involves increased muscle-specific gene expression, including contractile/sarcomeric proteins, e.g., myosin heavy chain (MHC), myosin light chain (MLC), and enzymes involved in muscle energy metabolism, e.g., muscle creatine kinase (MCK), through increased transcriptional activity of the muscle regulatory factors (MRFs) (32). Myogenesis can be stimulated by anabolic steroids (53), and their potential to restore muscle function during pulmonary rehabilitation has been studied in COPD patients (10, 26, 42).

The effects of GC on myogenesis and muscle recovery have received limited attention. Therefore, we investigated the effects of GC and the interaction with anabolic signaling on muscle regrowth using a translational research approach. First, the effect of GC on and interaction with IGF-I signaling on myogenesis was evaluated in differentiating myocytes. As proof of principle, a randomized controlled trial (RCT) assessing the effect of ND supplementation on muscle recovery in wasted male COPD patients, of which a subpopulation was on chronic GC medication (10), was reanalyzed.
MATERIALS AND METHODS

Cell culture. The murine skeletal muscle cell line C2C12 (ATCC no. CRL1772) was cultured in growth medium (GM). This was composed of low-glucose Dulbecco’s modified Eagle’s medium (DMEM) containing antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) and 9% (vol/vol) fetal bovine serum (from Gibco, Rockville, MD). C2C12 cells were plated at 10^4/cm^2 on Matrigel (BD Biosciences, Bedford, MA) coated (1:50 in DMEM low glucose) dishes, as described previously (25), over night in GM. To induce spontaneous differentiation by growth factor withdrawal (56), GM was replaced with differentiation medium (DM), which contained low-glucose DMEM with 1.0% heat-inactivated fetal bovine serum and antibiotics. As indicated additionally, differentiation was induced in the presence of murine IGF-I (Calbiochem, La Jolla, CA), with or without the GxS dexamethasone (Dex) or prednisolone (Pred) added directly with induction of differentiation, and again 24 h later when in the presence of murine IGF-I (Calbiochem, La Jolla, CA), with or without Dex (5, 6, 15 μM) was used. To obtain mature myotubes, myoblasts were cultured in DM to differentiate, with medium changes at 24 and 72 h for 5 days. At day 5, medium was changed with or without the presence of murine IGF-I and with or without the GC Dex.

Transfections and plasmids. Transient transfections were performed using nanofectin (PAA, Pasching, Austria) and in all cases included cotransfection with pSV-β-gal to correct for differences in transfection efficiency (Promega, Madison, WI). According to manufacturer’s instructions, 1.0 μg plasmid per 3.2 μl nanofectin was used. Per transfection, 1.0–2.5 μl luciferase and β-galactosidase (Tropix, Bedford, MA) were measured according to the manufacturer’s instructions. For SDS-PAGE 0.5–15 μg protein were loaded per lane and separated on a Criterion XT Precast 4–12% Bis-Tris gel (Bio-Rad, no. 3450124), followed by transfer to a 0.45-μm Whatman Protran Nitrocellulose Transfer membrane (Whatman, no. 7324007) by electro-blotting using a Bio-Rad Criterion Blotter (Bio-Rad, Hercules, CA). The membrane was blocked for 1–2 h at room temperature in 5% (wt/vol) nonfat dried milk (ELK, Campina, the Netherlands) diluted in Tris-buffered saline (TBS)-Tween 20 (0.05%). Nitrocellulose blots were washed in TBS-Tween 20 (0.05%) on a rotating platform, followed by overnight incubation at 4°C with specific antibodies directed against the following: phosphorylated (p)-Akt (Ser473) (no. 9271), Akt (no. 9272), GAPDH (no. 2118), p-mTOR (no. 2971), mTOR (no. 2972), p-p70-S6K1 (no. 2906), p70-S6K1 (no. 2902), total 4E-BP1 (no. 9452) (all from Cell Signaling Technology, Danvers, MA), and MLC-1 and -3 (no. F310) (Developmental Studies Hybridoma Bank, Iowa City, IA), all diluted in TBS/0.05% Tween 20, with or without 5% BSA/nonfat dried milk. After three washing steps of 10 min each, the blots were probed with a peroxidase conjugated secondary antibody (Vector Laboratories, no. PI-1000) and visualized by enhanced chemiluminescent Substrate (Pierce Biotechnology), according to the manufacturer’s instructions and exposed to film (Biomax light film, KODAK) or live imaged (Bio-Rad chemidoc XRS). Western blot images were quantified using the Quantity One analysis software from Bio-Rad.

