Progranulin compensates for blocked IGF-1 signaling to promote myotube hypertrophy in C2C12 myoblasts via the PI3K/Akt/mTOR pathway

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ABSTRACT

It is well known that growth hormone (GH)-induced IGF-1 signaling plays a dominant role in postnatal muscle growth. Our previous studies have identified a growth factor, progranulin (PGRN), that is co-induced with IGF-1 upon GH administration. This result prompted us to explore the function of PGRN and its association with IGF-1. In the present study, we demonstrated that, similar to IGF-1, PGRN can promote C2C12 myotube hypertrophy via the PI(3)K/Akt/mTOR pathway. Moreover, PGRN can rescue the muscle atrophy phenotypes in C2C12 myotube when IGF-1 signaling is blocked. This result shows that PGRN can substitute for IGF-1 signaling in the regulation of muscle growth. Our findings provide new insights into IGF-1-modulated complicated networks that regulate muscle growth.

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1. Introduction

The growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis plays an important role in the regulation of muscle growth [1]. GH-mediated growth has been postulated to be stimulated by GH–induced hepatic IGF-I secretion (endoctrine mode) and local IGF-1 production (autocrine and/or paracrine mode) [2]. However, a comparative analysis has shown that GH receptors (GHR) and IGF-1 double mutant mice exhibit a more severe growth defect than single mutant mice. This finding suggests that the effects of GH on muscles may not depend exclusively on IGF-1 [3]. To investigate other growth factors involved in muscle growth, downstream effectors of GH signaling have been studied extensively. We previously have shown that the epithelial tissue growth factor progranulin (PGRN) is induced along with IGF-1 by GH administration. This result suggests that PGRN participates in GH/IGF-1 axis-regulated growth and may serve functions similar to those of IGF-1 signaling [4]. IGF-1 is a critical regulator of skeletal muscle development. It participates in many stages of muscle growth from the proliferation and differentiation of myoblasts to myotube hypertrophy [5,6]. The binding of IGF-1 to IGF-1 receptors (IGF-1R) not only causes muscle hypertrophy through activations of phosphatidylinositol-3 kinase (PI3K)/Akt and its downstream targets mTOR, p70S6K, and PHAS-1/4E-BP1[7], but it also leads to the mitogenesis of muscle cells via the mitogen-activated protein kinase (MAPK) pathway [5]. PGRN and IGF-1 act on similar downstream signaling pathways; for instance, the MAPK and PI3K/Akt pathways are both involved in PGRN-mediated cell migration and growth [8,9]. In addition, PGRN is connected to IGF-1 in various manners. PGRN-induced proliferation effects are observed in IGF-1 receptor null 3T3 cells via activation of the MAPK and PI3K/Akt/p70S6K pathways [10,11]. Although the regulatory roles of IGF-1 signaling in muscle development are well known, the function and regulatory roles of PGRN remain unclear.

PGRN is a secreted growth factor that has been implicated in a wide variety of biological processes, including wound healing, embryo development, morphogenesis, and cancer diseases [12]. Genetic analysis has recently shown that mutation within the PGRN gene is a major cause of frontotemporal lobe degeneration (FTLD), which is one of the most common form of neurodegenerative disease accounting for 5~10% of all patients diagnosed with dementia [13–15]. This result suggests that PGRN is an emerging target for FTLD therapies. Furthermore, gene expression analysis elucidated that PGRN is upregulated in various neuroinflammatory conditions, and increased PGRN expression by microglia potentially plays a pivotal role in responses to neuroinflammation, neurodegeneration, and brain injury [16]. Regarding zebrafish, knockdown of the PGRN gene using antisense morpholinos lead...
to impaired liver morphogenesis and a small liver phenotype, indicating PGRN plays a vital role in embryonic liver morphogenesis [17]. In addition, PGRN promotes PC12 cells, which is a pheochromocytoma-derived neuronal cell line lacking responses to most nerve growth factors. IGF-1, which is similar to PGRN, is a growth factor capable of effecting on PC12 cells [18]. In embryonic fibroblasts, PGRN mediated similar signaling pathways similar to those mediated by IGF-1 [19]. Although these results suggest that PGRN possesses a role similar to IGF-1, potential importance and subtle differences would exist between PGRN and IGF-1. The main objective of this study is to clarify the function and roles of PGRN in muscle growth and its association with IGF-1 signaling. We demonstrated that PGRN stimulates mouse C2C12 myotube hypertrophy and also proved that PGRN-induced myotube hypertrophy is performed via the PI3K/Akt/mTOR signaling pathway. Furthermore, we blocked IGF-1 signaling with an IGF-1-specific inhibitor and found that PGRN could compensate partially for the loss of IGF-1 signaling in muscle hypertrophy. Taken together our findings demonstrate the function of PGRN in muscle growth and indicated a partially compensatory role of PGRN for failed IGF-1 signaling.

