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Functional muscle hypertrophy by increased insulin-like growth factor 1 does not require dysferlin

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Abstract

Introduction: Dysferlin loss-of-function mutations cause muscular dystrophy, accompanied by impaired membrane repair and muscle weakness. Growth promoting strategies including insulin-like growth factor 1 (IGF-1) could provide benefit but may cause strength loss or be ineffective. The objective of this study was to determine whether locally increased IGF-1 promotes functional muscle hypertrophy in dysferlin-null (Dysf−/−) mice.

Methods: Muscle-specific transgenic expression and postnatal viral delivery of Igf1 were used in Dysf−/− and control mice. Increased IGF-1 levels were confirmed by enzyme-linked immunosorbent assay. Testing for skeletal muscle mass and function was performed in male and female mice.

Results: Muscle hypertrophy occurred in response to increased IGF-1 in mice with and without dysferlin. Male mice showed a more robust response compared with females. Increased IGF-1 did not cause loss of force per cross-sectional area in Dysf−/− muscles.

Discussion: We conclude that increased local IGF-1 promotes functional hypertrophy when dysferlin is absent and reestablishes IGF-1 as a potential therapeutic for dysferlinopathies.

KEYWORDS

dysferlin, insulin-like growth factor 1, Miyoshi myopathy, muscle hypertrophy, skeletal muscle function

1 | INTRODUCTION

Loss-of-function mutations in the dysferlin gene (Dysf) in humans results in a spectrum of muscular dystrophies known as dysferlinopathies. The dysferlinopathies manifest as distal anterior compartment myopathy, limb-girdle muscular dystrophy type 2B (LGMD2B), and...
FIGURE 1  Legend on next page.
Dysferlin is a 230-kDa type II transmembrane protein localized at the sarcolemma of mature skeletal muscle but also at transverse tubules in developing myotubes. Dysferlin contains seven C2 domains and a transmembrane domain. The first C2 domain (C2A) can bind several phospholipids in a calcium-dependent manner. There is a plethora of evidence that implicates dysferlin as an essential regulator of vesicle fusion at the sarcolemma and an important player in muscle membrane repair. However, dysferlin is not limited to sarcolemma repair but is also involved in muscle regeneration, focal adhesion formation, adenosine triphosphate-dependent intercellular signaling, and insulin-like growth factor 1 (IGF-1) receptor recycling.

There is no effective treatment for increasing muscle strength or for preventing further muscle damage in these diseases. A potential treatment for dysferlinopathies is the use of IGF-1. Insulin-like growth factor 1 (IGF-1) is a circulating autocrine/paracrine factor that regulates many aspects of muscle development, repair, and growth. Increasing IGF-1 in muscles causes hypertrophy, enhances regeneration after injury, and can improve many diseased muscle phenotypes and functions. However, systemic delivery of recombinant IGF-1 was ineffective at promoting the hypertrophic responses in dysferlin null (Dysf−/−) mice, and the failure was ascribed to the lack of properly localized IGF-1 receptors.

A second potential treatment for dysferlinopathies is myostatin inhibition. As a negative regulator of muscle growth, a number of strategies have been developed to block myostatin activity and boost muscle mass. Indeed, transgenic expression of follistatin and delivery of a soluble form of the myostatin receptor led to significant hypertrophy of muscles in the Dysf−/− mouse; however, this was accompanied by progressive deficits of muscle function due to acceleration of degeneration.

Because of the lack of efficacy for systemic IGF-1 delivery and the risks of any progrowth strategy to potentially cause degeneration of dysferlin-deficient muscles, the objective of this study was twofold. First, we sought to determine whether local expression of IGF-1 could overcome the limitations of systemic delivery and promote gains in muscle mass in dysferlin-deficient mice. Second, we sought to determine whether any increase in muscle mass was associated with loss of force generating capacity.

