Endogenous substance P production in the Achilles tendon increases with loading in an in vivo model of tendinopathy – peptidergic elevation preceding tendinosis-like tissue changes

L.J. Backman1,2, G. Andersson1, G. Wennstig1, S. Forsgren1, P. Danielson1

1Dept. of Integrative Medical Biology, Anatomy, Umeå University, Umeå, Sweden; 2Dept. of Surgical and Perioperative Sciences, Sports Medicine, Umeå University, Umeå, Sweden

Abstract

Objectives: To quantify the intratendinous levels of substance P (SP) at different stages of overload in an established model for Achilles tendinopathy (rabbit). Also, to study the distribution of the SP-receptor, the NK-1R, and the source of SP, in the tendon. Methods: Animals were subjected to the overuse protocol for 1, 3 or 6 weeks. One additional group served as unexercised controls. Immunoassay (EIA), immunohistochemistry (IHC), and in situ hybridisation (ISH) were performed. Results: EIA revealed increased SP-levels in the Achilles tendon of the exercised limb in all the experimental groups as compared to in the controls (statistically significant; p=0.01). A similar trend in the unexercised Achilles tendon was observed but was not statistically significant (p=0.14). IHC and in ISH illustrated reactions of both SP and NK-1R mainly in blood vessel walls, but the receptor was also found on tenocytes. Conclusions: Achilles tendon SP-levels are elevated already after 1 week of loading. This shows that increased SP-production precedes tendinosis, as tendinosis-like changes occur only after a minimum of 3 weeks of exercise, as shown in a recent study using this model. We propose that central neuronal mechanism may be involved as similar trends were observed in the contralateral Achilles tendon.

Keywords: Neuropeptides, Neurokinin-1 Receptor, Animal Model, Overuse Injuries, Rabbit

Introduction

The larger tendons of the body, such as the Achilles tendon, are often afflicted by chronic pain (‘tendinopathy’), a condition that is often accompanied by tissue changes (‘tendinosis’), including hypercellularity and vascular proliferation1. It is, however, unclear why tendinosis occurs. To study the events leading to these tissue changes, we have recently re-established and validated an in vivo animal (rabbit) model for tendon overuse2, based on the studies by Backman and collaborators3,4. In the referred study, we subjected animals to the overuse protocol for one, three or six weeks, and one additional group served as unexercised controls. We hereby demonstrated that tendinosis-like tissue changes (statistically significant cellular proliferation and angiogenesis) occur in the rabbit Achilles tendons after a minimum of three weeks of the exercise protocol2. At one week of exercise, no such changes could be detected2. Surprisingly, the same changes as those seen in the tendons of the exercised legs, were also noted for the tendons of the contralateral, resting, legs, leading to speculations of the involvement of pathways on a central neuronal level2.

The mechanisms leading to the tendinosis changes in tendinopathy are unclear. Recently, a new hypothesis suggesting that neurochemical mediators, produced locally in the tendon tissue, are involved in tendinosis pathology has been proposed5,6. This hypothesis is based on findings of a non-neuronal production of traditionally neuronal signal substances by the tendon cells (often collectively named ‘tenocytes’) of human Achilles tendon tissue; findings mainly seen in tissue from patients with tendinopathy7,9. Particularly interesting are findings suggesting such an endogenous, non-neuronal, production of the neuropeptide substance P (SP) in human Achilles...
tendons. Studies have furthermore shown that tenocytes and blood vessels of the tendons express the preferred receptor for SP, the neurokinin-1 receptor (NK-1 R), making these structures susceptible to SP-stimulation. Actually, aside from its well-known role in pain physiology, SP can also stimulate cell proliferation in tendon tissue, and promote angiogenesis. The findings favouring local, non-neuronal, production of SP and presence of NK-1 R in tendon tissue were most distinct in tendinosis tendons displaying tenocyte hypercellularity and vascular proliferation. Moreover, increased immunoreactivity for nerve-related SP has been noted in a rat model of Achilles tendon overuse, and other studies have demonstrated increased circulating levels of SP post-exercise.

Nevertheless, the exact role of SP in tendinosis is still unclear, although the role of neuropeptides in tendon pathology is increasingly discussed. It is not known whether there is a statistically significant increase of the SP-production in tendinosis tissue, or if such an increase precedes the tissue changes or if it is just a mere side-effect of the latter; questions that require monitoring of dynamic events in order to be answered. Therefore, in this study, samples of Achilles tendon tissue from rabbits in all groups (unexercised, as well as exercised for one, three or six weeks) used in our previous study on the established tendinosis model were analyzed regarding endogenous SP production; keeping in mind that the tendinosis-like changes (cellular proliferation and angiogenesis) were only noted in the three and six weeks groups in this material (see Anderson et al. for details). Furthermore, the study aimed at investigating the source of the SP produced in the rabbit tendons, and the possible targets of the neuropeptide in the tissue.

