Yin Yang 1 Expression and Localisation in Quadriceps Muscle in COPD

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Abstract

Introduction: Yin Yang 1 (YY1) is a transcriptional repressor that inhibits muscle gene expression and myogenesis. YY1 has not previously been investigated in the skeletal muscle of patients with COPD. The aims of this study were to investigate YY1 expression and localisation in the quadriceps muscle of COPD patients compared to healthy age-matched controls, and to examine the relationship between YY1 expression and localisation and quadriceps muscle fibre cross-sectional area (CSA) in COPD patients.

Patients and methods: 15 COPD patients and 8 age-matched controls underwent lung and quadriceps function assessments and a percutaneous quadriceps biopsy. Quadriceps muscle fibre CSA and fibre proportions and YY1 localisation were determined by immunofluorescence. YY1 was immunoprecipitated from muscle and YY1 levels assessed by western blotting.

Results: YY1 levels were inversely correlated with type IIx and type I fibre CSA in patients and controls, though YY1 levels were not significantly different between the groups. Nuclear localisation of YY1 was demonstrated in the patients but not in controls.

Conclusion: YY1 expression is associated with smaller quadriceps fibre CSA in COPD and nuclear localisation of YY1 was found in muscle of patients but not controls. Regulation of YY1 appears altered in COPD and may be implicated in COPD-related muscle atrophy.

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Expresión y localización del factor de transcripción Yin Yang 1 en el músculo cuádriceps en la enfermedad pulmonar obstructiva crónica

Resumen

Introducción: El Ying Yang 1 (YY1) es un factor de transcripción represor que inhibe la expresión génica muscular y la miogénesis. Este factor no se ha investigado previamente este factor no se ha investigado en el músculo esquelético de pacientes con enfermedad pulmonar obstructiva crónica (EPOC). Los objetivos del presente estudio fueron investigar la expresión de YY1 y su localización en el músculo cuádriceps de pacientes con EPOC, comparado con individuos control sanos, emparejados por edad, y examinar la relación entre la expresión y localización de YY1 en las áreas transversales (AT) de las fibras musculares del cuádriceps en pacientes con EPOC.

Pacientes y métodos: Se sometió a 15 pacientes con EPOC y a 8 individuos de control, emparejados por edad, a evaluaciones de función pulmonar y del cuádriceps. YY1 se determinó mediante inmunofluorescencia y YY1 se inmunoprecipitó a partir del músculo y se evaluaron mediante inmunotransferencia.

Palabras clave:
Atrofia
Regeneración muscular
Debilidad muscular

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In order to maintain muscle mass, regeneration of the lung volumes with plethysmography and, therefore, a greater expression of YY1. For instance, the activation of NF-κB and muscle creatine kinase, or in the muscle. However, in patients compared with control subjects, the expression increases in the lung tissue of COPD patients when compared with control subjects. YY1 suppresses the muscle differentiation and gene transcription of the skeletal muscles, such as skeletal alpha actin and muscle creatine kinase, binding with the pertinent promoters and blocking the binding of a transcription activator, serum response factor. For instance, the activation of the nuclear factor kappa B (NF-κB) pathway by tumour necrosis factor-alpha (TNF-α) can inhibit muscle regeneration through an increase in YY1 expression. Furthermore, the localization of YY1 affects its activity, as does its expression. When YY1 is limited to the cytoplasm of the muscle cells, it is inactive, which allows for the differentiation and synthesis of the contractile proteins. YY1 is activated by the transport to the nucleus, for example, as a response to the presence of depolymerised actin.

There are at least two mechanisms due to which the muscle YY1 activity could increase in COPD. First of all, the patients with COPD and muscle wasting may present an increase in the DNA-NF-κB binding in the muscle and, therefore, a greater expression of YY1. The activation of NF-κB in the quadriceps of COPD patients could be a consequence of the stimulation secondary to the rise in TNF-α in blood or in the muscle. However, in patients compared with control subjects, a decrease in TNF-α values in the quadriceps muscle has been reported in comparison with the findings in the intercostal muscles. Given the fact that TNF-α can also stimulate the activation of satellite cells through the activation of the serum response factor, its reduction in the muscles could inhibit muscle regeneration regardless of YY1. In the second place, in the muscle of COPD patients, the activity of YY1 could increase due to the increase in the nuclear transport of YY1 in the presence of a rise in depolymerised actin, a consequence of the accelerated degradation of proteins through the ubiquitin-proteasome pathway.