RNA isolation and assessment of mRNA abundance by RT-quantitative PCR. C2C12 cells were washed twice with ice-cold 1× PBS, after which RLT solution containing 1% β-mercaptoethanol was added. Cells were scraped and DNA shearing was achieved by 8–10× passing the lysate through a 20-G needle, after which samples were stored at –80°C. Samples were further processed according to manufacturer’s instructions of the RNeasy Mini Kit of Qiagen, including the on-column DNase treatment. RNA was reconstituted in 30- to 50-μl RNase free water and stored at –80°C. The RNA concentrations were measured using a Nanodrop ND-1000 UV-Vis spectrophotometer. RNA was diluted to >5× in double-distilled H2O, and 400 ng of RNA were reverse transcribed to cDNA using the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) with anchored oligo-dT primers, according to the manufacturer for generating cDNA fragment of 4 kb with a final reaction volume of 20 μl. RNA of genes of interest (Table 1) was determined by reverse transcription quantitative PCR (qPCR). qPCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA), checked for both primer and amplicon secondary structures, and then obtained from Sigma Genosys (Haverhill, UK). qPCR reactions (2 μl final volume) contained 10 μl Sensimix SYBR Fluorescein (Bioline, Alphen a.d. Rijn, the Netherlands) 0.6 μl of forward and reverse primer, and 3.8 μl H2O. Relative cDNA starting quantities for

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the samples were derived by the standard curve method. Standards curve samples were generated by serial dilution of pooled cDNA samples and had at least an $R^2 > 0.98$ and an efficiency between 90 and 110%. The expression of the genes of interest (Table 1) were normalized with a correction factor derived by GeNorm, which is based on a combination of the expression levels of GAPDH and RPL13A, ARBP, calnexin, and R2M. RT-qPCR reactions were performed on a MyiQ single-color real-time thermal cycler (Bio-Rad, Hercules, CA).

Proof of concept randomized clinical trial. Sixty-three muscle-wasted male COPD patients (mean age, 66 ± 8 yr) with severe COPD (forced expiratory volume in 1 s, 36 ± 14% predicted) were randomized to receive either ND or placebo treatment as adjunct to a 8-wk intervention. Fat free mass (FFM) was determined by deuterium-wasted male COPD patients (mean age, 66 yr) with severe COPD (1.63 ± 1.75 vs. 0.66 ± 0.70, $P < 0.01$). For a full description of the trial, we refer to the original report (10). The trial was approved by the medical ethical committee of the University Hospital Maastricht, and all subjects gave their informed consent in writing. Baseline characteristics for the four groups after stratification are shown in Table 2. All subjects gave their informed consent in writing.

Statistical analysis. Statistical analysis of muscle cell culture experiments was performed by $t$-test for independent samples with unequal variance. Significance was determined at the level of $P < 0.05$, and data are expressed as means ± SD. Statistical analysis of the clinical trial was performed in the per-protocol group ($n = 29–32$). Differences between the groups at baseline were analyzed by $t$-test for independent samples. Changes within the groups between baseline and week 8 were tested by paired $t$-test. Differences in the treatment response after 8 wk of ND or placebo were tested using one-way ANOVA with post hoc least significant difference. Significance was determined at the level of $P < 0.05$. All data were analyzed using SPSS/PC+ (Statistical Package for the Social Sciences, version 17.0 for Windows; SPSS, Chicago, IL).

