Eur J Appl Physiol (2013) 113:2101–2109
DOI 10.1007/s00421-013-2638-3

ORIGINAL ARTICLE

No inflammatory gene-expression response to acute exercise in human Achilles tendinopathy

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Received: 22 October 2012 / Accepted: 30 March 2013 / Published online: 16 April 2013

Abstract Although histology data favour the view of a degenerative nature of tendinopathy, indirect support for inflammatory reactions to loading in affected tendons exists. The purpose of the present study was to elucidate whether inflammatory signalling responses after acute mechanical loading were more pronounced in tendinopathic versus healthy regions of human tendon and if treatment with non-steroidal anti-inflammatory medications (NSAID's) reduces this response. Twenty-seven tendinopathy patients (>6 months) were randomly assigned to a placebo ($n = 14$) or NSAID (Ibumetin NYCOMED GmbH Plant Oranienburg Germany (600 mg) × 3/day/1 week) group ($n = 13$) in a double-blinded-fashion. Tendon biopsies were taken from the painful and a healthy region of the same tendon 2 h after 1 h running. Gene-expression of several targets was analysed in the sampled Achilles tendon biopsies. The mRNA for TGF-β, collagen-I and collagen-III were significantly higher expressed, and decorin, CTGF, IL-6 and IL-10 were significantly lower expressed in the tendinopathic versus healthy tendon area. Only IL-10 was lower in expression in experiments with NSAID administration, while all other determined parameters were unaffected by NSAID. All ultrasonographic outcomes were unchanged in response to acute exercise and not influenced by NSAID. The signalling for collagen and TGF-beta was upregulated after acute loading in tendinopathic tendon. In contrast to the hypothesis, inflammatory signalling was not exaggerated in tendinopathic tendon 2 h after acute mechanical loading.

Keywords Tendinopathy · Inflammation · NSAIDs · Gene-expression · Collagen · Exercise

Communicated by Martin Flueck.

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Introduction

Tendon overuse, and the subsequent development of tendinopathy, represents a common disorder that is difficult to treat (de Jonge et al. 2011; Jain et al. 2011; Malvankar and Khan 2011). The tissue pathology associated with tendinopathy is dominated by degenerative and regenerative changes in the matrix structure (Kannus et al. 2002; Riley 2005; Riley et al. 1994) and with an upregulation of gene expression for structural proteins like collagen and associated growth factors. From histological analyses of tendinopathic tissue in humans, there has been no support for the existence of any inflammatory component associated with

tendinopathy (Puddu et al. 1976; Riley 2005). However, some data support that inflammation is present in tendinopathy (Cetti et al. 2003), and it has been documented that anti-inflammatory treatment with glucocorticoids in tendinopathic conditions does have a curative effect, at least in the short term (Hart 2011). Thus, it has been suggested that tendinopathic tendons may not display any inflammatory signs in the resting unloaded state, but, when subjected to loading, by muscular exercise, these will display some inflammatory characteristics. In healthy tendon subjected to exercise it has been demonstrated that the concentrations of inflammatory markers such as prostaglandins (e.g. PGE2) and interleukins (e.g. IL-6) rise in the interstitial space of the peritendinous tissue (Heinemeier and Kjaer 2011; Langberg et al. 2002), and it has been demonstrated that this rise in inflammatory activity regulates both blood flow (PGE2) (Langberg et al. 2003) and collagen formation (IL-6) (Andersen et al. 2011). Thus, we hypothesise that mechanical loading of a tendinopathic tendon will result in an exaggerated signalling in inflammatory pathways, compared with mechanically loaded healthy tendon tissue. In accordance with this, we also hypothesise that administration of anti-inflammatory medication prior to mechanical loading will diminish the inflammatory response. In the present study we have used a human tendinopathy model, in which we were allowed to obtain tendon biopsy samples after an acute bout of exercise both in a clinically and an ultrasonographically verified affected tendon area, as well as in a region proximal to this in the same tendon that appeared healthy. This was done in a blinded randomised design where individuals received either anti-inflammatory medication or placebo prior to the exercise bout.

Available data on collagen synthesis/turnover in and around the tendon (Fredberg 1997; Heinemeier et al. 2013; Langberg et al. 2003) suggest that the peritendinous tissue, and potentially the surface layer of the tendon, have a higher protein turnover than tissue in the core of the tendon. In the present study, the biopsy method provided full-diameter tendon tissue and enabled the splitting of the biopsies into regional parts. Thus in addition to comparing diseased and healthy tendon regions, we used the biopsy from the healthy tendon region to compare mRNA expression between regions. It was hypothesized that heterogeneity with regard to RNA expression exists within a tendon and that superficial regions of the tendon could display greater levels total RNA and of mRNA expression for matrix-related components compared with the core region.

