Tendon protein synthesis rate in classic Ehlers-Danlos patients can be stimulated with insulin-like growth factor-I

Rie Harboe Nielsen, Lars Holm, Jacob Kildevang Jensen, Katja Maria Heinemeier, Lars Remvig, and Michael Kjaer

1Institute of Sports Medicine, Department of Orthopaedic Surgery, Bispebjerg Hospital, and Centre for Healthy Aging, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; 2Institute of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; and 3Department of Infectious Medicine and Rheumatology, Rigshospitalet, Copenhagen, Denmark

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THE EHLERS-DANLOS SYNDROME (EDS) is a heterogenic group of inherited connective tissue disorders. Patients with the classic form of EDS (cEDS) are clinically diagnosed according to the Villefranche criteria from 1998, where the three major criteria are generalized joint hypermobility, skin hyperextensibility, and atrophic scarring (2). Although, the syndrome often affects many organ systems, symptoms from the musculoskeletal system such as chronic pain from joints, muscles, and tendons, as well as severe fatigue and acute risks of joint dislocations, are most often the dominant ones (11, 19, 20). Recent genetic research in cEDS patients shows that at least 90% of all patients harbor a mutation in one of the two type V collagen genes, COL5A1 or COL5A2 (18). Since all mutations in these genes are not yet known, negative results cannot be used to fully rule out mutations in the individual patient, and the genetic tests are not currently used in normal clinical practice.

Type V collagen is a fibrillar forming collagen that coassembles with type I collagen and regulates the assembly and lateral growth of the type I collagen fibrils (4, 17, 22). Mutations in the type V collagen-encoding genes in cEDS patients are therefore thought to affect the assembly and lateral growth of fibrils, and hereby the structure and function of these collagen I/V fibrils. The amount of type V collagen compared with type I collagen is in healthy individuals around 1:80 (16). Type I collagen constitutes the major part of protein in most connective tissues such as tendon, dermis, and cornea (7). An altered structure of collagen with large, irregular collagen fibrils has been shown in dermis of type V collagen haploinsufficient mice (col5a1+/−) and in patients with cEDS (21, 24). More recently, we have shown that tendons of cEDS patients also contain these large, irregular fibrils (13). Based on these observations it is plausible that COL5A1/COL5A2 mutations in cEDS lead to structural changes in collagen fibrils due to lower amounts of type V collagen, but how the mutations affect overall collagen and connective tissue turnover remains unknown. The purpose of this study was therefore to investigate whether the overall basal collagen synthesis rate in cEDS patients is diminished, and to what extent this synthesis rate can be increased by growth factor stimulation.

As a growth factor for stimulation of collagen synthesis we chose insulin-like growth factor-I (IGF-I), since earlier studies from our laboratory have shown that IGF-I can stimulate tendon protein synthesis in young, middle-aged, and old healthy men (8, 14). In addition systemic growth hormone administration, which led to increased IGF-I levels in circulating blood and increased IGF-I expression in musculotendinous tissue, also stimulated collagen synthesis in tendon and muscle in young males (5). Furthermore, IGF-I is known as a potent stimulator of collagen synthesis in in vitro studies (1, 6, 15). We therefore hypothesized that IGF-I potentially would affect tendon tissue in cEDS patients by enhancing the protein synthesis rate.

In this study, we measured the in vivo protein fractional synthesis rate (FSR) in connective tissue isolated from skin and tendon in patients with cEDS and in healthy, matched controls. In one patellar tendon of each participant we injected IGF-I directly into the tendon to investigate whether IGF-I had any effect on tendon protein synthesis rate in cEDS patients. To do this, we used the flood-primed, continuous stable isotope tracer approach where the fractional synthesis rate of proteins in skin and tendon was measured with the stable isotope technique using a flood-primed, continuous infusion over 6 h. After the infusion one skin biopsy and one tendon biopsy (one from each patellar tendon) were obtained. We found similar baseline FSR values in skin and tendon in the cEDS patients and controls [skin: 0.005 ± 0.002 (cEDS) and 0.007 ± 0.002 (CTRL); tendon: 0.008 ± 0.001 (cEDS) and 0.009 ± 0.002 (CTRL) %/h, mean ± SE]. IGF-I injections significantly increased FSR values in cEDS patients but not in controls (delta values: cEDS 0.007 ± 0.002, CTRL 0.001 ± 0.001%/h). In conclusion, baseline protein synthesis rates in connective tissue appeared normal in cEDS patients, and the patients responded with an increased tendon protein synthesis rate to IGF-I injections.
were given a standardized meal containing 32.2 kJ/kg LBM [20-

tendon. Age, mean (range) 39 (25–61) 39 (23–70)

Table 1. Participant characteristics

<table>
<thead>
<tr>
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<th>cEDS Patients</th>
<th>Healthy Controls</th>
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<td>6/4</td>
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<td>Age, mean (range)</td>
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<td>39 (23–70)</td>
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<td>BMI, mean (range)</td>
<td>23 (17–28)</td>
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cEDS, classic Ehlers-Danlos syndrome; BMI, body mass index.