Material and methods

Animals

In this study, 24 adult female New Zealand white rabbits, with an age ranging from 6 to 9 months to obtain a weight of approximately 4 kg, were used. The animals were randomly divided into four groups of six rabbits. One group served as a control group whereas three groups were exposed to an overuse protocol involving the right leg of the animal. The rabbits in the experimental groups were sacrificed after 1, 3 and 6 weeks of exercise, respectively. The animals were caged in ordinary cages allowing freedom of movement in a 12:12 light-dark cycle.

The experiments were approved by the local ethical committee for research on animals.

Experimental design

A kicking machine, originally designed by Backman and collaborators, which has been shown to induce objectively verified tendinosis-like changes, was used to achieve passive dorsiflexion and plantarflexion of the exercised ankle joint. The right leg was attached to a pneumatic piston, which was set to alter a movement of 9.5 cm, a range from 20-25° dorsiflexion to 35-40° plantarflexion. To constrain excessive movements, a band was tied around the pelvis. The left leg was not attached to the piston and the ankle of this leg was at rest throughout the session.

Electrical stimulation using surface electrodes (pediatric electrodes 40 426A; Hewlett Packard, Andover, Massachusetts, USA) was used to accomplish an active contraction in the triceps surae synchronized to plantarflexion movement by a microswitch (type 14E 10; Disa Elektronik A/S Herlev, Denmark) in the piston. The microswitch was set to trigger the muscle contraction with an impulse of 0.2 ms duration, 85 ms after the initiation of the plantarflexion. This was done in order to mimic the action of the Achilles tendon during running. An amplitude of 35-50 V was used.

Throughout the 2-hour training sessions, the rabbits were kept under anaesthesia induced by intramuscular injection of fentanylfluanison (0.2-0.3 mL/kg) and diazepam (5 mg/mL, 0.2 mL/kg). To uphold anaesthesia fentanylfluanison (0.1 mL/kg) was injected each 30-45 min. After each training session buprenorphine (0.01-0.05 mg/kg) was given subcutaneously for analgesia.

Sampling, fixation, and sectioning

The animals were sacrificed one day after the last exercise with an overdose of pentobarbital. The Achilles tendon was collected and biopsies were harvested from the exercised, and contralateral, unexercised, side from three different parts of the tendon; the distal part (adjacent to calcaneus), the midportion, and the muscle-tendon junction.

All biopsies intended for immunohistochemistry were fixed by placing them in 4% formaldehyde in 0.1 M phosphate buffered saline pH 7.0 overnight in 4°C following a wash in Tyrode’s solution containing 10% sucrose at 4ºC overnight. Unfixed biopsies were used for the in situ hybridization (post-fixation according to the protocol, see further text). All samples were mounted on cardboard in transverse orientation to the longitudinal axis of the tendons in optimal cutting temperature (OCT) compound (TissueTek®, Miles Laboratories, Naperville, IL, USA), and frozen by submersion in propane chilled with liquid nitrogen. Storage was done at -80ºC until sectioning.

For enzyme immunoassay (EIA) biopsies with an average weight of 0.030 g were collected from the midportion of the tendon. Focus was thus given to the midportion biopsies, since this is the part of the Achilles tendon known to be afflicted by tendinosis in patients. These specimens were, directly after being weighed, frozen in liquid nitrogen, without prior chemical fixation, and stored at -80ºC. Total amount of biopsies for EIA in each group were 5, 5, 4 and 6 in the 0 week, 1 weeks, 3 weeks and 6 weeks group, respectively.

Immunohistochemistry

Immunohistochemistry was performed to evaluate substance P (SP) and NK-1 R immunoreactions in the tissues. A goat polyclonal antibody directed towards SP (sc-14104, Santa Cruz, CA, USA) and a goat polyclonal anti NK-1 R antibody (sc-5220, Santa Cruz, CA, USA) were used at concentrations of 1:50 on sections of fixed tissue. Briefly, sections were
placed in 1% triton X-100 (Merck, Darmstadt, Germany) 20 min for permeabilisation which was followed by 3x5 min washes in phosphate buffered saline (PBS). Incubation with normal donkey serum at a concentration of 5% (1:20) was done for 15 minutes prior to incubation with primary antibody 60 min in 37°C. Additional washes 3x5min was done in the washing buffer before another incubation with 5% normal donkey serum. This was followed by 30 min incubation in 37°C with the secondary antibody: Alexa Fluor 488 (Invitrogen, CA, USA), a green dye conjugated to a donkey anti-goat antibody, used at a concentration of 1:300. Washes, as mentioned earlier in the text, were carried out before the sections were finally mounted in Vectashield H-1000 mounting medium (Vector Laboratories, Burlingame, California, USA).