Materials and methods

The subjects were fully informed and gave their written consent to participate in adherence to the declaration of Helsinki. The study was approved by the Ethical Committee of the capital region of Copenhagen, Denmark (H-1-2009-114).

Table 1 Subject characteristics

	Placebo	NSAIDs
Gender	(3 female; 11 male)	(5 female; 8 male)
Age (years)	50 ± 11	47 ± 12
Height (cm)	179 ± 9	177 ± 13
Weight (kg)	93 ± 12	89 ± 13
BMI	29 ± 4	30 ± 5
History of pain (months)	39 ± 10	49 ± 19
VISA-A	44 ± 2	45 ± 2

Values shown as mean ± SD

Experimental design

Subjects

Twenty-seven Achilles tendinopathy patients were recruited and randomly divided into two groups $n = 14$ PLC and $n = 13$ NSAIDS. All subjects were recreational athletes or workers with a long-term history of chronic Achilles tendon pain (detailed subjects characteristics shown in Table 1), and conventional conservative treatments, like eccentric training programmes, NSAIDs and corticosteroid injections, had been tried with no effect. A window of at least 6 months between the most recent last intake of NSAID or corticosteroid injection and the present study was ensured in all cases. All subjects were recruited from the Diagnostic Centre, Silkeborg Region Hospital, Denmark, and the biopsies from the Achilles tendons were taken as part of a standard procedure to examine for deposits of cholesterol, uric acid, and amyloid in the injured Achilles tendons.

Design

All subjects received either placebo or NSAID [Ibumetin NYCOMED GmbH Plant Oranienburg Germany (600 mg)] pills and were instructed to take one pill three times a day during the week prior to the experimental day. The last pill was taken on the morning of the experimental day. The intake of the pills was recorded by the subjects in a diary. On the experimental day the subjects arrived in the morning and the Achilles tendons of both legs were ultrasound scanned, and the tendon thickness, real-time sonoelastography, and Doppler activity were analysed. Blood samples were taken and sent to the local department of biochemistry immediately, where they were analysed for

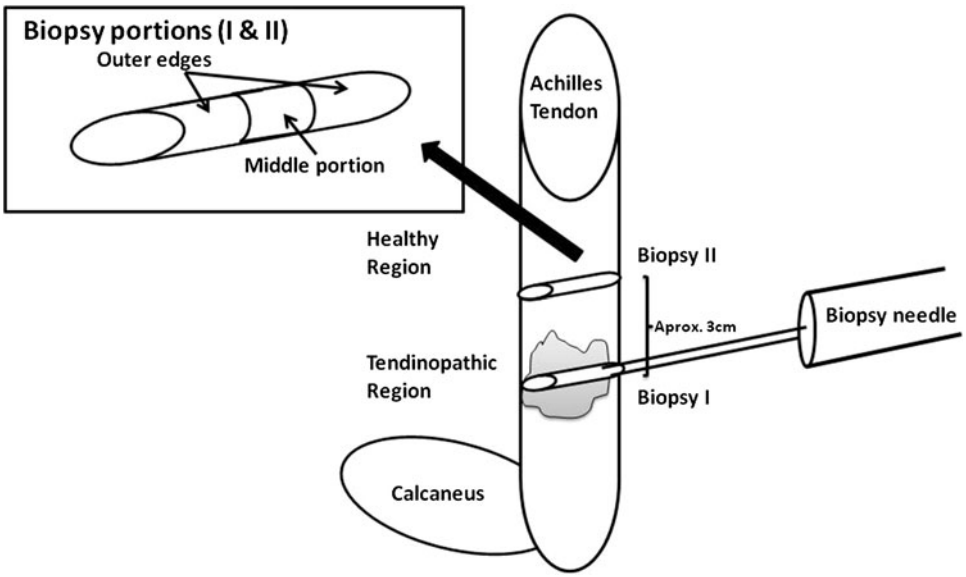

Fig. 1 Biopsy procedure. Schematic overview: the procedure of portioning tendon biopsies for further PCR analysis