Table 1. Quantitative PCR primer sequences of genes of interest and reference genes

<table>
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<tr>
<th>Target</th>
<th>Full Target Name</th>
<th>Target Type</th>
<th>Genbank Identifier</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<tr>
<td>REDD1</td>
<td>Ddit4</td>
<td>GOI</td>
<td></td>
<td>CCGGCCGCGAGGAGAAAGACT</td>
<td>CTGCATCGAGTTGCGACACAGA</td>
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<td>Glut</td>
<td>Glutamate ammonia ligase, glutamine synthetase</td>
<td>GOI</td>
<td></td>
<td>GGCCATGGGAGGAGGAAGAA</td>
<td>GTCGCTCTTGCTGCACTTGTGCA</td>
</tr>
<tr>
<td>KLF15</td>
<td>Kruppel-like factor 15</td>
<td>GOI</td>
<td>NM_00710</td>
<td>TGGAGAGATGACGCAGGAG</td>
<td>ATGCAGCGCTGCTGAC</td>
</tr>
<tr>
<td>MCK</td>
<td>Muscle creatine kinase</td>
<td>GOI</td>
<td></td>
<td>AGGGTTTCCGGCGCTCTCT</td>
<td>CGGTCGAGTCCCTGGA</td>
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<tr>
<td>MyoD1</td>
<td>Myoblast determination protein 1</td>
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<tr>
<td>MyoD1</td>
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<td>GOI</td>
<td></td>
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<td>MHC IIB</td>
<td>Myosin heavy chain 2B</td>
<td>GOI</td>
<td></td>
<td>ACAAGCTCGGGTGAAAAGCC</td>
<td>AGCAAGCTCGGAAGAA</td>
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<tr>
<td>MHC peri</td>
<td>Myosin heavy chain perinatal</td>
<td>GOI</td>
<td></td>
<td>ACAAGCTCGGGTGAAAAGCC</td>
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<td>ARBP</td>
<td>60S acidic ribosomal protein P0 (L10E)/Rppp0</td>
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<td></td>
<td>GGACCCGAGAAGACGACTCTCTG</td>
<td>GCACTACGAGAATTTGAAGG</td>
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<tr>
<td>RPLA13A</td>
<td>60S ribosomal protein L13a (transplantation antigen P198)(Tum-P198 antigen)</td>
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<td>NM_009438.4</td>
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<td>GACAGCATGAGAAAGAACAGTGC</td>
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<td>β2M</td>
<td>Beta-2 microglobuline</td>
<td>RG</td>
<td></td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>RG</td>
<td></td>
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<td>TGCCAGTGGCATTG</td>
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<tr>
<td>Cyclophilin</td>
<td>Peptidylprolyl isomerase A</td>
<td>RG</td>
<td>X52803</td>
<td>TGCCCTGGTACACAAGAATTTCACA</td>
<td>GGCCAGTGCGCATTATG</td>
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</tbody>
</table>

GOI, genes of interest; RG, reference genes.

Table 2. Baseline patient characterization after post hoc stratification according to glucocorticoid usage

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No Glucocorticoid</th>
<th>Glucocorticoid</th>
<th>$P$ Value</th>
</tr>
</thead>
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<tr>
<td>Age, yr</td>
<td>65.31 ± 7.41</td>
<td>67.55 ± 8.39</td>
<td>0.266</td>
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<tr>
<td>FEV1, %predicted</td>
<td>31.93 ± 9.20</td>
<td>40.05 ± 16.68</td>
<td>0.019</td>
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<tr>
<td>FVC, %predicted</td>
<td>84.92 ± 15.38</td>
<td>83.98 ± 16.61</td>
<td>0.817</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21.65 ± 3.48</td>
<td>21.41 ± 3.55</td>
<td>0.789</td>
</tr>
<tr>
<td>FFM index by D₂O, kg/m²</td>
<td>15.87 ± 1.71</td>
<td>15.60 ± 1.36</td>
<td>0.500</td>
</tr>
<tr>
<td>Inspiratory muscle strength, cmH₂O</td>
<td>79.81 ± 20.19</td>
<td>77.81 ± 21.51</td>
<td>0.704</td>
</tr>
<tr>
<td>Peak cycling workload, %predicted</td>
<td>38.43 ± 15.69</td>
<td>38.36 ± 16.41</td>
<td>0.986</td>
</tr>
<tr>
<td>CRP, mg/ml</td>
<td>20.74 ± 4.18</td>
<td>22.21 ± 25.25</td>
<td>0.804</td>
</tr>
<tr>
<td>Total testosterone, mmol/l</td>
<td>13.89 ± 4.18</td>
<td>14.38 ± 5.34</td>
<td>0.693</td>
</tr>
<tr>
<td>Reported recent weight loss, %</td>
<td>56</td>
<td>55</td>
<td>0.912</td>
</tr>
<tr>
<td>No. of hospitalizations during last year</td>
<td>0.66 ± 0.70</td>
<td>1.63 ± 1.75</td>
<td>0.005</td>
</tr>
<tr>
<td>Oral glucocorticoid dose, mg/24 h</td>
<td>0 ± 0</td>
<td>7.46 ± 2.42</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SD; $n$, no. of subjects. FEV₁, forced expiratory volume in 1 s; FVC, inspiratory vital capacity; BMI, body mass index; FFM, fat free mass, CRP, C-reactive protein.
RESULTS