2. Materials and methods

2.1. Cells and culture medium

C2C12 cells were maintained in Dulbecco’s modified minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 3.7 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Gibco) at 37 °C in air with a humidified atmosphere of 5% CO2. The myoblasts were fused into myotubes at 50% confluence by shifting the growth medium to a differentiation medium consisting of DMEM supplemented with 2% horse serum. C2C12 cells were incubated in a differentiation medium for 4 days to complete myotube formation. The differentiation medium was renewed every 2 days. The time at which the differentiation medium was first introduced was referred to as day 0. All chemical treatments, including recombinant human GH (hGH) (GenWay, CA, USA), R3-IGF-1 (Sigma, St. Louis, MO, USA), recombinant mouse PGRN (R&D Systems, Minneapolis, MN), recombinant human granulin C (Adipogen, CA, USA) and human granulin E (Abnova, CA, USA) were administered either on day 2 or day 4 after fusion as described. PQ401 (Sigma Aldrich), granulin E (Abnova, CA, USA) were administered either on day 2 or day 4 after fusion as described. PQ401 (Sigma Aldrich), LY294002 (Sigma-Aldrich) and rapamycin (Calbiochem, San Diego, CA) were dissolved in DMSO (Sigma-Aldrich); an equal amount of DMSO was administered as a negative control.

2.2. Quantitative PCR

RNA was extracted from C2C12 cells using RNA-Bee™ (Tel-Test, Friendswood, TX, USA) according to the manufacturer’s instructions. RNA samples were reverse transcribed using the SuperScript™ first-strand synthesis system (Invitrogen, Carlsbad, CA). The qDNAs corresponding to various genes were amplified using the Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and quantified using a Roche LightCycler 480 II (Roche, Mannheim, Germany). The reaction conditions were 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were analyzed at least in triplicate. The following mouse primers were used: PGRN sense 5'-CTGCTCCTGCCGAGGAAAGAT-3' and antisense 5'-CTCAGCCACACAGCATTGG-3'; IGF-1 sense 5'-CTCAGCCATACTGTGATC-3' and antisense 5'-TACAGTGCCACTACAAG-3'; GAPDH sense 5'-CTCCTCAAGGGCGAATTCAGC-3' and antisense 5'-TACAGTGCCACTACAAG-3'. The expression of each transcript was measured using the threshold cycle (CT) method; GAPDH transcription was used as an internal control. The amplification of specific transcripts was further confirmed by melting curve profile analysis and agarose gel electrophoresis.