Therefore, the hypothesis of this present study was that the deregulation of YY1 signalling is involved in the atrophy of the quadriceps muscles of patients with COPD. We have investigated the expression and location of YY1 in the quadriceps muscles of a small group of patients with COPD and control individuals, paired for age, and we have examined the relationship between the expression and location of YY1 and the cross-sectional area (CSA) of the quadriceps fibres.

### Results

Los niveles de YY1 se correlacionaron inversamente con el AT de las fibras del tipo IIx y de tipo I en pacientes e individuos de control, aunque los niveles de YY1 no fueron significativamente diferentes entre ambos grupos. En los pacientes, pero no en los individuos control, se demostró la localización nuclear de YY1.

### Conclusion

La expresión de YY1 se asocia a un AT más pequeña de las fibras del cuádriceps en pacientes con EPOC, en cuyo músculo también se observa una localización nuclear del factor, a diferencia de los individuos control. La regulación de YY1 parece alterada en la EPOC y podría estar implicada en la atrofia muscular relacionada con la enfermedad.
in PBS for the entire night at 4 °C. After an incubation of 1 h at room temperature in the dark with secondary antibodies marked with fluorescence (A11008 Invitrogen and A11005 Invitrogen, dilution 1:250 in PBS), the slices were treated with diamino-phenylinol (DAP). The images (two fields per sample, two cuts per individual) were obtained using a wide-field Zeiss Axiosvert microscope, with a 10× lens and Improvision Volocity software. Using a Leica SP2 microscope, we obtained confocal images with a 63× oil immersion lens and they were analysed with LCS Lite software (Leica Microsystems, Germany).

Quantification of the Yin Yang 1 Protein

YY1 is not abundant in the skeletal muscle of adults and, consequently, before immunoblotting, immunoprecipitation was necessary. From nine patients and seven control individuals, for this analysis there were 3–6 mg of protein.

Immunoprecipitation of Yin Yang 1

Between 3 to 6 mg of homogenised protein were incubated in a solution with Nonidet P40, cocktails of protease inhibitor and phosphatase inhibitor (Sigma, Poole Dorset, United Kingdom) in ice for 30 min with 30 μl of Protein G-Sepharose beads. After centrifuging for 2 min (8,000 rpm), the supernatant was incubated for 1 h in ice with 1 μg of rabbit anti-YY1 antibody (sc-281, Santa Cruz Biotechnologies, United States). For each sample, another 30 μl Protein G-Sepharose beads were added and they were re-incubated in ice for 1 h. The samples were centrifuged again and the supernatant was discarded. The beads were washed in Nonidet P40 and boiled with 30 μl of the sample buffer (loading buffer and 2-mercaptoethanol) for 5 min at 100 °C. The process was optimised to guarantee that the supernatant would not have a detectable quantity of YY1 in the Western blot.

Western Blot Analysis

The samples were analysed using electrophoresis in polyacrylamide gel with sodium dodecyl sulphate (SDS/PAGE) (10% gel) and a semi-dry immunoblotting technique (LKB). After blocking (5% BSA in PBS), the membranes were incubated with mouse anti-YY1 antibody (ab58066, dilution 1:200, Abcam, United Kingdom) in 3% BSA in PBS for the whole night at 4 °C, and immediately followed with an IgG murine antibody bound with horseradish peroxidase (ab 6728, Abcam, United Kingdom, 1:5000 in 3% BSA in PBS) for 1 h at room temperature. The proteins were visualised with Supersignal (Pierce, Rockford, IL, United States), determined the density of the band with a densitometry 1D analysis (AIDA, Raytek, Sheffield, United Kingdom) and was normalised for the quantity of proteins used in the immunoprecipitation.

Determination of the Transversal Area of the Muscle Fibres

From all the subjects, the 10 μm frozen transverse muscle slices were incubated with primary antibodies for type I myosin, type Ila myosin and laminin (A4.840 and N2.261 Developmental Studies Hybridoma Bank, University of Iowa, Unites States, and L-9393 Sigma, Zwijndrecht, Netherlands, respectively) followed by secondary antibodies marked with fluorescence (A-21121, A-21426 and A-11069, Invitrogen).23 The epifluorescence signal was registered using a Texas Red excitation filter (540–580 nm) for type I myosin, an FITC excitation filter (465–495 nm) for type Ila myosin and a UV DAPI excitation filter (340–380 nm) for the laminin using a Nikon Eclipse 800 microscope. The fibres were classified into type I, Ila, IIX (without staining) and hybrid I/Ila (dual staining) and we used the laminin edge of the fibres to calculate the CSA of each fibre (and with this the mean CSA for each type of fibre) and the proportions of fibres using Lucia 4.81 statistical software (Nikon, Japan).24 For each individual, we analysed an average of 207 fibres, with a minimum of 103 fibres.