Synergistic stimulation of myotube formation by GCs and IGF-I. The synthetic GC Dex induces muscle atrophy and was used to induce GC signaling. Dex resulted in diminished morphological differentiation of C2C12 myocytes, whereas differentiation was enhanced by IGF-I compared with control (Fig. 1A). Combined, Dex and IGF-I enhanced morphological differentiation compared with IGF-I alone. Quantification of morphological differentiation using the myogenic index (Fig. 1B) clearly showed Dex reduced myoblast fusion. In contrast, IGF-I increased fusion, which was further exceeded by the combination of IGF-I and Dex. This not only resulted in more, but also larger, myotubes (Fig. 1C). Similar effects on myotube morphology were observed with equimolar as well as equipotent doses of Pred (Fig. 2).

GC receptor signaling is not impaired in presence of IGF-I. To evaluate the effects of IGF-I on GC signaling, GC receptor (GR) transcriptional activity was determined. A more than eightfold increase was observed by Dex alone, while, combined with IGF-I, an additional (45%) increase was detected (Fig. 3A). Endogenous GC-sensitive GR target genes Glul (glutamate ammonia ligase, glutamine synthetase), KLF-15 (Kruppel-like factor 15), and REDD1 (Ddit4) (Fig. 3, B–D) were all significantly induced by Dex, ranging from 2.5- to 55-fold increases, regardless of absence or presence of IGF-I, confirming that active GC-induced GR signaling is slightly attenuated, but not abrogated by IGF-I.

Synergism between GCs and IGF-I on muscle protein abundance. MCK is increasingly expressed during differentiation (51), which was suppressed by Dex (−50%) and increased with IGF-I (3.8-fold) or IGF-I/Dex (4.2-fold) treatment (Fig. 4A). Total protein after 72 h (data not shown) was lower after Dex (40%), while it was increased with IGF-I (2.4-fold) and IGF-I/Dex (2.0-fold). Sarcomeric protein content (Fig. 4B) revealed a decrease (−50%) in response to Dex compared with control (Fig. 4, C–E). Conversely, IGF-I increased sarcomeric protein content (≥2-fold) according to expectation. Dex and IGF-I combined increased MLC-1 and -3 abundance by 15 and 20%, respectively. MCK and MLC-1 and -3 tended to be increased for IGF-I/Dex compared with IGF-I. Similar results were also observed on muscle and in particular myofibrillar proteins when IGF-I was combined with Pred at equimolar as well as equipotent doses compared with Dex (Fig. 5). In addition, in differentiating myoblasts, MHC fast isoform abun-
dance increased significantly, more than twofold when comparing IGF-I/Dex to IGF-I alone. Importantly, this synergistic effect on myotube size (not shown), and in particular myofibrillar proteins, was not observed when identical concentrations of IGF-I/Dex were applied to fully differentiated myotubes (Fig. 4F). Altogether, these data reveal a potentiating effect of combined Dex and IGF-I on myotube size and myofibrillar protein expression selectively during myogenic differentiation.