2.3. Western blot analysis

Cells were washed briefly with PBS and collected in a Mammalian Cell-PE LB buffer (Geno Technology Inc., St. Louis, MO). Cell debris was removed by centrifugation at 12,000×g for 10 min, and the supernatants were used for immunoblotting analysis. The Bradford assay (Bio-Rad, Hercules, CA) was used to measure protein concentration. Aliquots of 50 μg protein were subjected to 12% SDS-PAGE, and the proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA) at 25 V for 45 min in a buffer containing 192 mM glycine and 25 mM Tris-HCl. Membranes were blocked with 0.25% gelatin and 0.05% Tween-20 in Tris-buffered saline for 2 h at room temperature. The membranes were then probed with a 1:2000 dilution of the indicated antibody for 2 h at room temperature. The used antibodies were anti-mouse PGRN (R&D Systems), anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-GSK-3β (Ser21/26), anti-phospho-p70S6K (Thr389) (cell signaling Technologies, Beverly, MA), and anti-α-tubulin (Abcam, Cambridge, MA). After washing, membranes were incubated with horseradish peroxidase-linked anti-rabbit, anti-mouse, or anti-rat secondary antibodies for 2 h. Membranes were then incubated with a chemiluminescence reagent (Immobilon Western, Millipore) with gentle agitation and exposed to Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK). Films were scanned and specific bands were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The band intensity was normalized to the α-tubulin band in the same sample.

2.4. WST cell proliferation

Assay C2C12 cells proliferation in response to IGF-1 and PGRN treatments was measured using a BioVision cell proliferation kit (Mountain View, CA) according to the manufacturers’ instructions. Cells were first seeded into 96-well plates, and IGF-1 or PGRN was administered on either day 2 or day 4 after fusion as described. Following the treatments, 10 μl of WST reagent was added to each well, and cells were incubated for 1 h at 37 °C. Absorbance at 480 nm was measured using a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA).

2.5. Immunocytochemistry and quantitative analyses of myotubes

C2C12 cells were seeded on a sterile glass coverslip in a 6-well plate, and the cells were fixed and permeabilized with cold acetone for 10 min. The slides were then washed 3 times with PBS and blocked with 5% BSA in 0.1% PBST for 1 h at room temperature. Cells were stained with 1:100 anti-myosin heavy chain (MHC) antibody (R&D Systems) for 1 h. After rinsing with PBS, cells were incubated with 1:2000 Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Invitrogen) for 1 h and counterstained with 1.5 μg/ml DAPI (Invitrogen) for nuclei visualization. The slides were then viewed and photographed with an inverted fluorescence microscope and a CCD camera using MetaMorph image analysis software (Molecular Devices, Downingtown, PA). The fusion index was defined as the ratio of nuclei within the myotubes (>2 nuclei) to the total number of nuclei. One-thousand nuclei per culture were counted in 3 independent cultures. The average number of nuclei per myotube was determined by counting over 500 nuclei from randomly chosen MHC-positive cells, and the average diameter per myotube was calculated as the mean of 5 measurements taken along the entire tube. At least 50 myotubes per well were measured in 3 independent experiments.
2.6. Statistical analysis

Data are expressed as mean ± S.E. Between-groups results were compared using the Student's t test. Statistical significance among multiple experimental treatments was determined by one-way analysis of variance (ANOVA). Tukey's test was used to evaluate differences among means (SAS Institute, Cary, North Carolina, USA). Statistical significance was determined with P values <0.05.

Fig. 1. IGF-1 decreased PGRN expression in C2C12 cells through the IGF-1 receptor. PGRN or IGF-1 mRNA expression levels were determined by real-time quantitative PCR. Myotubes were treated with (A) 100 ng/ml hGH; (B) 20 ng/ml IGF-1; (C) 20 ng/ml or 100 ng/ml IGF-1; (D) 200 ng/ml, 500 ng/ml, or 1000 ng/ml mouse PGRN; (E) PGRN protein expression levels were analyzed by Western blotting. Myotubes were treated with 20 ng/ml IGF-1 with or without 30 μM PQ401 at day 4 for 24 h. Relative protein levels were quantified using ImageQuant software. Data are shown in comparison with untreated cells. Data are expressed as means ± S.E. (n = 3). Star symbol indicates significant differences. * indicates p < 0.05; ** indicates p < 0.01.
3. Results