Technique followed by tissue samples from both skin and tendon.

MATERIALS AND METHODS

Participants. We included five cEDS patients who met all three major diagnostic criteria for cEDS (2). Other disease-characteristic data on the included patients have been published earlier (13), and mutation analyses from that study showed that three of the five patients in this study had a demonstrable COL5A1 mutation. Each patient was individually matched with two healthy controls (CTRL, n = 10) on sex, age, body mass index (BMI), and self-reported physical activity level. Six of the 10 controls were also part of the previously published study where biomechanical data were published (13). Persons with present or historical injuries in the patellar tendons or present systemic diseases (other than cEDS in the patient group) were not included in the study. We aimed to include 10 participants in each group, but despite extensive advertisement we were only able to include 5 patients. The study was approved by Ethical Committee of Capital Region of Denmark (H-1-2011-010) and conducted in accordance with the Helsinki Declaration. All participants signed a written consent form before entering the study. The study was registered as a clinical trial (ClinicalTrials.gov Identifier: NCT01446783).

Intratendinous IGF-I injections. The injection protocol in this study was identical to the one used earlier with healthy participants in our laboratory (14). Briefly, recombinant human IGF-I (Increlex, Ipsen, 10 mg/ml, 0.1 ml) was injected into one patellar tendon of all participants at 24 and 6 h before tendon biopsies were obtained. The contralateral tendon was injected at the same time points with an identical volume of isotonic saline as control. All injections were given with use of ultrasound guidance into the proximal, middle part of the patellar tendon in a double-blinded manner. The participants rested supine for 30 min after injections and were instructed not to perform any exercise between the injections and the biopsy sampling.

Measurements of serum IGF-I. Blood samples for IGF-I measurements were taken in the morning after an overnight fast at the day of experiments. Blood was drawn from an antecubital vein and serum was stored at −80°C until analysis. Total serum IGF-I was measured with an ELISA kit (RMEE20, Biovendor) following the manufacturer’s manual. We have previously shown that similar intratendinous IGF-I injections lead to a small rise in systemic IGF-I values (14), and since the blood samples for IGF-I measurements in this study were taken 17 h after the first IGF-I injections we have presumably overestimated baseline values by ~20%.

Study design to measure protein synthesis in skin and tendon. Before the experimental day all participants had a whole body dual-energy X-ray absorptiometry (DXA) scan (Lunar, DPX-QX software v. 4.6c; Lunar, Madison, WI) performed to precisely determine lean body mass (LBM), which was used for individual meal preparation and tracer infusion rate. On the day before the experimental tendon samples were obtained, all participants had received the first IGF-I and saline injections. On the experimental day the participants came into the lab in the morning after an overnight fast. Two antecubital catheters were placed on each participant for blood sampling and tracer infusion, respectively. A morning blood sample was obtained in the fasting state for IGF-I concentration measurements and to determine [13C]/[12C]proline background abundance. Then participants were given a standardized meal containing 32.2 kJ/kg LBM [20-

energy (E)% fat, 23E% protein and 57E% carbohydrates]. Hereafter, the second IGF-I and saline injections were given, and the tracers, which were purchased from Cambridge Isotope Laboratories (An-
dover, MA, USA), solubilized in sterile 9 g/l saline, and subsequently sterilized through a disposable 0.20-μm filter (Sartorius, Hannover, Germany), were then infused to determine the fractional synthesis rate (FSR) of connective tissue/collagen proteins isolated from skin and tendon specimens. By applying the flood-primed [420 mg (48.8 mol) [U-13C3]proline (>99 atom %) and 3,500 mg (389.0 mol) unlabeled proline], continuous (12.0 μmol [U-13C3]proline kg LBM⁻¹ h⁻¹) infusion, we were able to use the precursor-product approach to measure the protein FSR over a 6-h period (10). Blood samples were obtained at 10, 30, 60, 120, 240, and 360 min. After 6 h of infusion a skin biopsy and two tendon biopsies (from each patellar tendon) were obtained from each participant.