Quantification of the Levels of Tumour Necrosis Factor α mRNA

The TNF-α mRNA transcripts were determined by a chain reaction of the quantitative polymerase, in real time, using SYBR® Green PCR Master Mix (Applied Biosystems) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The transcripts normalised to a geNorm factor derived from two internal genes, acidic ribosomal phosphoprotein (RPLPO) and beta-2 microglobulin (β2M), as previously reported.25 The following are the primer sequences:

| Primer         | Sense Sequence
<table>
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<tr>
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<tbody>
<tr>
<td>TNF-α</td>
<td>TCAATGGCCCGACTATCCT</td>
</tr>
<tr>
<td>RPLPO</td>
<td>CAGCGCAATGCTCAAGACT</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>GCCCGCTTCACATAGCGCTGCA</td>
</tr>
<tr>
<td>β2M</td>
<td>GCCCGCTTCACATAGCGCTGCA</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>TACATCTTGTCACAGCCAAGATA</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>AAATCCGGCGGATCTGACACT</td>
</tr>
</tbody>
</table>

Statistical Analysis

The data did not have normal distribution (according to the histogram and the test of symmetry), so they are reported as averages (25th percentile and 75th percentile). The group differences in the continuous variables were analysed with the Mann–Whitney U-test, while the Fisher’s exact test was used to test the group differences in the categorical variables. Spearman’s rank correlation coefficient (ρ) was calculated to determine the relationship among the variables (Statview 1.0, Abacus Instruments). To define the statistical significance, a two-tailed P value ≤ .05 was used.

Results

Table 1, shows the physiological data of the patients and controls. As was expected, the patients presented a deterioration in lung function and a decrease in the strength of the quadriceps, in comparison with the control individuals [maximal voluntary contraction 22 (17.34) kg compared with 34 (28.42) kg in control subjects, P = .03].
Table 2
Levels of YY1, HDAC 5, and HDAC 5 Bound With YY1 and Mean CSA of the Type I, I/IIa, IIa and IIx Fibres in the Quadriceps of Patients With COPD and Control Individuals.

<table>
<thead>
<tr>
<th></th>
<th>COPD Patients (n=15)</th>
<th>Control Subjects (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YY1, AU/mg protein</td>
<td>3.25 (1.95, 7.17) (n=9)</td>
<td>3.84 (2.18, 6.01) (n=7)</td>
</tr>
<tr>
<td>Levels of mRNA-TNF-α, AU</td>
<td>0.08 (0.03, 0.11) (n=15)</td>
<td>0.04 (0.03, 0.05) (n=8)</td>
</tr>
<tr>
<td>CSA type I fibre, µm²</td>
<td>5352 (4151, 6783) (n=15)</td>
<td>5.644 (4.111, 7.411) (n=8)</td>
</tr>
<tr>
<td>CSA type I/IIa fibre, µm²</td>
<td>4971 (3292, 7309) (n=15)</td>
<td>5754 (5005, 5956) (n=3)</td>
</tr>
<tr>
<td>CSA type IIa fibre, µm²</td>
<td>4221 (2996, 5170) (n=15)</td>
<td>3314 (3002, 5408) (n=15)</td>
</tr>
<tr>
<td>CSA type IIx fibre, µm²</td>
<td>3439 (2348, 3739) (n=14)</td>
<td>5015 (3618, 6303) (n=8)</td>
</tr>
<tr>
<td>Percentage of type I fibres</td>
<td>34 (11, 38) (n=15)</td>
<td>52 (47, 63) (n=8)</td>
</tr>
<tr>
<td>Percentage of type I/IIa fibres</td>
<td>3 (1, 6) (n=15)</td>
<td>0 (0, 4) (n=8)</td>
</tr>
<tr>
<td>Percentage of type IIa fibres</td>
<td>60 (52, 70) (n=15)</td>
<td>40 (35, 46) (n=8)</td>
</tr>
<tr>
<td>Percentage of type IIx fibres</td>
<td>3 (1, 16) (n=15)</td>
<td>1 (0, 4) (n=8)</td>
</tr>
</tbody>
</table>

The values are means (25th percentile, 75th percentile). The percentages of fibres in each individual reach 100% (the sum of the mean values in each group does not necessarily equal 100% due to the variance around the mean). The levels for mRNA normalise for the transcripts of RPLPO (internal gene).

* p < 0.005 for the group comparisons (analysed with the Mann–Whitney U test).

† p < 0.05 for the group comparisons (analysed with the Mann–Whitney U test).