3.1. IGF-1 regulates PGRN expression in C2C12 cells through the IGF-1 receptor

In our previous study, we showed that PGRN is induced along with IGF-1 in liver tissue after GH administration. To clarify whether GH also induced PGRN in muscle cells, differentiated C2C12 cells were treated with recombinant hGH to estimate PGRN and IGF-1 mRNA expression levels. The finding showed that, similarly to the results in the liver, GH also induces PGRN expression in muscle cells (Fig. 1A). To further elucidate the relationship between PGRN and IGF-1, differentiated C2C12 cells were treated with IGF-1 and PGRN separately. We found PGRN mRNA expression was decreased significantly at 12 h and 24 h after treatment with either 20 ng/ml or 100 ng/ml IGF-1 (Fig. 1B and 1C). In contrast, PGRN treatments did not affect IGF-1 mRNA expression, even at concentrations of 200 ng/ml to 1000 ng/ml (Fig. 1D). These results suggest that the IGF-1 is an upstream modulator of PGRN. To further explore whether IGF-1 regulates PGRN expression through the IGF-1 receptor (IGF-1R), IGF-1 signaling was blocked using the specific IGF-1R inhibitor PQ401. PQ401 has been reported to inhibit IGF-1R autophosphorylation effectively, thus suppressing IGF-1-stimulated cell growth [20]. In contrast to IGF-1 administration alone, the simultaneous addition of PQ401 and IGF-1 mitigated the IGF-1-mediated suppression of PGRN expression from 48 ± 2.1% to 15 ± 5%. When PQ401 only was administrated to block endogenous IGF-1 signaling, protein expression of PGRN was elevated significantly. The amount of granulin (GRN) proteins cleaved from PGRN did not change suggesting the variation of PGRN is due to expression not processing (Fig. 1E). These results indicate that PGRN suppression occurs downstream of IGF-1 signaling and that IGF-1 regulates PGRN expression through IGF-1R.

3.2. PGRN stimulates C2C12 myotube hypertrophy

IGF-1 is known to promote proliferation and myotube hypertrophy in C2C12 myoblasts; however, PGRN effects on myoblasts have been unclear. To examine the functional properties of PGRN on myoblast proliferation, PGRN was administrated on day 2 after fusion. As found previously, myoblast proliferation was stimulated significantly in the presence of 100 ng/ml IGF-1 (Fig. 2A). However, the effects of PGRN on myoblast proliferation were slight, even at concentrations of 500 ng/ml and 1000 ng/ml (Fig. 2B). These results suggest that the critical role of PGRN in myoblast function may not be related to its proliferation. Therefore, we further evaluated the effects of PGRN on C2C12 myotube hypertrophy. Several criteria, including the measurements of the mean number of nuclei per myotube, myotube fusion index, and myotube diameter, were used to evaluate the state of muscle hypertrophy in response to PGRN treatment. Immunochemistry using an anti-myosin heavy chain (MHC) antibody showed that PGRN and IGF-1 both caused hypertrophic phenotypes (Fig. 3A). The myotube diameters, the fusion index and the mean number of nuclei per myotube also increased, corresponding with the hypertrophic effects of IGF-1 and PGRN treatment (Fig. 3B, C, and D). These results show that PGRN is able to induce myotube hypertrophy in a similar manner to IGF-1. To further understand whether PGRN mediated myotube hypertrophy is due to GRN fragments or not, commercially available GRN C and E were used to estimate the myotube hypertrophy effect. According to the criteria of phenotype, fusion index and the mean number of nuclei per myotube, GRN C and E did not affect myotube hypertrophy (Supplementary Fig. S1). These results suggest that the PGRN mediated myotube hypertrophy potentially through PGRN alone.

3.3. PGRN induced myotube hypertrophy by the PI(3)K/Akt/mTOR pathway

To determine whether PGRN-induced myotube hypertrophy is mediated through the PI(3)K/Akt/mTOR pathway as is IGF-1, the effects of pharmacological inhibitors of the PI(3)K/Akt/mTOR signaling pathway on myotube hypertrophy were evaluated. Treatment with 20 μM LY294002, which is a specific inhibitor of PI(3)K/Akt, caused myotube atrophy reduction in the fusion index and the mean number of nuclei per myotube. Co-treatment of differentiated myotubes with PGRN and 20 μM LY294002 blocked the PGRN-induced myotubes completely. These results suggest that the PI(3)K/Akt pathway is essential for PGRN-induced myotube hypertrophy (Fig. 4A, B and C). The blockage of the mTOR kinase, a downstream effector of the PI(3)K/Akt pathway, with 100 nM rapamycin also blunted PGRN-induced hypertrophy (Fig. 4). These results suggest that the PI(3)K/Akt/mTOR pathway participates in PGRN-induced myotube hypertrophy.