Skin and tendon biopsies. The skin biopsy was obtained from the gluteal region. The skin was anesthetized with 2 ml lidocaine (1%), and the skin biopsy was obtained with a 4-mm Derma Biopsy Punch needle (Miltex, York, PA) under sterile conditions. The skin biopsies weighed ~20 mg. Hereafter, a tendon biopsy from each patellar tendon was obtained. The skin overlying the tendon was anesthetized with 2 ml lidocaine (1%), and the skin was opened under sterile conditions in a 5-mm incision and the tendon biopsy obtained with a 14-gauge automatic needle (Bard Magnum Biopsy Instrument, Bard, Covington, GA). The tendon specimens weighed ~10 mg and were taken at the same knee position of the participant as the injections were given, and the angle of the biopsy needle mimicked that of the injection needle to sample from the injected area of the tendon. After sampling, skin and tendon specimens were visually inspected and fatty tissue removed under microscope. All biopsies were stored at −80°C until further analyses.

Sample preparation and analysis for calculation of the FSR. Calculations of FSR values were performed as described earlier (14). To determine the tracer enrichment in the precursor pool for protein synthesis, the plasma free proline was used. Amino acids were isolated from 200 μl plasma by pouring the acidified plasma (1 ml 50% acidic acid) over AG 50W-X8 resin (Bio-Rad Laboratories; Hercules, CA) columns preconditioned with 1 ml 50% acidic acid. After five washes with Milli-Q water the amino acids were eluted with 2 × 1 ml 4 M NH4OH. The purified and dried amino acids were derivatized using N-methyl-N-(tert-butylidimethylsilyl)-trifluoroacetamide (MtBSTFA) + 1% tert-butyl-dimethylchlorosiliane (tBDMCS) (Regis Technologies, Morton Grove, IL) and analyzed as its t-butyldimethylsilyl derivative using a capillary column (CP-SIL 8, CB Low Bleed, 30 m × 0.32 mm, coating 0.25 μm, Chrompack, Varian, Palo Alto, CA) on a triple stage quadrupole mass spectrometer, TSQ Quantum (Thermo Scientific; San Jose, CA) operated in electron ionization (EI) mode. The settings for the mass spectrometer were positive polarity; profile mode; selected reaction monitoring for

![Fig. 1. Serum IGF-I levels in classic Ehlers-Danlos (cEDS) patients and healthy controls. Total serum IGF-I was measured in cEDS patients (n = 5) and in healthy, matched controls (CTRL) (n = 9). There were no significant differences in serum IGF-I levels between the 2 groups. Error bars represent SD.](http://jappl.physiology.org/ by 10.220.231.9, on July 9, 2017)
tBDMCS-derivatized fragments of proline with 286.150 m/z for unlabeled proline and 291.150 m/z for the [U-13C5]proline tracer, corresponding to product ions 258.150 and 262.150 m/z, respectively; scan time 0.033 s; collision energy 10 V; collision gas (Ar) pressure 1.0 mTorr. Data processing, including peak areas and the tracer-ratio (TTR) enrichment values, was carried out by MassRatio 4.15 (FBU Engineering). The enrichment was calculated by subtracting the background TTR from all samples obtained during the infusion. Skin and tendon specimens were homogenized in buffer (0.15 M NaCl, 0.1% Triton X-100, 0.02 M Tris·HCl, 5 mM EDTA, pH 7.4), left for 3 h, and centrifuged (1,600 g, 20 min, 4°C). The pellet was washed once in 70% ethanol and hydrolyzed overnight in 6 M HCl at 110°C. The liberated amino acids were then purified over cation-exchange resin columns and derivatized as their N-acetyl-n-propyl (NAP) esters. The proline 13C abundance was determined by gas chromatography-combustion-isotope ratio mass spectrometry (Delta Plus XL, Thermo Finnigan, Bremen, Germany) using a CP-Sil 19 CB column, 60 m × 0.32 mm, coating 0.25 μm (ChromPack, Varian, Palo Alto, CA) to isolate the NAP-derivatized proline compound. Skin and tendon protein FSR was calculated according to the precursor-product method: 

\[
\text{FSR} = \frac{\Delta_m^{product}}{\Delta_m^{precursor}} \times \Delta_t, 
\]

where \(\Delta_m^{product}\) is the difference in the tracer enrichment between the tissue samples obtained after the tracer infusion (skin or tendon) and the background proline 13C abundance determined from plasma proteins in the background blood sample, \(\Delta_m^{precursor}\) is the weighted average of tracer enrichment measured in plasma throughout the tracer infusion period, and \(\Delta_t\) is the tracer incorporation time, that is, from the start of tracer exposure until tissue harvesting, measured in hours.