Preabsorption overnight of the primary antibodies with blocking peptides (SP: s6883, Sigma, St Louis, MO, USA; NK-1R: sc-5220P, Santa Cruz, CA) was performed as control stainings, using a peptide concentration of 50 μg/mL.

A Zeiss Axioscope 2 plus microscope equipped with epifluorescence and an Olympus DP70 digital camera was used for analysis.

**In situ hybridisation**

Sections of unfixed tissue were used for this procedure. Custom designed digoxigenin (DIG)-hyperlabelled oligonucleotide anti-sense probes (ssDNA) for detection of SP and NK-1 R (also known as TACR1) mRNA were applied. The DIG-molecule was attached to the 3’ end of the probe. In the case of detection of TACR1 mRNA, a “triple probe cocktail” (GD1001-DS custom designed; GeneDetect, New Zealand) was used rather than a single probe, since the rabbit TACR1 sequence has not yet been cloned. This cocktail contained three antisense probes directed towards both rat and human TACR1 mRNA, and is considered to have a very high probability of detecting the rabbit TACR1 mRNA. The antisense probe sequences were:


and probe #3:

5’-GCA-GCC-GAA-AGT-GTG-TAG-GAT-TGG-GGG-3’.

The antisense probe for detection of rabbit SP mRNA (γ-preprotachykinin I) (GD1001-CS custom designed; GeneDetect, New Zealand) had the following sequence:


The procedure, previously used in our laboratory10, is a modification of an earlier established protocol16. Basically, freshly 10 mm thick sections were air-dried and fixed in 4% paraformaldehyde (PFA). Sections were incubated in 0.2 M HCl at room temperature (RT) for inhibition of endogenous alkaline phosphatase (AP)-activity. To reduce background-staining, sections were acetylated at RT in a mixture including diethylylpyrocarnlate (DEPC)-water, triethanolamine, HCl, and acetic anhydride. After denaturation of the ssDNA probes they were administrated on the slides with totally 50 ng in 15 µL of hybridization solution and incubated overnight in 56°C followed.

Blocking with serum at RT was performed, after several washes to eliminate excess of probes. This was followed by incubation with an AP-labelled anti-DIG antibody (Roche, Germany, 11 093 274 910) for detection. Subsequently, the substrate solution was added and incubated overnight in 4°C. Finally the colour reaction was stopped before the slides were mounted using pertex.

The entire procedure was done under sterilized condition.

As positive controls a Poly(dT) probe (GD4000-OP; GeneDetect, New Zealand) and a β-actin antisense probe (GD5000-OP, Gene Detect, New Zealand) were used. As negative controls, the corresponding sense DIG-hyperlabeled oligonucleotide ssDNA probes/probe cocktails were applied.

**Enzyme immunoassay (EIA)**

**Sample preparation**

The frozen biopsies were mechanically homogenized in a 100 Mm Tris-HCl buffer pH 7.0 containing 1 M NaCl, 2% Bovine Serum Albumine (BSA), 4 mM EDTA, 0.2% Triton X-100, 0.02% sodium azide buffer. Protease inhibitors Pepstatin A (P4265, 0.1 μg/mL), aprotinin (A1153, 5 μg/mL), antipain (A6191, 0.5 μg/mL), benazamid (B6506, 167 μg/mL) and PMSF (P7626, 5.2 μg/mL) were included, all from Sigma, Steinheim Germany. The buffer and biopsies were mixed in a 1:20 ratio before mechanical homogenization using 5000 rpm, 2 periods with duration of 1 min for each period, repeated twice. Subsequently, the samples were centrifuged at 13 000 rcf at 4°C for 15 min. Aliquots were stored at -80°C until assay. The entire procedure was carried out on ice.

**EIA procedure**

The concentration of SP was analyzed using a commercially available enzyme immunoassay kit with a detection range of 0-0.25 ng/mL (EK-061-05 Phoenix Pharmaceuticals, CA, USA). The instructions of the suppliers were followed accordingly. Briefly, samples were diluted in a 1:5 ratio with the 1x assay buffer before added into each well. All wells were precoated with the secondary antibody and the nonspecific binding sites were blocked. Prior to 2 hours incubation on an orbital shaker (300-400 rpm) in RT, the rehydrated primary antibody and the rehydrated biotinylated peptides were added in all wells except the blank wells. After washing 4 times, the SA-HRP solution was added followed by 1 hr incubation on the orbital shaker. After washing 4 times the TMB substrate solution was added into each well. The plate was protected from light throughout the 1 hr incubation on the orbital shaker. Adding 2N HCl stopped the reaction before the absorbance was read at 450 nm.
Statistics

Kruskal-Wallis test (K-W), followed by pair-wise Mann-Whitney U test (M-W U), with Bonferoni correction, was performed on the data obtained from the EIA to compare the levels of intratendinous SP between groups. To compare tendons of the same rabbits, the Wilcoxon Signed Rank test was performed. Values with a CV% >15 were excluded.