To further confirm that PGRN, similarly to IGF-1, induces myotube hypertrophy via the PI(3)K/Akt/mTOR pathway, the activation of downstream targets, such as Akt, GSK-3β, and p70S6K, were analyzed by Western blotting. Results show that the phosphorylation of Akt, GSK-3β, and p70S6K in C2C12 myotubes increased in
response to PGRN treatment (Supplementary Fig. S2). Consistent with the effects of PI(3)K/Akt/mTOR inhibitors on myotube hypertrophy, treatment of C2C12 myotubes with 20 nM LY294002 or 100 nM rapamycin blocked PGRN-induced activations of GSK-3α/β and p70S6K (Fig. 4D). These results further demonstrate that PGRN-mediated myotube hypertrophy occurs via the PI3K/AKT/mTOR pathway.

3.4. PGRN rescues myotube atrophy induced by blocked IGF-1 signaling

Blockage of IGF-1 signaling leads to increased PGRN expression, and PGRN and IGF-1 share similar functions and signaling pathways in myotube hypertrophy, suggesting that PGRN might be able to compensate for IGF-1 functions when IGF-1 signaling is blocked. To test this hypothesis, myotube atrophy was induced by treating differentiated C2C12 myotubes with 30 µM PQ401 for 2 days. The addition of PQ401 resulted in an atrophic phenotype with a decrease in the fusion index and the mean number of nuclei per myotube. To determine whether PGRN could rescue this myotube atrophy, which is induced by IGF-1 signaling blockage, atrophic myotubes were co-treated with 500 ng/ml PGRN and 30 µM PQ401 for 2 days. These results show that myotube atrophy induced by PQ401 was rescued by PGRN (Fig. 5A, B and C). Increases in the fusion index and the mean number of nuclei per myotube were observed after PQ401 and PGRN co-treatment, compared to PQ401 treatment alone (Fig. 5B and C). These results show that PGRN plays a compensatory role in the maintenance of myotube hypertrophy when IGF-1 signaling is blocked (Fig. 5D).

4. Discussion

Numerous in vivo and in vitro studies have demonstrated that IGF-1 is a critical signaling factor involved in the regulation of postnatal muscle growth [10,21,22]. In skeletal muscle, IGF-1 serves multiple functions, including stimulating the proliferation and differentiation of myoblasts and promoting protein synthesis [11]. Although the liver is the principal source of circulating IGF-1, several reports have demonstrated the importance of locally produced IGF-1 in muscle growth. Liver-specific targeted disruption of the IGF-1 gene does not influence postnatal growth [23,24], and muscle-specific miGF-1 transgene mice display profound hypertrophy in trunk and limb musculature [25]. In vitro experiments have also shown that C2C12 myoblasts differentiation relies strongly on the elevated expression of endogenous IGF-1 [26]. Several lines of evidence suggest that GH may directly regulate the IGF-1 expression.
in muscles, as well as in the liver. First, GH receptors are found in skeletal muscles [27]. Second, GH treatment increases IGF-1 mRNA expression in skeletal muscle tissues and in the myoblast cell line C2C12 [28,29]. These results support the notion that GH promotes postnatal body growth through the local production of IGF-1 in muscle tissues.