**Statistics.** Group differences in serum IGF-I and baseline skin FSR were assessed with unpaired t-tests. Effect of IGF-I on tendon FSR in the two groups was assessed with a two-way ANOVA with IGF-I/placebo (repeated measurements) and cEDS/CTRL as variables. When interaction was found a Sidak’s post hoc test was used for multiple comparisons. All statistical analyses and graphs were done in Prism (version 6.0c, GraphPad Software). \(P\) values below 0.05 were considered significant.

**RESULTS**

**Participants.** There were no differences with regard to age or BMI between the cEDS and the CTRL group (Table 1). Further, the groups were also successfully matched on sex (Table 1) and self-reported physical activity (data not shown), and all participants in the patient as well as in the control group had a low to moderate physical activity level. All cEDS patients had serum IGF-I levels within the normal range, and no difference was found in serum IGF-I values between patients and healthy controls (Fig. 1).

**FSR at baseline and in response to IGF-I.** The baseline FSR values did not differ between cEDS patients and healthy controls in either skin (cEDS 0.005 ± 0.002, CTRL 0.007 ± 0.002%/h, mean ± SE, Fig. 2) or tendon (cEDS 0.008 ± 0.002, CTRL 0.009 ± 0.002%/h, mean ± SE, Fig. 3). All five cEDS patients showed an increase in tendon FSR values in response to IGF-I (Fig. 3). In the control group the effect of IGF-I on tendon protein synthesis rates was less clear, and there was a relatively large variation in individual responses (Fig. 3). Statistical testing showed a significant increase in FSR in the cEDS patients in response to IGF-I \((P < 0.05)\), and no effect of IGF-I on FSR in the control group (group delta values between IGF-I and placebo: cEDS 0.007 ± 0.002, CTRL 0.001 ± 0.001%/h, mean ± SE).

**DISCUSSION**

In this study we found that cEDS patients have a baseline protein synthesis rate within the normal range in both tendon and skin. This indicates that overall protein synthesis in tendons and skin were not chronically diminished. Furthermore, all cEDS patients responded locally to intratendinous IGF-I injections with a significant increase in tendon protein synthe-

![Fig. 2. Fractional protein synthesis rate in skin in cEDS patients and healthy controls. The FSR was measured in skin proteins with the stable isotope approach in cEDS patients \((n = 5)\) and healthy controls \((n = 10)\). We used a flood-primed, continuous infusion \((6 h)\) with tracer [U-13C5]proline. After the tracer infusion a skin biopsy was obtained from the gluteal region. Error bars represent SD.](http://jap.physiology.org/)

![Fig. 3. Fractional protein synthesis rate in tendon in response to IGF-I in cEDS patients and healthy controls. The FSR was measured in tendon proteins in response to IGF-I with the stable isotope approach in cEDS patients \((n = 5)\) and healthy controls \((n = 10)\) using a flood-primed, continuous infusion \((6 h)\) with tracer [U-13C5]proline. Prior to tracer infusion one patellar tendon of each participant was injected with IGF-I twice \((24 and 6 h before tendon biopsy)\), and the contralateral tendon was injected in a similar manner with saline as control. Tendon biopsies from patellar tendons were obtained after tracer infusion. A shows the individual FSR values, while B shows group means with SE. *Significant difference in placebo FSR and IGF-I FSR values in the cEDS group.](http://jap.physiology.org/)
It is known that cEDS patients have hyperextensibility of the skin, and further we have recently found that cEDS patients have markedly lower tendon stiffness but a normal tendon diameter and cross-sectional area (13). These findings indicate that the type V collagen mutations in cEDS patients lead to altered tissue quality but not to overall size change in collagen-rich tissues such as skin and tendon. The demonstrated low tissue stiffness is probably related to a genetically based decreased ratio between type V and type I collagen, which experimentally has been shown to lead to collagen fibrils that are larger and more irregular in shape than normal fibrils (3, 22). It is currently unknown how a reduction in type V collagen will affect type I collagen or overall connective tissue turnover. A reduction solely in type V collagen synthesis would be impossible to detect in the overall tendon protein synthesis, since type V collagen constitutes such a small amount of the total collagen in tendon. It could, however, be speculated that a mutation in type V collagen-encoding genes, which reduces tissue quality, would lead to a compensatory increase of overall collagen synthesis. In the present study, no differences were found in baseline protein synthesis rates in either tendon or skin between the cEDS patients and healthy controls. We should nevertheless be careful to conclude on the baseline measurements in this study because of the relatively small amount of participants. Collagen turnover in relation to cEDS has, to our knowledge, only been measured in one previous cell study with skin fibroblasts from cEDS patients harboring known COL5A1 mutations (23). In this study they found that cEDS cells only deposited around half of the amount of total collagen compared with normal cells over 21 days. However, no difference in the rate of collagen synthesis was seen when comparing cEDS and healthy cells. This implies that measures of collagen synthesis rates do not represent collagen deposition. Thus our findings do not rule out that cEDS patients synthesize the same amount of tendon collagen as healthy persons, but are not able to incorporate an adequate amount of collagen into structural tissue proteins. Furthermore, we found that protein synthesis was increased in cEDS patients with IGF-I stimulation, but we cannot state how much of this increased synthesis will lead to any true incorporation of new collagen into fibrils of the tendon.