2 | MATERIAL AND METHODS

2.1 | Animal studies

Animal studies were performed in accordance with and were approved by the University of Pennsylvania animal care committee. For transgenic studies, muscle-specific IGF-1 transgenic (mIgf1−/−) male mice that had been backcrossed to the C57BL/6 J strain were bred with female Bla/J (JAX No. 012767). Male and female mice from the F2 generation were used, and mice with the desired genotypes were used for the experiments. These included mice homozygous for the loss of dysferlin and transgenic expression of Igf1 (Dysf−/−;mIgf1+/+), mice homozygous for the loss of dysferlin with no transgenic expression of Igf1 (Dysf−/−;mIgf1−/−), mice with wildtype levels of dysferlin and transgenic expression of Igf1 (Dysf−/−;mIgf1+/+), and mice with wildtype for both genes (Dysf−/−;mIgf1−/−). Male and female mice 16 weeks of age were used for analysis.

In postnatal viral delivery experiments, controls were male and female dysferlin-deficient A/J mice (JAX No. 000646) and A/WySnJ mice (JAX No. 006647), which is a commercially available inbred strain that is a suitable control for A/J mice. A/J mice are referred to as Dysf−/−, and A/WySnJ mice are referred to as Dysf+/+. Both Dysf−/− lines bear an early ETn retrotransposon insertion in intron 4 of the dysferlin gene, resulting in disruption of the dysferlin gene expression.

2.2 | Viral injections

Recombinant self-complementing adenoassociated virus (scAAV) serotype 2/8 vectors expressing the Igf1 cDNA of murine class I IGF-1A under the control of the chicken β-actin promoter and cytomegalovirus enhancer were generated by the Children’s Hospital of Philadelphia Vector Core, as previously described. Self-complementing adenovirus associated virus was diluted with phosphate-buffered saline to 1 × 10^5 viral particles/μL, and 50 μL was injected into each of the anterior and posterior compartments of one lower hind limb of anesthetized 3-week-old Dysf−/− and Dysf+/+ mice, targeting the tibialis anterior (TA), extensor digitorum longus (EDL), and soleus muscles. The contralateral muscles of male and female Dysf−/− and Dysf+/+ mice were significantly higher in Dysf−/−;mIgf1+/+ mice compared with Dysf−/−;mIgf1−/− mice.

**FIGURE 1** Transgenic Igf1 expression increases skeletal muscle mass independent of dysferlin with no functional decrement. A,B, Bodyweights of male (A) and female (B) mice show significant increases in male Dysf−/− and females of both mIgf1+/+ strains. C,D, TA muscle mass in male mice increased ~30% in both mIgf1+/+ and ~20% in female mice. Only Dysf−/− mice displayed significant hypertrophy in EDL and soleus. EDL muscle tetanic forces from male (E) and female (F) mice were significantly higher in Dysf−/−;mIgf1+/+ mice compared with Dysf−/−;mIgf1−/− mice. EDL muscle-specific forces from male (G) and female (H) mice showed no significant differences among groups. Diaphragm-specific forces from male mice (I) show significant reduction between Dysf−/−;mIgf1+/+ and Dysf−/−;mIgf1−/− muscles of both mIgf1+/+ strains. Diaphragm-specific forces in female mice (J) were not different across all strains. Data are means ± SEM for males (Dysf−/−;mIgf1+/+, n = 6–7; Dysf−/−;mIgf1−/−, n = 4–9; Dysf−/−;mIgf1+/+, n = 6–9) and females (Dysf−/−;mIgf1+/+, n = 5–6; Dysf−/−;mIgf1−/−, n = 4–6; Dysf−/−;mIgf1+/+, n = 4–9). **P < .05, ***P < .001, ****P < .0001 for comparisons between mIgf1+/+ and mIgf1−/−, two-way ANOVA with Tukey’s multiple-comparison test. ANOVA, analysis of variance; DP, diaphragm; Dysf−/−, dysferlin null; Dysf−/−;mIgf1+/+, mice homozygous for the loss of dysferlin and transgenic expression of Igf1; Dysf−/−;mIgf1−/−, mice with wildtype levels of dysferlin and transgenic expression of Igf1; Dysf−/−;mIgf1−/−, mice with wildtype for both genes; EDL, extensor digitorum longus; IGF-1, insulin-like growth factor 1; mIgf1−/−, muscle-specific IGF-1 transgene; TA, tibialis anterior [Color figure can be viewed at wileyonlinelibrary.com]
limb was used as control. After injection, mice were housed in the animal facility until time of analysis. They were humanely killed 1 week, 1 month, or 2 months after injection, and tissues were obtained for functional, biochemical, and morphological analysis.