However, locally produced IGF-1 may not be the only growth factor responsible for postnatal muscle growth. Studies on MKR mice, which express a dominant negative IGF-1 receptor specifically in skeletal muscles, show that additional regulatory mechanisms (i.e., outside IGF-1) must exist. MKR mice have lower muscle weight compared to wild type mice, but the weight difference diminishes gradually with aging. Meanwhile, the protein content of the muscle tissue and the number of nuclei per myofiber are both increased in MKR mice [30,31]. MKR mice clearly adapt to the IGF-1R-free condition through compensatory hyperplasia. PGRN was initially discovered as a mitogenic growth factor that could stimulate DNA synthesis and the growth of R02/C0 fibroblasts derived from mouse embryos with a targeted disruption of the IGF-1R gene. This finding demonstrates that PGRN could abrogate the requirement for IGF-1R in mouse embryonic fibroblasts to progress through the cell cycle via the MAP kinase pathway [32]. These results are similar to those presented in the current study, which also showed that PGRN circumvent IGF-1 signaling to stimulate muscle growth, although the revealed phenotypes are muscle cell hypertropy, rather than cell proliferation. The findings suggesting that PGRN is a growth factor can be used to mediate an adaptive strategy for sustaining partial muscle growth via the PI3K/Akt/mTOR pathway in the absence of IGF-1 signaling. Recently, sortilin has been identified as a PGRN binding partner to regulate PGRN trafficking [33]; moreover, PGRN acting as a ligand of TNF receptor has also been found to plays a critical role in the pathogenesis of inflammatory arthritis [34]. Although the receptor which mediated by PGRN cannot be clarified in the present study, the receptor of PGRN signaling participating as compensatory mechanism in case of IGF-1 absence is worth investigating further.

In our previous studies, we showed that hepatic PGRN and IGF-1 expressions are both up-regulated by GH administration [4]. In the present study, PGRN expression induced by GH administration

![Fig. 4. PGRN induced muscle hypertrophy via the PI(3)K/Akt/mTOR pathway.](image-url)
was proven in muscle cells as well; moreover the time-course profile of GH-induced PGRN expression is earlier than that of IGF-1. We speculated that PGRN is upregulated prior to IGF-1, securing its preparation to serve as a compensatory mechanism in the event of IGF-1 failure. After correct IGF-1 expression, PGRN is no longer required and its expression is therefore suppressed. To further enhance our understanding the role of PGRN plays in the presence of IGF-1, we also estimated the C2C12 myotube phenotype in the simultaneous presence of PGRN and IGF-1. Results showed that if IGF-1 and PGRN are combined, the phenotype of myotube hypertrophy is more evident. However, although pERK 1/2 is also activated by the combined addition of IGF-1 and PGRN, the phenotype of myotube hyperplasia is too slight to discern. We speculated that extra PGRN addition might synergistically contribute to IGF-1-induced myotube hypertrophy (Supplementary Fig. S3).

Fig. 5. PGRN rescued PQ401-induced muscle atrophy. (A) Muscle atrophy was induced by adding 30 μM PQ401 to the differentiation medium at the beginning of differentiation (from day 0 to day 4). At day 4, the atrophic myotubes were treated continuously with 30 μM PQ401 with or without 500 ng/ml PGRN for 48 h. Myotubes were detected by immunostaining with an anti-myosin heavy chain antibody, and nuclei were stained with DAPI; (B) fusion index; (C) Average number of nuclei per myotube was determined to validate myotube atrophy and hypertrophy. Data are expressed as means ± S.E. (n = 3). Star symbol indicates significant differences. * indicates p < 0.05; ** indicates p < 0.01. (D) A model diagram showing PGRN compensates for blocked IGF-1 signaling during muscle hypertrophy. The red dotted line indicates the PGRN-induced muscle hypertrophy pathway during IGF-1 signaling blockage. The question mark is used to indicate that the PGRN receptor remains unclear.
responsible for muscle growth in both endocrine and autocrine/paracrine modes. Other GH downstream targets are believed to be involved in the regulation of postnatal muscle growth because muscle growth is maintained even after the loss of IGF-1. Based on similar functions and the downstream of PGRN and IGF-1, we proposed a compensatory model in which PGRN mediates muscle growth and helps to sustain muscle growth in the absence of IGF-1. We expected our findings to provide new insights into the IGF-1-mediated complicated network in regulating muscle growth.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.febslet.2012.07.077](http://dx.doi.org/10.1016/j.febslet.2012.07.077).

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