CK and CRP. Additionally a VISA-A score and the bodyweight and height of the patient were recorded. Thereafter, the patient was supervised while running on a treadmill for 1 h at a self-decided velocity and a treadmill slope of 2 %. A VAS score was obtained every 10 min during the run and the heart rate (HR) was recorded during the run to estimate the Achilles pain level of the patient (Vas scale range from 0 to 10 AU). Immediately after the run the patients were ultrasound scanned again and the same parameters were analysed as before the run, and blood samples were taken. After that the subjects were allowed to rest for 2 h, and then two tendon biopsies were taken (Fig. 1). The ends of each biopsy were cut off and analysed together, separately from the middle part of the biopsy, to investigate whether there were any differences in the amount of RNA or various gene targets within the biopsy (Fig. 1).

Biopsy procedure

The subjects were locally anaesthetized, in the peritendinous space from both the medial and lateral side of the tendon with injections of 2 × 10 ml 1 % Lidocaine, using ultrasound guidance. Biopsies were taken, ultrasound (US)-guided, with semi-automatic biopsy needle (14 GA 9 cm (Angiotech)). An initial tendon biopsy was taken in the tendinopathic area as indicated by US (defined as the area with maximal increased tendon thickness, neovascularisation, and/or hypo-echogenicity). This area was usually 3–5 cm above the attachment of the Achilles tendon to the calcaneus bone. A second biopsy was taken from the same tendon 3 cm proximal to the first biopsy in a region of the tendon tissue that was deemed normal using US (Fig. 1). The biopsies obtained ranged from 19 to 25 mg wet weight. The samples were snap-frozen and stored at −80 °C for later gene expression analysis.

RNA extraction and real time–PCR analysis

Total RNA isolation

Total RNA was extracted from frozen tendon samples from 27 subjects using 1 ml of TriReagent (MRC) containing five stainless steel balls of 2.3 mm diameter (BioSpec Products, Inc., Bartlesville, Oklahoma, USA), and four silicon-carbide sharp particle of 1 mm (BioSpec Products, Inc.), by shaking in a FastPrep®-24 instrument (MP Biomedicals, Inc., Illkirch, France) at speed level four for 15 s. In order to obtain complete homogenization of tissue, the shaking process was repeated six times with cooling on ice between each shaking step (to avoid heating of the sample). Following homogenization, bromo-chloropropane was added to separate the samples into an aqueous and an organic phase. Glycogen was added (120 µg per ml of TriReagent) to the tendon samples to improve RNA precipitation. Following isolation of the aqueous phase, RNA was precipitated using isopropanol. The RNA pellet was then washed in ethanol and subsequently dissolved in RNAse-free water. The RNA concentration was determined using a RiboGreen RNA Quantitation kit, Molecular Probes USA. The RNA samples were stored frozen at −20 °C until subsequent use in real-time RT-PCR procedures. The total amount of RNA was too low for reliably evaluating the quality. However, in general the standard

Table 2 PCR primers

mRNA	Sense primer	Antisense primer
Collagen1A1	GGCAACAGCCGCTTCACCTAC	GCGGGAGGACTTGGTGGTTTT
Collagen3A1	CACGGAAACACTGGTGGACAGATT	ATGCCAGCTGCACATCAAGGAC
Decorin	GGTGGGCTGGCAGAGCATAAGT	TGTCCAGGTGGGCAGAAGTCA
CTGF	TGCGAAGCTGACCTGGAAGAGA	GCCGTCGGTACATACTCCACAGAA
TGFb-1	GAGGTCACCCGCGTGCTAATG	CACGGGTTCAGGTACCGCTTCT
IL-1b	TCCAGGGACAGGATATGGAGCA	AGGCCCAAGGCCACAGGTATTT
IL-6	GAGGCACTGGCAGAAAACAACC	CCTCAAACTCCAAAAGACCAGTGATG
TNF-a	TTCCCCAGGGACCTCTCTCTAATC	GAGGGTTTGCTACAACATGGGCTAC
RPLP0	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
GAPDH	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
IL-10	CGCTGTCATCGATTTCTTCCCTGT	TGGCTTTGTAGATGCCTTTCTCTTGG
COX-2	AACTGCGCCTTTTCAAGGATGG	TGCTCAGGGACTTGAGGAGGGT
Substance-P	TGGTACGACAGCGACCAGATCAA	TCTCTGCAGAAGATGCTCAAAGGG

errors for the measured targets were low, indicating that the measurements were not influenced by a potential reduction in mRNA quality.

cDNA synthesis

100 ng RNA was reverse transcribed for each tendon sample in a total volume of 20 µl using the Qiagen Omniscript RT Kit (with random hexamers) at 37 °C for 1 h followed by 70 °C for 15 min. The resulting cDNA was diluted 20 times in dilution buffer (10 mM Tris EDTA buffer: Sigma Germany) + Salmon Testes DNA (1 ng/µl; Sigma Germany).