2.3 | Functional analysis of isolated muscles

Muscle function testing was performed in EDL, soleus, and diaphragm muscles according to previously published protocols. Maximum isometric tetanic forces were measured at fusion frequency and supramaximal stimulation. After completion of the mechanical procedures, muscles were blotted, weighted, and rapidly frozen for subsequent assays. Muscle-specific forces (N/cm²) were calculated by normalizing the maximum muscle tension to the muscle cross-sectional area.

2.4 | Immunohistochemistry

Fiber type, size, and number analyses were performed in EDL and soleus cryosections with anti-laminin (FB-082A; ThermoFisher Scientific) and anti-myosin heavy chain IIA (Sc-71; Developmental Studies Hybridoma Bank), as previously described. Stained sections were visualized with a Leica DMR microscope. Fiber type and size quantitation were performed in SMASH software.

2.5 | Muscle IGF-1 content

Muscle IGF-1 levels were determined with the rat/mouse IGF-1 enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. MG100; R&D Systems) in accordance to the manufacturer’s instructions and as
previously described.\textsuperscript{32} Data are expressed as nanograms IGF-1 per gram of tissue.

2.6 Immunoblotting

Tissues that had been extracted for immunoblot analysis were snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until further processing. Tibialis anterior muscles were mechanically ground by mortar and pestle in dry ice and homogenized in radioimmunoprecipitation assay buffer, with the addition of phenylmethylsulfonyl fluoride, protease, and phosphatase inhibitors. Proteins were quantitated by Bradford assay, and equal amounts were loaded in 12\% precast acrylamide gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. Primary antibodies for phospho-protein kinase b (Akt; Ser473; cat. No. 9271; Cell Signaling Technology) and total Akt (pan; 40D4; cat. No. 2920; Cell Signaling Technology) were used to measure the changes in Akt phosphorylation, which was evaluated in ImageJ.

2.7 Statistical analysis

All data are presented as mean ± SEM. All analysis was performed in Prism 8 (GraphPad Software). Student’s \(t\) test was performed for comparison of two groups, and two-way analysis of variance (ANOVA) was performed for comparison of three or more groups to determine significance, followed by Tukey’s multiple-comparisons post hoc testing. \(P < .05\) was considered significant.

3 RESULTS

3.1 Transgenic IGF-1 expression mediates hypertrophy in the absence of dysferlin

Mice with transgenic expression of \(Igf1\) were compared with age- and sex-matched littermate controls in terms of body and muscle mass. In male mice, there was a significant effect of increased IGF-1 and absence of dysferlin on body weight according to two-way ANOVA, with increased body weight evident in \(Dysf^{-/-}\) mice according to post hoc testing.