CSA: cross-sectional area; HDAC: histone deacetylase; RPLPO: acidic ribosomal phosphoprotein; TNF-α: tumour necrosis factor-alpha; AU: arbitrary units; YY1: Yin Yang 1.

The CSA in the type IIx fibres diminished and the quotient type I fibres: type II fibres decreased in patients with COPD in comparison with control individuals.

In patients, a significantly smaller CSA was detected in the type IIx fibres than in control individuals [3.49 (2.348, 3.739) µm² compared with 4.628 (4.087, 6.303) µm², respectively, P<0.05], but there was no significant difference in the CSA of the other types of fibres between groups, as had been previously reported.26 In patients, we detected a significantly lower proportion of type I fibres and a greater proportion of type IIa fibres than in control individuals [34 (14, 39)% compared with 52 (47, 63)% and 60 (49, 70)% compared with 40 (35, 46)% respectively], as previously described27 (Table 2).

The levels of YY1 in the quadriceps were associated with a decrease of the CSA in the muscle fibres in COPD.

The quantity of YY1 protein in the quadriceps was not significantly different in patients compared with the control subjects (Fig. 1, Table 2). In patients and controls, the levels of YY1 protein inversely correlated with the CSA of the type IIx fibres (ρ = −0.83, P<0.001); likewise it was true with the CSA of the type I fibres (ρ = −0.59, P=0.017), and there was also an observed tendency towards a negative correlation with the CSA of the type IIa fibres (ρ = −0.43, P=0.09) (Fig. 2). Only in the group of patients did we identify a correlation between the levels of YY1 and in the muscle the CSA of the type IIx fibres (ρ = −0.88, P<0.002). The muscle levels of YY1 did not correlate with the forced expiratory volume in one second or the CO₂ diffusing capacity (TlCO₂) as a percentage of the reference value, maximal voluntary contraction of the quadriceps or the lean mass index in patients and control subjects (P=0.69, 0.43, 0.94 and 0.21, respectively) or correlation was only identified in the patient group.

The muscle TNF-α mRNA levels were higher in patients than in control subjects (0.08 [0.03, 0.1] arbitrary units [AU] compared with 0.04 [0.03, 0.05] AU, P=0.05). The TNF-α mRNA transcripts and the levels of YY1 did not correlate in patients and control subjects (P=0.38) or in the patients alone.

The levels of YY1 in the quadriceps were associated with a decrease of the CSA in the muscle fibres in COPD.

![Figure 1](image1.png)

**Figure 1.** Western blot analysis for YY1 from the quadriceps muscle of COPD patients and healthy control subjects. Immunoblotting representative of YY1 in muscle samples of a control subject (left) and three COPD patients (right), subjected to immunoprecipitation using an anti-YY1 antibody. The bands are observed in position 50 kDa. Statistically significant differences in the YY1 protein levels were not identified in the muscle of the patients compared with the control individuals.

![Figure 2](image2.png)

**Figure 2.** Point diagrams of the YY1 protein levels and CSA of the type I, I/IIa, IIa and IIx fibres in the quadriceps of patients with COPD and healthy control subjects. There were correlations between the YY1 protein levels and the CSA of the type I and IIx fibres and a tendency towards a negative correlation between the levels of the factor and the CSA of the type IIa fibres when the patients and the control subjects were combined (ρ = −0.83, P<0.001; ρ = −0.59, P=0.017 and ρ = −0.43, P=0.09, respectively), and a negative correlation between the levels of YY1 and the CSA of the type IIx fibres was only detected in patients (ρ = −0.88, P=0.002).
Fig. 3. Image from a wide-field microscope of a longitudinal slice of the quadriceps muscle of a healthy control subject demonstrating the localisation of YY1 regarding the muscle nuclei by means of immunohistochemistry. The muscle was stained for YY1 (green), the heavy chain of the fast myosin (red) and the nuclei using 4′,6-diamidino-2-phenylindole (blue). The YY1 staining is observed along the sarcomeres, not co-localised with the myosin, and the absence of nuclei is also observed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Nuclear Localisation of Yin Yang 1 in the Muscle of a Patient Subgroup With Chronic Obstructive Pulmonary Disease but not in Control Individuals

YY1 was absent from the nucleus but present in the cytoplasm in the quadriceps of the healthy control subjects (Figs. 3 and 4C, D, G, H). YY1 in the cytoplasm aligned with the sarcomeres, but it did not co-localise with myosin (Fig. 3).