Polymerase chain reaction

The Real-time PCR-method using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 60S acidic ribosomal protein P0 (RPLP0) as reference genes to study specific mRNAs of interest was applied. The primers were purchased from MWG biotech. For each target cDNA the PCR reactions were carried out under identical conditions using 5 µl diluted cDNA in a total volume of 25 µl QuantiTect SYBR Green PCR Mix (Qiagen) and 100 nM of each primer (Table 2). The amplification was monitored in real-time using a MX3005P real-time PCR machine (Stratagene, CA). The threshold cycle (C_t) values were related to a standard curve made with cloned PCR products to determine the relative difference between the unknown samples, accounting for the PCR efficiency. The specificity of the PCR reaction was confirmed by melting curve analysis after amplification. The real-time PCR conditions were as follows: to denaturate the DNA strands the reaction mix was heated above the melting temperature of DNA (95 °C) for 10 min, followed by 50 cycles each of 15 s at 95 °C, followed by the annealing step where optimal primer hybridization conditions were obtained by lowering the temperature to 58 °C for 30 s, and the extension step, where the reaction mix was heated to 63 °C for 90 s. The RPLP0 gene had been chosen as an internal control for normalization, assuming RPLP0 to be constitutively expressed. To validate this assumption, GAPDH mRNA was measured as another unrelated "constitutive" mRNA and normalized with RPLP0, showing no difference between the healthy and the tendinopathic region of the tendon. Data are shown as fold differences from the mean of healthy tendon in the placebo group.

Ultrasonography

For the ultrasonography, and the power Doppler and real-time sonoelastographic (real-time SE) examinations, a high-resolution Hitachi 900 ultrasonograph with a 16-MHz linear transducer were used. Patients were in a prone position and their heels overhanging the examination couch.

The ankle was in 90-degree flexion when tendon thickness was measured and in relaxed position when the power Doppler and real-time SE were done. The Achilles tendons were scanned both longitudinally and transversally. The thickness of the tendon was measured perpendicular to the greatest width of the tendon at the thickest point of the tendon and was recorded in both longitudinal and transversal scan plane as described by Fredberg et al. (2008). If the difference between the two measurements was more than 0.3 mm, both measurements were repeated until the difference was 0.3 mm or less. Then the longitudinal measurements were taken. The power Doppler ultrasound signal in the Achilles tendon was measured with standardized settings (transmit power <500 mW/cm^2,

low-pass wall filter, medium persistence) that remained fixed throughout the study (Torp-Pedersen and Terslev 2008). These settings were chosen to maximize the sensitivity to low-velocity and low-volume blood flow. The power Doppler ultrasound gain was optimized with an increase in gain until noise appeared; then the gain was reduced slightly, only enough to suppress the noise (usually ~60–70 % gain). We applied the appropriate colour velocity scale using the musculoskeletal programme of our ultrasound unit. The window (colour box) was restricted to the vascular area studied.

Sonoelastography

Sonoelastography measurements were made of the tendinopathic area of the tendon before and after 1 h of running exercise. The tissue elasticity in real-time SE was represented by a colour-coded image overlaid on the conventional B-mode image. The force applied to the tendon was adjusted appropriately, according to the visual indicator seen on the screen which showed optimal pressure in the region of interest (Palle et al. 2011). Each real-time sonoelastographic scan was repeated by compression and relaxation of the scan area several times until the findings were confirmed to be reproducible. The values were given after a scale ranging from 1 to 5 (Table 3).

Statistics

The PCR data were log transformed and a three-way Anova followed by a Sidak post hoc test was made to compare the tendinopathic- and the healthy area of the tendon, the placebo or NSAID treatment, and the position of the sample either the middle part or the outer edges of the biopsy. All PCR data are presented as the geo mean ± backtransformed SEM. The subject characteristics are shown in mean ± SD; the subsequent data are shown in mean ± SEM. The statistical calculations of the three-way Anova were made in SAS 9.1. The VAS data and the CK and CRP levels of the blood were analysed in Graphpad Prism 4.0. (GraphPad software, San Diego California USA) using a two-way Anova with Bonferroni correction. All graphs were prepared in Graphpad Prism 4.0. (GraphPad software, San Diego California USA). The level of significance was taken as being $p < 0.05$ for all analyses.