\begin{figure}[h]
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\caption{Transgenic \(Igf1\) expression increases IGF-1 content in both male and female muscles independent of strain. IGF-I content of TA muscles was measured by ELISA in male (A) and female (B) mice. Muscles from \(mlgf1^{-/-}\) mice had IGF-1 levels greater than 10-fold higher compared with strain and sex-matched \(mlgf1^{-/-}\) mice. Mean ± SEM for \(n = 4\)–9 for all groups. C.D. Correlations between IGF-1 content and mass for TA muscles are shown for male and female mice. \(R^2\) calculated for low and high IGF-1 content separately. Analysis by two-way ANOVA, followed by Tukey’s multiple comparison test. *\(P < .05\), **\(P < .01\), ***\(P < .001\), ****\(P < .0001\) for comparisons between \(mlgf1^{-/-}\) and \(mlgf1^{-/-}\); \(11p < .01\) for comparison between \(Dysf^{-/-}\) and \(Dysf^{-/-}\). ANOVA, analysis of variance; Dysf”, dysferlin; Dysf”, null; Dysf”, \(mlgf1^{-/-}\), mice homzygous for the loss of dysferlin and transgenic expression of \(Igf1\); Dysf”, \(mlgf1^{-/-}\), mice homozygous for the loss of dysferlin with no transgenic expression of \(Igf1\); Dysf”, \(mlgf1^{-/-}\), mice with wildtype levels of dysferlin and transgenic expression of \(Igf1\); Dysf”, \(mlgf1^{-/-}\), mice with wildtype for both genes; ELISA, enzyme-linked immunosorbent assay; IGF-1, insulin-like growth factor 1; \(mlgf1^{-/-}\), muscle-specific IGF-1 transgene; TA, tibialis anterior [Color figure can be viewed at wileyonlinelibrary.com]}
\end{figure}
hoc analysis (Figure 1A). In contrast, female mice displayed significant responses of body weight to increased IGF-1 in both strains (Figure 1B). Male mice exhibited hypertrophy in TA muscles with transgenic expression of Igf1 regardless of strain, and EDL and soleus muscles were also larger in Dysf−/−:mlgf1+/− mice (Figure 1C). Female mice displayed a less robust hypertrophic response to IGF-1, with significantly increased mass in only fast twitch muscles (EDL, TA) of Dysf−/− mice (Figure 1D), which was similar to what has been reported in previous studies.²⁸ Thus, the effects of IGF-1 on muscle do not require dysferlin, and, in fact, there is an enhanced hypertrophic effect in the absence of dysferlin.

Isolated muscle function testing was performed on the EDL and diaphragm to determine whether changes in muscle mass in mlgf1+/− mice were accompanied by changes in the force-generating capacity of the muscles. Isometric tetanic force significantly increased in EDL muscles of male and female Dysf−/− mice (Figure 1E,F). No changes in the specific force in response to high IGF-1 were observed in EDL muscles (Figure 1G,H), indicating that total tetanic force increased in proportion to mass. For diaphragm muscles (Figure 1I,J), there was significance for both IGF-1 and strain by two-way ANOVA. In post hoc comparisons, the diaphragm from the male Dysf−/− mice displayed an ~30% deficit in force compared with muscles from Dysf+/− mice, which was less pronounced than previous findings in the A/J strain of mice in terms of diaphragm function.²⁹ Increased IGF-1 combined with dysferlin loss did not rescue the specific force deficit compared with Dysf−/−:mlgf1−/− mice. No deficits in diaphragm function were apparent in the female mice regardless of strain.

To investigate whether the observed gain of muscle mass was due to increased muscle fiber numbers or fiber size, EDL muscle morphology was analyzed by immunostaining with anti-laminin. Muscle fiber analysis revealed that there were no differences in fiber number across the genetic conditions (Figure 2A,B). Instead, minimum Feret analysis revealed that there were no differences in fiber number to increased muscle fiber numbers or fiber size, EDL muscle morphologies were similarly evaluated. Transgenic expression of IGF-1 provides a growth stimulus initiated before birth. To determine whether postnatal modulation of IGF-1 could also drive hypertrophy in the absence of dysferlin, we used viral mediated gene transfer into muscles of young Dysf−/− and Dysf−/− mice with scAAV vectors encoding IGF-1A. At 1 week postinjection, IGF-1 content was ~10-fold higher in the injected muscles compared with contralateral control muscles in both genotypes (Figure 4A). There was no significant difference in basal IGF-1 or postinjected IGF-1.

**FIGURE 4** Viral delivery of IGF-1 leads to increased signaling of IGF-1 pathways. A, IGF-1 content was significantly higher after viral delivery in TA muscles from Dysf−/− and Dysf−/− mice (n = 3 pairs for each genotype). There was no significant difference in basal IGF-1 or in postinjected IGF-1 between mouse strains. B, Immunoblot of P-Akt and T-Akt in TA muscle lysates with and without AAV–IGF-1 injection. C, P-Akt levels were increased in the scAAV–IGF-1A injected muscles (+AAV) compared with contralateral control muscles (−AAV) from both Dysf−/− and Dysf−/− mice (n = 3 pairs for each genotype). *P < .05 between injected and uninjected muscles, two-tailed paired Student’s t test. Dysf−/−, dysferlin; Dysf−/−, dysferlin null; IGF-1, insulin-like growth factor 1; P-Akt, phospho-protein kinase b; scAAV, self-complementing adenoviral vector; TA, tibialis anterior; T-Akt total-protein kinase b [Color figure can be viewed at wileyonlinelibrary.com]
between mouse strains. We examined phosphorylation of Akt as a downstream index of IGF-1 receptor activation. Phospho-Akt increased in the scAAV–IGF-1A-injected muscles compared with noninjected control muscles from both Dysf+/+ and Dysf−/− mice (Figure 4B,C).