In all the cEDS patients we found that IGF-I stimulated tendon protein synthesis (Fig. 3). This was in contrast to the responses in the matched healthy controls where no significant effect of IGF-I on tendon protein synthesis was found in the present study (Fig. 3). We were surprised not to find an effect of IGF-I in our healthy control group, since we have earlier found that men at a large age range (from 20 to 75 yr of age) show increased tendon protein synthesis significant in response to local injections of IGF-I (8, 14). It can, however, be difficult to compare different FSR protocols (5, 8). Furthermore, in the present study our control group consisted of six women and four men. The overall lack of significant response to IGF-I in our control group could potentially therefore be due to a sex difference in response to IGF-I. As estrogen is believed to have an inhibitory effect on IGF-I signaling and responses in collagen synthesis, this could explain why women would not react to IGF-I as men do (9, 12). However, since the two groups in the present study were matched on sex and both consisted of 60% females, this cannot explain why cEDS patients responded to IGF-I and healthy controls did not.

In the current study the tendon biopsy from each participant was obtained at one time point, which was 6 and 24 h after the two injections with IGF-I. The design with two timely interspersed IGF-I injections prior to measurement of tendon protein FSR was chosen to improve the likeliness of detecting the stimulatory effect of IGF-I if at all present. Tendon protein FSR was only measured once due to the fact that carryover effects from earlier biopsies possibly will affect protein synthesis rates. The drawback, however, is that we cannot conclude anything about the temporal pattern of the stimulatory effect of IGF-I, which must be up to further studies to investigate. The fact that IGF-I stimulated collagen synthesis in cEDS patients is encouraging, since no treatment to this disease currently exists. Further studies are needed to determine the long-term effects of this stimulus on tendon tissue. Also growth hormone (GH) injections should be tested as possible treatment, because of the practical advantages in availability and costs of GH compared with IGF-I.

In conclusion, we found that cEDS patients had baseline serum IGF-I concentration within normal range, that they had baseline protein synthesis rates in skin and tendon tissue similar to that in healthy controls, and that their tendon protein synthesis was increased in response to intratendinous IGF-I injections. From these results we conclude that the overall collagen synthesis in tendon and skin appears unaffected by type V collagen mutations in cEDS patients, but that the collagen synthesis can be stimulated in these patients with IGF-I injections. This could be clinically relevant for cEDS management since no treatment currently exists. A stimulation of collagen synthesis by IGF-I administration could potentially result in tissue remodeling and thereby a mechanical stiffening of collagen-rich tissues. However, further studies are needed to address whether the increased collagen synthesis is followed by altered tissue structure and function.

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GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: R.H.N., L.H., K.M.H., L.R., and M.K. conception and design of research; R.H.N., L.H., and J.K.J. performed experiments; R.H.N. and M.K. interpreted results of experiments; R.H.N. prepared figures; R.H.N. drafted manuscript; R.H.N., L.H., J.K.J., K.M.H., L.R., and M.K. edited and revised manuscript; R.H.N., L.H., J.K.J., K.M.H., L.R., and M.K. approved final version of manuscript.

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