Table 3 Sonoelastography scaling

Numeric scale	Value description
1.0	Blue
1.5	More blue than green
2.0	Equal blue and green
2.5	More green than blue
3.0	Green
3.5	More green than red
4.0	Yellow (equal green and red)
4.5	More red than green
5.0	Red

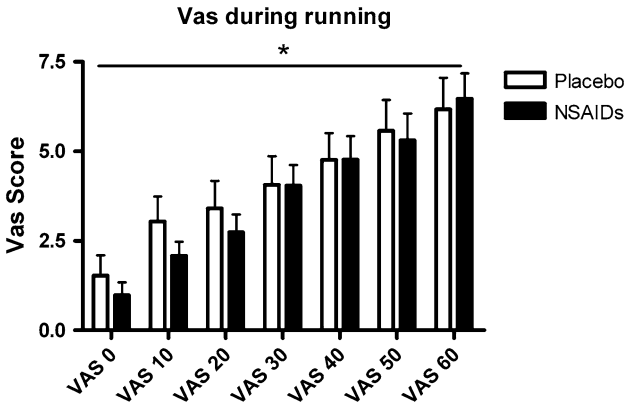

Fig. 2 VAS score during 1 h running. The Vas score values taken each tenth minute increasing significantly in both groups throughout the run. *Error bars* represent SEM. *$p < 0.05$. PLC $n = 14$; NSAIDs $n = 13$

Results

Pain

The VISA-A score was 44 ± 1 and 42 ± 1 in the PLC and NSAIDs groups, respectively, and there was no significant difference between the two groups ($p = 0.83$). The VAS score increased significantly in both groups during the run (1.5–6.2; $p = 0.0001$), but there was no significant difference between the groups ($p = 0.09$; Fig. 2).

Exercise parameters

The heart rate during the run was mean HR: 129 ± 6 bpm; max HR: 152 ± 9 bpm in the PLC group and mean HR: 133 ± 6 bpm and max HR: 162 ± 7 bpm in the NSAIDs group with no significant difference between the groups ($p = 0.68$; $p = 0.90$, respectively). The distance the patients ran was 6.2 ± 0.6 km in the PLC group and 6.2 ± 0.8 km in the NSAIDs group.

Ultrasonography

The results of the ultrasound investigations, including tendon thickness colour Doppler and real-time sonoelastography, were not significantly different, either over time or between the two groups (Table 4).

Table 4 Ultrasonography and blood sample outcomes

	Placebo		NSAIDs		Values
	Pre run	Post run	Pre run	Post run	
Doppler activity (AU)	2.0 ± 0.1	2.2 ± 0.2	2.3 ± 0.3	2.6 ± 0.2	$p > 0.05$
Sonoelastography (AU)	3.0 ± 0.2	2.9 ± 0.3	3.0 ± 0.2	2.8 ± 0.2	$p > 0.05$
Tendon thickness (mm)	10.4 ± 0.6	10.1 ± 0.6	10.0 ± 0.8	9.4 ± 0.8	$p > 0.05$
CK (U/l)	225 ± 40	270 ± 42[a]	104 ± 22	131 ± 30	$p < 0.0005$
CRP (mg/l)	2.2 ± 0.7	2.3 ± 0.7	1.6 ± 0.3	1.5 ± 0.3	$p > 0.05$

All values shown as mean ± SEM

p values indicates differences between the placebo and the NSAIDs group

[a] Indicates a p value $p < 0.002$ when pre run was compared with post run in the PLC group

In blood samples, no significant differences were found in the CRP concentrations, either between the groups ($p = 0.21$) or as a result of the running exercise ($p = 1.0$). The CK values in the blood increased significantly in the PLC group only ($p < 0.002$). Furthermore, a significant difference between the groups was observed ($p = 0.0005$) (Table 4).

Gene expressions

No significant changes were observed in any gene target, comparing the placebo and the NSAIDs groups, apart from the expressions of IL-10 which was significantly down regulated in the NSAIDs group compared with the placebo group ($p = 0.001$; Fig. 3). With regard to the comparison of the middle versus the outer region of the tendon tissue only IL-b was differentially regulated and showed significant interaction between treatment and region. However, the post hoc test showed no significant differences between the groups for IL-1b. For all other targets, no differences were observed between the outer edges of the tendon and the middle part of the tendon, neither in the tendinopathic region nor in the healthy region of the Achilles tendon (data not shown). As the outer and middle parts of the tendon were so similar, all figures just show the average values.