YY1 staining was demonstrated in the nuclear region in none of the control individuals but in 5 of the 10 patients. This difference between groups was statistically significant (Fisher’s exact test, \(P=.04\)). Compared with 6 of the 8 control subjects, only in 2 of the 10 patients was there a demonstrated exclusively cytoplasmic YY1 distribution that, once again, was statistically significant (Fisher’s exact test, \(P=.05\)). No significant difference was identified between patients with or without nuclear YY1 localisation regarding muscle or lung function, quadriceps fibre CSA or YY1 protein levels.

Discussion

We have made a new finding: that the YY1 transcription factor, a repressor of muscle-specific gene expression and myogenesis, is expressed in a proportion inverse to CSA of the type Ix and type I fibres in the quadriceps muscle of patients with COPD. In a significant proportion of these patients, the nuclear localisation of the factor was evident, which contrasts with the distribution in the muscle of healthy adults.

Critique of the method

Naturally, the present study presents limitations. First of all, the size of the sample was small and the associations detected had to stand out; therefore it is possible that no other associations or group differences were identified due to the lack of power. The control subjects included in the present study were characterised by a relatively low lean mass index and a wide variance in their degree of physical activity, for which there is no clear explanation, which increases the threshold for the statistical significance of the group differences. Although the study of patients in stage II, III, and IV of the GOLD initiative should have made it easier to find correlations between the muscle and lung function and the YY1 levels (because the data was not expected to gather), the exclusive use of patients in stage IV could have maximised the probability of finding a significant difference in YY1 expression between patients and control subjects. Second, the observational design does not allow for the extraction of conclusions about the effect of the differences in YY1 expression between patients and control subjects. Second, the observational design does not allow for the extraction of conclusions about the effect of the differences in YY1 expression between patients and control subjects. Lastly, we used the

Fig. 4. Microscope images of quadriceps muscle slices from COPD patients and control subjects, demonstrating in the myotubules the localisation of YY1 with immunohistochemistry with regard to the muscle nuclei. (A–D) Wide-field microscope images. E–H: images from a confocal microscope with cross-sectional quadriceps muscle cuts that demonstrate the YY1 stain in green and for the nuclei with 4′,6-diamidino-2-phenylindole in blue (B, D, E, and G) and the same cuts that show staining for YY1 only in green (A, C, F, and H). (A, B, E, and F) The slices of a patient in whom the anomalous staining pattern of the factor was identified in the nucleus as well as in the cytoplasm. (C, D, G, and H) From a control subject showing exclusively cytoplasmic YY1 staining.
localisation and expression of the factor as an indirect variable of the activity because YY1 is activated by transport from the cytoplasm to the nucleus. Our findings could have been corroborated by the quantification of YY1 bound to DNA (immunoprecipitation of chromatin, a technique that has not been previously published as being used in human muscle) or the quantification of YY1 able to bind with DNA (for example, by means of electrophoretic analysis of the change in mobility).

It could also be argued that the YY1 factor detected in the nuclei is not present in the periphery of the myofibrils, but is instead in the latent, satellite cells. However, we consider that this is improbable as the satellite cells only represent 2–5% of the muscle nuclei. Even supposing a massive increase in the population of satellite cells in the tissue of the patients, the number of nuclei that stained for the factor considerably surpasses the number that could be justified by the satellite cells. Furthermore, in the samples of the present study, the absence of centralised nuclei suggests a limited activation of the satellite cells in the period of time when the samples were taken.

Significance of the Findings

The implication of a powerful inverse correlation between the levels of the YY1 factor and the size of the fibres is that YY1 could participate in the mechanism of fibre atrophy, particularly as the levels of the YY1 factor and the size of the fibres is that YY1 could be regulated by the size of the fibres. Furthermore, we consider that this is improbable as the satellite cells only represent 2–5% of the muscle nuclei. Even supposing a massive increase in the population of satellite cells in the tissue of the patients, the number of nuclei that stained for the factor considerably surpasses the number that could be justified by the satellite cells. Furthermore, in the samples of the present study, the absence of centralised nuclei suggests a limited activation of the satellite cells in the period of time when the samples were taken.

Conflict of Interest

S.A. Natanek (born in Sathyapala) receives funding from a Wellcome Trust Clinical Research Fellowship and previously received a grant from GlaxoSmithKline. G.S. Marsh and J. Riddoch-Contreras received funding through a scholarship at the Imperial College by GlaxoSmithKline. W.D.-C. Man receives funding from the National Institute for Health Research Clinician Scientist Award. GlaxoSmithKline took no part in either the compiling of the data or preparation of the manuscript. The NIHRR Respiratory Biomedical Research Unit of the Royal Brompton Hospital and Imperial College contribute to a part of M.I. Polkey’s salary.

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References