Dex does not affect increased protein synthesis signaling by IGF-I. Considering that GC decreases protein synthesis (27, 54), it was surprising to find that increased MHC-fast accretion was observed during differentiation by the combination of IGF-I/Dex compared with IGF-I alone. Therefore, regulators of

Fig. 2. Synergistic stimulation of myotube formation by prednisolone (Pred) and IGF-I. C2C12 cells were differentiated for 72 h and treated with vehicle, IGF-I (5 nM), or Pred at equimolar (10 μM) or equipotent (50 μM) concentrations alone or in combination with IGF-I. Shown are representative data of three independent experiments.

Fig. 3. Glucocorticoid (GC) receptor (GR) signaling is not impaired in the presence of IGF-I. A: C2C12 myoblasts were transfected with a GRE-luciferase-reporter construct (0.9 μg) and plasmid encoding β-gal (0.1 μg), cultured for 48 h, treated with IGF-I (5 nM), Dex (10 μM), or VC, as indicated, and harvested for GC-induced GRE-luciferase activity. B–D: C2C12 cells [Glu1 (B), KLF-15 (C), REDD1 (D)] differentiated for 48 h and treated with IGF-I (5 nM), Dex (10 μM), or VC, as indicated, were harvested for mRNA quantification. Values are means ± SD. Shown are representative data of three independent experiments. Two-way t-test with unequal variance: *Ctrl vs. Dex; #Ctrl vs. IGF-I, P < 0.05. AU, arbitrary units.

A

B

C

D

Fig. 3. Glucocorticoid (GC) receptor (GR) signaling is not impaired in the presence of IGF-I. A: C2C12 myoblasts were transfected with a GRE-luciferase-reporter construct (0.9 μg) and plasmid encoding β-gal (0.1 μg), cultured for 48 h, treated with IGF-I (5 nM), Dex (10 μM), or VC, as indicated, and harvested for GC-induced GRE-luciferase activity. B–D: C2C12 cells [Glu1 (B), KLF-15 (C), REDD1 (D)] differentiated for 48 h and treated with IGF-I (5 nM), Dex (10 μM), or VC, as indicated, were harvested for mRNA quantification. Values are means ± SD. Shown are representative data of three independent experiments. Two-way t-test with unequal variance: *Ctrl vs. Dex; #Ctrl vs. IGF-I, P < 0.05. AU, arbitrary units.
protein synthesis signaling during differentiation in the presence of IGF-I and/or Dex were investigated next (Fig. 6A). Phosphorylation of 4E-BP1 was decreased by Dex (Fig. 6C), whereas it was increased by IGF-I with or without Dex. p70-S6K1 phosphorylation was strongly (70%) decreased by Dex (Fig. 6B). IGF-I, even in presence of Dex, was able to increase S6K1 phosphorylation more than twofold.

GCs upregulate muscle-specific gene expression. As a mere restoration of protein synthesis signaling by IGF-I did not explain the synergistic effects of IGF-I and GC on myotube formation and myosin expression, muscle-specific mRNA abundance during differentiation was assessed. After 48 h of differentiation, muscle-specific mRNA was quantified (Fig. 7, A–C). MCK expression was increased by IGF-I (90%), but surprisingly also by Dex...
Fig. 6. Dex does not affect increased protein synthesis signaling by IGF-I. C2C12 cells were serum starved for 18 h and then treated with IGF-I (5 nM), Dex (10 μM), or VC, as indicated, after 6 h. A: cells were harvested for Western blot analysis, and indicated phosphor (p) and total (t) proteins were detected to determine protein synthesis signaling status with induction of myogenic differentiation. p70S6K phosphorylation (B) and determination of 4E-BP1 phospho-isoform distribution (C) were quantitatively assessed. Two-way t-test with unequal variance: *Ctrl vs. Dex; #Ctrl vs. IGF-I, P < 0.05. mTOR, mammalian target of rapamycin.