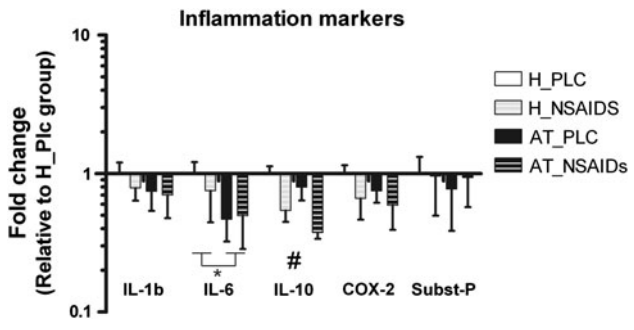

Fig. 3 Inflammatory markers. Inflammatory markers, shown as a relative ratio. The healthy Placebo region equals 1. Healthy NSAIDs, Tendinopathic Placebo and the Tendinopathic NSAIDS group are shown relatively to the healthy Placebo group. *Data* shown as GeoMean ± backtransformed SEM. *Asterisk* Refers to a main difference between the healthy and the tendinopathic area of the tendon ($p < 0.05$), and [#] indicates a main difference between the placebo and NSAIDs treatment ($p < 0.05$). PLC $n = 14$; NSAIDs $n = 13$

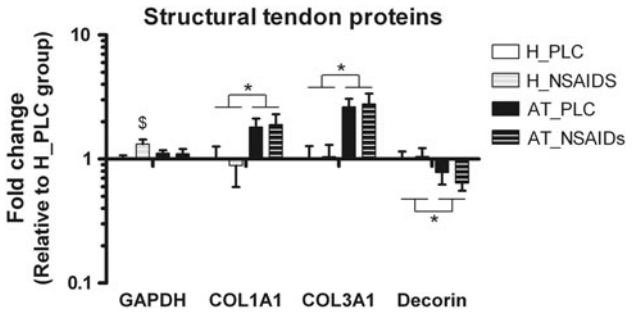

Fig. 4 Structural proteins of the tendon. Gene expressions of collagens, and non-collagenous matrix components, shown as a relative ratio. The healthy Placebo region equals 1. Healthy NSAIDs, Tendinopathic Placebo and the Tendinopathic NSAIDS group are shown relatively to the healthy Placebo group. *Data* shown as GeoMean ± backtransformed SEM. *Asterisk* Main difference between the healthy and the tendinopathic area of the tendon ($p < 0.05$), [$] difference between the placebo and NSAIDs treatment in healthy region ($p < 0.05$). PLC $n = 14$; NSAIDs $n = 13$

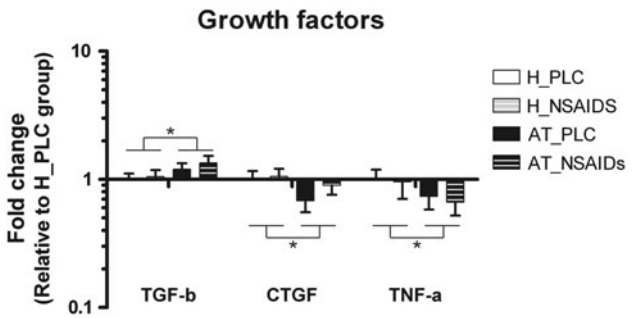

Fig. 5 Growth factors in the tendon. Gene expressions of Growth factors shown as a relative ratio. The healthy Placebo region equals 1. Healthy NSAIDs, Tendinopathic Placebo and the Tendinopathic NSAIDS group are shown relatively to the healthy Placebo group. *Data* shown as GeoMean ± backtransformed SEM. *Asterisk* Main difference between the healthy and the tendinopathic area of the tendon ($p < 0.05$). PLC $n = 14$; NSAIDs $n = 13$

Several significant differences were observed when comparing the healthy area of the tendon with the tendinopathic area. A significant increase in Collagen1A1 ($p < 0.0001$), Collagen3A1 ($p < 0.0001$) (Fig. 4) and TGF-β ($p = 0.03$; Fig. 5) was observed in the tendinopathic area. Furthermore, a significant decrease of decorin ($p = 0.002$; Fig. 4), CTGF ($p = 0.02$; Fig. 5), TNF-α ($p = 0.02$; Fig. 5) and IL-6 ($p = 0.002$; Fig. 3) was observed in the tendinopathic area, compared with the healthy area of the tendon. No differences in the expressions of COX-2 and substance-P were observed between healthy and tendinopathic area (Fig. 3).