To determine the effect of increased IGF-1 production on muscle mass, limb muscles were dissected from male and female animals 1 month after adenoassociated virus (AAV) injection. In all mice, the EDL muscles exhibited a 10%–15% increase in mass (A) and a similar increase in tetanic force (B) in treated limbs in both Dysf+/+ and Dysf−/− mice. Specific force (C) did not differ between treated and untreated limbs. At 2 months postinjection, there was a 17%–22% increase in EDL muscle mass (D) in injected limbs, with tetanic force increasing 8%–40% (E). Similarly to the 1-month timepoint, specific force (F) was not different between treated and untreated limbs. Muscles obtained from n = 6–8 mice per strain and timepoint. Male and female mice were combined for analysis. Values are depicted as individual data points, with lines connecting the untreated and treated muscles from the same mouse. **P < .01, ***P < .001 between injected and uninjected muscles, two-tailed paired Student’s t test. AAV, adenoassociated virus; Dysf+/+, dysferlin; Dysf−/−, dysferlin null; EDL, extensor digitorum longus; IGF-1, insulin-like growth factor 1, ns, not significant.
potential for strain-dependent genetic modifiers. With the development of a dysferlin-deficient mouse on the C57Bl/6 background, more closely matched comparisons were possible, although it was unclear whether the same pathological phenotypes would be retained. Thus, for our transgenic crosses, it provided an opportunity to examine functional outcome measures of dysferlin loss on the C57Bl/6 background. The male Dysf−/− mice exhibited a 30% decrease in specific force only in the diaphragms, with no evidence of decreased specific forces in other muscle groups due to the absence of dysferlin. Our previous findings in A/J mice, in which there were deficits of ~50% in specific force in the A/J diaphragm, suggest that the physiological phenotype is altered in the C57Bl/6 strain. Previous researchers have also compared disease severity with respect to dysferlin mutation and background strain and found that the A/J mouse C57Bl/6 background displayed highly variable recovery from strain injury in contrast to three other mouse lines lacking dysferlin. These findings suggest that background strain may affect the pathogenesis associated with the absence of dysferlin, as with other disease models. Whether a single mouse strain can capture the essence of disease progression in humans is an open question, but our observations, among others suggest that genetic modifiers are likely contributing to variability in pathology of dysferlinopathies. The effects of IGF-1 appear to be independent of background strain, which suggests that a therapeutic strategy including a boost of IGF-1 may be generally effective in this disease.

We were struck by the differential response to transgenic expression of IGF-1 in male and female mice. Male mice displayed clear muscle hypertrophy regardless of the presence of dysferlin in the TA muscles, similarly to what has been reported in previous studies in which this approach was used to boost muscle IGF-1 content in models of disease, yet only in Dysf−/− mice was this accompanied by increased body weight. In contrast, female mlgf+/+ mice exhibited increased body weight in the presence and absence of dysferlin, but muscle hypertrophy was not as extensive and was evident only in the absence of dysferlin. Multiple issues arise with this observation. First, it is clear that both males and females respond to high levels of IGF-I in skeletal muscle, but female mice have a blunted hypertrophic response. Second, a more speculative point is that, because increased muscle mass combined with a lack of changed body weight in the male Dysf+/+ : mlgf+/- mice occurs, it reflects findings in previous studies in which modulating muscle IGF-1 led to sex-specific alterations locally in muscle and globally in fat content. Thus, it is essential to evaluate male and female mice separately for global IGF-1 dependent changes in muscle mass.