Combined IGF-I/Dex increased MCK expression more than threefold compared with either IGF-I or Dex alone (Fig. 7A), indicating a synergistic interaction. This synergism between Dex and IGF-I during differentiation was consistently shown for MHC genes MYH-4 and MYH-8 mRNA transcripts (Fig. 7B, C). Since the differentiation status appeared to determine the sensitivity to this synergistic interaction, we next addressed the involvement of the MRFs, which are primarily active during myogenic differentiation. mRNA expression of myf-5 was increased by Dex and myogenin with Dex and IGF-I combined (Fig. 7D–F). MRF transcriptional activity was increased by IGF-I (2.4-fold) as expected, but even more pronounced increases were observed in response to Dex alone (3.4-fold) or Dex + IGF-I (7-fold) (Fig. 7G). Altogether, these data suggest that increased MRF activity in response to Dex and IGF-I, and subsequent restoration of mRNA translation by IGF-I, may lie at the basis of the potentiating interaction between IGF-I and GC on myogenic differentiation.

Synergy between nandrolone and GCs on muscle mass recovery in COPD patients with muscle wasting. Based on the observed results in myocytes with combined IGF and GC-induced signaling during myogenesis, a post hoc analysis stratifying for GC use of our ND RCT (10) was performed to address the influence of low-dose GC on ND efficacy. The ND treatment response was confirmed by reduced endogenous circulating total testosterone levels to 38 ± 21% of baseline levels, which was similar for GC and no-GC groups, whereas testosterone levels remained unchanged in the placebo (Ctrl) group. The gain in FFM (Fig. 8A) was greatest and only reached statistical significance in the ND/GC (2.27 ± 1.61 kg,
GC are frequently administered to COPD patients and are known to induce muscle atrophy (14, 20, 34), but little is known regarding their influence on muscle regrowth. Yet recovery of muscle mass is an important and main goal of pulmonary rehabilitation programs for muscle-wasted COPD patients. These pulmonary rehabilitation programs are often multimodal and typically include a personalized exercise program combined with nutritional support upon indication. The last two decades, several studies addressed complementation of this approach with muscle growth-stimulating pharmaceuticals, including anabolic steroids (20, 22). Many of the effects of anabolic steroids are a consequence of increased androgen receptor-mediated expression of IGF-I in skeletal muscle, which, in an auto/paracrine fashion, induces local IGF signaling and subsequent muscle growth (18, 23, 41, 44, 50). Therefore, we used IGF-I to model these steroid effects on myogenesis, which is an important component of muscle regrowth. In line with previous reports, IGF-I enhanced myotube formation and muscle-specific protein expression, including sarcomeric proteins (9, 30, 51). In contrast, the synthetic GC Dex potently inhibited all of these aspects of myogenesis. Although a few studies have reported stimulatory effects of GC on myogenic differentiation (29, 57), our findings are in agreement with most reports showing inhibitory effects of GC on myogenesis (27, 34, 54). These contradictory effects of GC on myogenesis may reflect a concentration-dependent biphasic response. Belanto et al. (2), for example, observed increased myoblast fusion in response to low GC levels, and we postulate that those results could be derived from an interaction between GC and anabolic factors, like IGF-I, present in the cell culturing serum. This notion is in line with the synergistic stimulation of myotube formation in response to simultaneous addition of GC and IGF-I during myoblast differentiation reported here and suggests that independent mechanisms lie at the basis of inhibition of myoblast fusion by GC and its synergistic stimulation when combined with IGF-I.