Discussion

The results from the present study indicate that a tendinopathic human tendon after acute exercise does not display any acute inflammation. This indicates that there is no exaggerated inflammatory signalling in tendinopathic human tendon after acute mechanical loading. Despite this, there were parameters, like collagen, decorin and IL-6, that revealed significant differences in signalling between tendinopathic and healthy parts of the same tendon.

We originally hypothesized that acute exercise would generate an inflammatory response in the tendon tissue and that a pronounced inflammatory signalling pattern would be present in a tendinopathic tendon area after exercise. Exercise has been shown to increase the concentrations of cytokines both in the blood (Fischer 2006), in human fibroblasts after stretching (Skutek et al. 2001a, b) and in the peritendinous tissue of healthy human Achilles tendon, determined by microdialysis (Langberg et al. 2002) and at mRNA expression levels in human tendon (Heinemeier et al. 2011). Additionally, exercise has been shown to increase the expression of growth factors in the tendon in both rats and rabbits (Heinemeier et al. 2007; Nakama et al. 2006). The absence of inflammation in the tendon tissue after acute running exercise in the patients of the present study does not indicate that anti inflammatory drugs should be used. In accordance with that, the intake of NSAID's for 1 week prior to the experiment did not affect the expression of any of the analysed targets, when comparing the tendinopathic with the healthy area of the same human tendon. Considering the wide use of anti inflammatory drugs among athletes, it is of concern how little is known about their effect on mechanical loading and tissue healing (Riley 2004). In the present study no effect of NSAIDs was observed, either between the two groups (PLC vs. NSAID) on the expression of inflammatory markers or structural proteins at the mRNA level, or between the two areas of the tendon. It has been shown that PGE2 is released by the peritendinous tissue after prolonged running exercise in healthy tendons (Langberg et al. 1999). Furthermore, a previous study has shown that NSAIDs blunted the exercise-induced increase in collagen synthesis in human patellar tendon (Christensen et al. 2011).

However, since the present study does not have any pre exercise measurements, we cannot evaluate to what extent exercise influenced the levels of inflammatory marker expression, but can only note that after exercise there were no markedly high levels of inflammatory markers.

Another reason for clinically using NSAIDs in relation to tendinopathy is the pain release. However, considering the VAS data of the present study, no significant difference between the level of pain during the run could be observed in the NSAID group compared with the PLC group ($p = 0.09$) (Fig. 2). In other words, the present study does not support the hypothesis of NSAIDs having a beneficial effect for AT patients in the form of pain reduction when performing exercise. It has been reported that an increased matrix turnover is one of the major mechanisms in the pathology of Tendinopathy (Riley 2004). The present finding of a higher expression of both collagen type 1 and collagen type 3 in the tendinopathic region of the tendon supports this notion. Additionally tendinopathy has been described as an ongoing degenerative condition rather than an inflammation (Riley et al. 1994). Furthermore, histological analyses have shown that inflammatory cells are absent in tendinopathy (Jarvinen et al. 1997; Khan et al. 1999). However, previous studies have also shown that anti-inflammatory treatments, like corticosteroid injection, are effective, at least in the short term (Fredberg 1997; Hart 2011; Paavola et al. 2002). The patients of the present study had a rather long history of tendon pain and were at a stage where inflammation might potentially have vanished. This does not rule out the possibility that these patients might have been in a more inflammatory state of the disease previously, when anti-inflammatory treatments might have had an effect. In the present study, no changes in Doppler activity and tendon thickness were observed after exercise in AT patients as compared with healthy individuals. Ultrasound Doppler is a very common clinical diagnostic method used to examine neovascularisation in AT patients, but previous results investigating the acute effect of exercise on Doppler activity are contradictory. Previous studies have shown that acute exercise either increased Doppler activity (Boesen et al. 2006) or decreased Doppler activity (Malliaras et al. 2012) while the present study shows no changes in Doppler activity compared with a healthy tendon region after 1 h running exercise. Recent studies have shown that the use of contrast-enhanced ultrasound increased the sensitivity and could visualize the microvascularisation in tendon tissue (Cadet et al. 2012; Klauser et al. 2010). The use of contrast-enhanced ultrasound might be a promising tool to