**FIGURE 6** Functional hypertrophy by viral delivery of IGF-1 on soleus muscles is blunted and delayed in Dysf−/− mice. At 1 month postinjection, treated soleus muscles exhibited a 10%–15% increase in mass in Dysf+/+ mice but no significant increase in Dysf−/− mice (A). Tetanic force was significantly increased in the treated soleus muscles only from Dysf+/+ mice (B). Specific force (C) did not differ between treated and untreated limbs in both strains. At 2 months postinjection, treated soleus muscles from both strains showed significant increases in mass (D), and only Dysf−/− mice had significant increases in tetanic force (E). Similarly to the 1-month timepoint, specific force (F) was not different between treated and untreated limbs. Muscles obtained from n = 6–8 mice per strain and timepoint. Male and female mice were combined for analysis. Values are depicted as individual data points, with lines connecting the untreated and treated muscles from the same mouse. *P < .05, **P < .01, ***P < .001 between injected and uninjected muscles, two-tailed paired Student’s t test. AAV, adenoassociated virus; Dysf+/+, dysferlin; Dysf−/−, dysferlin null; ns, not significant.
Complementary to lifetime exposure to IGF-1, postnatal viral delivery caused similar hypertrophy in male and female mice. However, the soleus muscles in the Dysferlin−/− mice were delayed in responding to IGF-1; hypertrophy was evident only 2 months after viral delivery. This suggests that there is a fiber type specificity in the role that dysferlin plays in mediating hypertrophy by IGF-1. In our previous study, we examined the dysferlin levels in several skeletal muscles, including the soleus.29 The soleus did not have a different dysferlin level compared with the TA or the EDL, which indicates that dysferlin content is independent of fiber type, and this cannot explain the delayed response to increased IGF-1. However, similarly to the negative findings after infusion of recombinant IGF-1 into dysferlin-deficient animals,16 it provides evidence that there is a blunted response to increased IGF-1. The remedy for the lack of hypertrophy appears to be time because 1 month of treatment was not sufficient for recombinant IGF-1 treatment or viral IGF-1 injection. However, 2 months after viral delivery and at 4 months of age with lifetime exposure to IGF-1, there are significant increases in muscle mass. In addition, the high levels of IGF-1 within the muscle are also a likely advantage compared with the delivery of IGF-1 through the circulation.

The strategy to inhibit the myostatin pathway resulted in significant hypertrophy27 but came at the expense of diminished functional capacity. Analysis of EDL muscle function at 16 weeks of age revealed a 30% decrease in specific force in dysferlin-deficient male mice with transgenic expression of follistatin. Hence, we sought to evaluate function in the EDL muscles at the same age. We observed that mass and force production increased in tandem, resulting in no differences in specific force and providing evidence that the IGF-1 and myostatin pathways are not driving a similar degenerative response.

From the context of a therapeutic strategy, it is important to determine an effective "dose" of IGF-1 for boosting muscle mass without off-target effects. In our hands, more is not better because IGF-1 levels five- to 25-fold above normal caused the same extent of hypertrophy, indicating that there is a plateau for efficacy. In contrast, systemic delivery of 2 mg/kg recombinant IGF-1, which is approximately 25-fold higher than the normal circulating levels, can drive hypertrophy only in the presence of dysferlin,16 suggesting that supraphysiologic levels are required to surpass the limitations of IGF-1 signaling in this disease when delivered through the circulation. From a safety standpoint, delivery of such high levels through the bloodstream is not clinically tenable, but, if an effective dose can be achieved locally, then the safety concerns may be reduced. There is no established method for boosting IGF-1 levels specifically in skeletal muscle other than with AAV. This could be combined with restoring dysferlin, similarly to the dystrophin replacement strategy demonstrated in mouse models for Duchenne muscular dystrophy,42 providing both correction of the genetic defect and an enhancement of functional hypertrophy.

In summary, we evaluated the therapeutic potential of IGF-1 for boosting functional muscle mass in the absence of dysferlin. We observed increased muscle mass and commensurate increases in force generation, providing evidence that the IGF-1 pathway is sufficiently intact to mediate functional hypertrophy. Going forward, we encourage our colleagues to reconsider growth promoting strategies for LGMD2B and Miyoshi myopathy patients.

CONFlict of interest
None of the authors have any conflicts of interest to disclose.

Ethical Publication Statement
We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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