We and others previously demonstrated segregation between morphological and biochemical parameters of myogenic differentiation (2, 3, 33). However, the abundance of muscle-specific and particularly MHC proteins was consistently increased in the presence of IGF-I/GC compared with IGF-I only, indicating that the regulatory cues in control of both muscle-specific gene expression and myoblast fusion corresponded. The effects of simultaneous GC and IGF-I signaling on myogenesis were not previously described, but in mature myotubes IGF-I prevents GC-induced muscle atrophy (17, 36). Although IGF-I and Dex at the concentrations applied in our study did not affect myofibrillar protein content in myotubes, importantly, no synergistic effect on myotube size or myofibrillar protein accretion in myotubes was observed, in contrast to differentiating myoblasts. In myotubes, prevention of GC-induced atrophy partly results from restoration of protein synthesis (17, 27, 54), as Dex-induced decreases in phosphorylation of 4E-BP1 and p70-S6K1, two key regulatory proteins of mRNA translation, is prevented in the presence of IGF-I. In differentiating myoblasts, Dex similarly decreased 4E-BP1 and p70-S6K1 phosphorylation (27, 54), and IGF-I restored 4E-BP1 and p70-S6K1 phosphorylation levels (17). Based on these studies and our results, regulation of protein synthesis signaling in response to GC and IGF-I appears to be conserved in myoblasts and myotubes.

As the synergism between GC and IGF-I signaling on muscle differentiation was not explained by a further stimulation of mRNA translation initiation, the stimulatory effect of simultaneous GC and IGF-I addition must stem from another regulatory level of myogenesis. Apart from inhibiting protein synthesis (signaling), GR activation also initiates the transcription of GC/GR-sensitive genes, including Glul, KLF-15, and REDD1 (43, 54). Although IGF-I decreased both Glul and KLF-15 induction by Dex, a strong upregulation compared with control was still detected. Furthermore, GC-induced REDD1 expression (54), as well as GR-dependent reporter gene activity, further increased with IGF-I addition. These differential responses of promoter transactivation and known mRNA targets

P < 0.05, compared with the other groups, e.g., ND/No-GC [1.10 ± 3.03 kg, P = nonsignificant (NS)], Ctrl/GC (0.39 ± 2.03 kg, P = NS), Ctrl/No-GC (0.27 ± 1.90 kg, P = NS). A similar trend was observed for inspiratory muscle strength (Fig. 6B). Irrespective of GC medication, the placebo-treated patients did not lose FFM, indicating that the rehabilitation program, which was devoid of resistance training to study the effects of anabolic steroids per se, may have prevented further muscle wasting, but did not induce muscle gain.

DISCUSSION

GC are frequently administered to COPD patients and are known to induce muscle atrophy (14, 20, 34), but little is known regarding their influence on muscle regrowth. Yet recovery of muscle mass is an important and main goal of pulmonary rehabilitation programs for muscle-wasted COPD patients. These pulmonary rehabilitation programs are often multimodal and typically include a personalized exercise program combined with nutritional support upon indication. The last two decades, several studies addressed complementation of this approach with muscle growth-stimulating pharmaceuticals, including anabolic steroids (20, 22). Many of the effects of anabolic steroids are a consequence of increased androgen receptor-mediated expression of IGF-I in skeletal muscle,
of GR-mediated gene transcription could reflect a change in specific GR-regulated genes in the presence of IGF-I, unrelated to effects on overall promoter transactivation. Nevertheless, these data reveal intact GC-induced, GR-mediated signaling, despite the presence of IGF-I.

IGF-I increases muscle-specific gene transcription during myogenesis (9, 30, 51), which was confirmed by a moderate increase in MCK and MYH mRNA levels in response to IGF-I. Considering the inhibitory effects of Dex on myogenesis, the elevated MYH-4, MYH-8, and MCK mRNA levels observed in response to Dex were rather surprising, although Dex-induced increases in MYH-4 and MYH-8 mRNA levels have been reported previously (8). These results further confirm that inhibition of myogenesis by GC likely results from impaired mRNA translation, as muscle-specific mRNA transcripts were higher rather than lower in response to GC. In fact, this effect may lie at the basis for the synergism observed between Dex and IGF-I on muscle-specific protein content, as increased accumulation of muscle-specific mRNA transcripts induced by Dex can be effectively translated to protein in presence of IGF-I.