detect the microvascularisation as such and minor changes in the microvascularisation after exercise in AT patients. Additionally, the present study examined the elasticity of the tendon tissue by using Sonoelastography, which has been considered previously to be a reproducible technique (De Zordo et al. 2009; Drakonaki et al. 2009). No significant changes of Sonoelastography during exercise were observed when comparing before and after exercise measurements. Previous findings have shown that AT patients have an increased stiffness in the symptomatically enlarged Achilles tendons, in comparison with normal tendons at rest (Saito et al. 2008). Due to the lack of a healthy control group in the present study, the authors cannot remark on the general tendon stiffness of the patients, but only on the present results which indicate that the tendon stiffness of AT patients does not change with acute exercise.

Regarding the secondary purpose of the present study, very few difference (only IL-1b) were found between the outer edges of the biopsy compared to the middle region of the tendon, either in the RNA concentrations or in the expression of mRNA for all of the selected targets. This is a very important finding in relation to future studies, where it can be used to separate tendon biopsies into several regions for different analyses. It has previously been found that mRNA expression differs significantly between the tendon sheath and the tendon proper (Berglund et al. 2006). The tendon is surrounded by a thin layer of loose connective tissue known as the endotenon or the tendon sheath (Kastelic et al. 1978). The tendon sheath carries blood vessels and nerves (Jozsa et al. 1991; Riley 2004). Even though the tendon is supplied by the tendon sheath, it seems that no systematic differences occur between the edges and the mid tendon.

Limitations of the study

The present study has several limitations. A major limitation is the lack of a non-running control group. Although the present design does not allow the authors to conclude anything regarding the effects of acute exercise in AT patients, it does allow for comparison between healthy region and tendinopathic region of the same human tendon. The present results are clearly just a snapshot of tendinopathic tendon tissue that has been loaded acutely. Furthermore, since the present results show relative differences between the healthy and the tendinopathic regions of the tendon, the present findings might indicate either that exercise does not result in an exaggerated signalling in inflammatory response in AT patients, at least on the mRNA level, or that it is only the healthy area of the tendon that reacts. This could indirectly explain the relatively lower gene expression levels of inflammatory markers in the tendinopathic area of the tendon. A further limitation of the study is the lack of measurements of protein levels of the selected targets, since changes in mRNA expression may not lead to similar changes in protein expression. Another limitation is that the biopsies were taken at one single time point. Although pre exercise biopsies would have been beneficial, due to ethical reasons, it was not possible to take tendon biopsies before exercise. Additionally, biopsies taken 2 h after exercise will most likely not be proven the optimal time point for all the gene targets that were selected. The use of ultrasonography can include several sources of error, such as different sensitivities of different ultrasound machines, different settings, probe pressure, joint position, etc. In the present study we tried to avoid these uncertainties. All investigations were made by a very experienced clinician, using the same machine, the same probe and the same settings. Despite that, no differences in any ultrasonographic parameters could be observed. This leads the authors to conclude that Doppler activity and sonoelastography are not sensitive enough to detect changes from pre- to post-exercise conditions in AT patients. Since previous studies have shown an increased sensitivity by using contrast-enhanced ultrasound, the authors suggest that future studies are needed to investigate the vascular changes in Achilles Tendinopathy after acute exercise using contrast-enhanced ultrasound.

In summary, the present study observed a focally increased collagen mRNA expression in the tendinopathic area of the tendon, compared with a healthy area of the tendon. Furthermore, a significantly lower response of several targets involved in inflammation and healing of connective tissue, e.g. IL-6; IL-1b and CTGF were observed in the tendinopathic area. When comparing the outer versus inner parts of the tendon, only IL-1b was differentially regulated, while no differences were detected in mRNA levels for the remaining target genes. Real-time SE, Doppler activity, and tendon thickness did not change with acute exercise. The findings of the present study cannot support the hypothesis that acute exercise generates an acute inflammation in tendinopathic Achilles tendon.

Acknowledgments This study was supported by grants from the Danish Rheumatism Association, The Novo Nordic Foundation, the Danish Ministry of Culture Committee for Sports Research, the Danish Medical Research Counsel (22-04-0191) and the Nordea foundation (Healthy Aging grant). We are grateful to Burt Farber, for reading and correcting this manuscript.

Conflict of interest All authors declare no conflicts of interests in the present results.

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