Still, in mature myotubes, IGF-I restores GC mRNA translation inhibition, but no synergic increases of myofibrillar protein content or myotube size have been reported (16, 27, 34, 45), nor observed in our own study. A striking difference between differentiating myoblasts and mature myotubes are the expression levels and activity of MRFs, which are essential to muscle-specific gene expression (32). In line with previous reports, Dex potently induced Myf-5 expression in differentiating myoblasts (31, 48). Combined Dex and IGF-I treatment synergistically increased myogenin expression, suggesting GC alone or combined with IGF-I may affect muscle-specific transcription by increasing the expression of specific MRFs. This coincided with increased MRF transcriptional activity, as we noted elevated transactivation of a MRF-responsive promoter containing E-box sequences by GC alone, or combined with IGF-I. Whether this is the consequence of increased MRF expression or activity resulting of posttranslational modification remains to be established, but these data imply positive regulation of MRFs by GC, which has not been reported previously. This increased MRF expression and activity when IGF-I and GCs are combined may also lie at the basis of their synergistic effect on myoblast fusion, considering the role of MRFs like MyoD (myoblast determination), Myf-5, and myogenin on this process (13). Importantly, as Dex is not frequently applied in the treatment of COPD, key findings were reproduced using the particularly relevant GC Pred at equimolar and equipotent concentrations.

Evaluating the potential clinical relevance of these findings, a post hoc analysis of a RCT (10) aimed at reversal of muscle wasting in male COPD patients with nandrolone was performed following additional stratification for GC use (mainly Pred). In line with its effects on myogenesis described above, and in agreement with a previous study (11), GC negatively
affected pulmonary rehabilitation outcome as no gain in FFM (indicative of impaired muscle regrowth) was detectable in ~55% of patients of the placebo/GC compared with only 29% of patients in the placebo/No-GC group.

ND administration in absence of GC did not result in significant gain of FFM or inspiratory muscle strength. Absence of detectable favorable androgenic steroid effects has been previously reported and may have resulted from the relatively low dose of ND and the type of pulmonary rehabilitation protocol that was used (28, 39, 46, 49). Alternatively, these findings may reflect a relative insensitivity of the measurements to detect changes in muscle mass, which may have been subtle based on the modest effects of IGF-I alone on myogenesis detected in the muscle cell culture experiments. Conversely, ND administration in the presence of GC did significantly improve gain in FFM as well as inspiratory muscle strength. This is in line with the synergistic effect of GC and IGF-I observed in cultured myocytes, which stimulated rather than inhibited myogenesis compared with GC only and was more potent than IGF-I alone. Furthermore, in only 13% of these patients, FFM was not increased upon completion of the protocol for ND/GC group, compared with a lack of response in 55% of the Ctrl/GC group.

Limitations of the present study include the use of a post hoc analysis of a previously conducted and reported RCT (10). Despite matched numbers for GC use, there was a slightly skewed distribution of the subjects over the GC-only and ND-only groups. In addition, the original design did not include the collection of muscle biopsies, which could have substantiated the accumulation of muscle-specific mRNA transcripts and inhibition of protein synthesis signaling in the presence of GC and restoration by ND. These considerations would certainly be included in the design of a prospective study to verify the synergistic effects of GC and increased IGF-I signaling on muscle regrowth. Moreover, although requirement for myonuclear addition in compensatory hypertrophy has been demonstrated (1), the relative contribution of myogenesis to muscle recovery from atrophy remains to be resolved. Nevertheless, the current results indicate that it might be worthwhile to further investigate the synergy of anabolic steroids and GCs on muscle regrowth in wasted COPD patients during recovery from a severe acute exacerbation.

In conclusion, and as schematically illustrated in Fig. 9, GC in the presence of increased IGF-I signaling may synergistically stimulate muscle regrowth as a consequence of increased muscle-specific mRNA transcription in the presence of restored protein synthesis signaling during myogenesis.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: N.A.P., R.C.L., and A.M.S. conception and design of research; N.A.P. performed experiments; N.A.P., R.C.L., and A.M.S. analyzed data; N.A.P. and R.C.L. interpreted results of experiments; N.A.P. prepared figures; N.A.P., R.C.L., E.W., and A.M.S. drafted manuscript; N.A.P., R.C.L., E.W., and A.M.S. edited and revised manuscript; N.A.P., R.C.L., E.W., and A.M.S. approved final version of manuscript.

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