Computer software (PASWStatistics18.0 for Macintosh) was used for all statistical calculations, with significance predetermined at p<0.05.

Results

SP-levels in tendon tissue as measured by EIA

The levels of SP in the midportion Achilles tendon biopsies, as quantified with EIA, were measured in all four groups. It was hereby revealed that the SP-levels of the exercised Achilles tendon of all three experimental groups (1, 3 and 6 weeks) were elevated as compared to the control group (K-W: p=0.01; Pair-wise Mann Whitney U test: *p<0.05, **p=0.01). Error bars indicate inter-quartile range (IQR).

SP-immunoreactions and SP mRNA

SP (Figure 2) and SP mRNA (Figure 3a) were extensively detected in the walls of several blood vessels, predominantly in the paratendinous tissue, in all groups using immunohistochemistry and in situ hybridisation, respectively. The immunoreactions were seen in both the endothelium and the smooth muscle cells, whereas the SP mRNA was mainly seen in endothelial cells.

Occasionally, fine nerve fascicles in the paratendinous tissue were seen to be immunostained for SP (Figure 2, inset). On the whole, nerve fascicles were not seen in the tendon tissue.
proper. No SP mRNA was detected in nerve structures.

There were no detectable immunoreactions for SP in tenocytes, and only occasional reactions in these by the in situ hybridisation method.

The immunoreactions for SP were abolished after preabsorption, and the in situ hybridisation showed no reactions using sense-staining (Figure 3b).

Similar findings were observed in the exercised and the contralateral, unexercised, Achilles tendons.

**NK-1 R immunoreactivity and NK-1 R mRNA**

Staining, using immunofluorescence as well as in situ hybridisation, revealed reactions for NK-1 R (Figure 4a) and NK-
1 R mRNA (Figure 4c), respectively, in the walls of the blood vessels of the tendon tissue proper as well as in the paratendinous tissue of tendons from all groups. The NK-1 R reactions at both protein and mRNA level were predominantly seen in the endothelium, but were also present in the smooth muscle cells. Also, nerves in the paratendinous tissue showed NK-1 R immunoreactions (not shown). The tenocytes of the tendon tissue proper displayed NK-1 R immunoreactivity (Figure 4a, inset), as well as NK-1 R mRNA by in situ hybridisation (not shown).

The immunoreactions were abolished after preabsorption (Figure 4b), and no reactions were seen in the sense stainings of the in situ hybridisation.

Similar findings were observed in the exercised and the contralateral, unexercised, Achilles tendons.

**Discussion**

This study shows that the endogenous production of SP in tendon tissue is elevated in response to loading in this animal model, thereby supporting theories on the involvement of SP in tendon pathology. Interestingly, this increase in SP levels is noticeable after only one week of exercise in the current animal model. A previous study of ours on the same model has shown that the tendinosis-like tissue changes in the rabbit Achilles tendon, i.e. hypercellularity and vascular proliferation (same as for human tendinosis), do not occur until a minimum of three weeks of exercise. This would indicate that the elevated SP production precedes tendinosis, a fact that is highly interesting for our hypothesis that SP is involved in the early stages of tendinopathy. This is particularly noteworthy if one bears in mind that it is previously reported that exogenously administered SP can stimulate angiogenesis and cell proliferation in the Achilles tendon during healing as shown in a rat model of tendon rupture. Neuropeptides have previously been reported to increase following certain trauma and also overuse/exercise. It should however be emphasized that the effects of the electrical stimulation given during the plantar flexion phase in the present model must also be taken into consideration, as it has previously been shown that electrical stimulation triggers release of SP from peripheral nerve endings.

The present study also provides a basis for potential widespread SP-effects in the rabbit tendon tissue, including effects on tenocytes and vascular effects, as both immunostainings and in situ hybridisation show that the preferred receptor for SP, the tachykinin receptor NK-1 R, is expressed on the tendon cells and in the tendon blood vessel walls, predominantly on endothelial cells but also on smooth muscle cells. These results are in keeping with studies of human Achilles tendons, thus further validating the relevance of this rabbit model when studying SP-effects in tendon pathology.

Regarding the possible source of SP produced in the Achilles tendon, the present study indicates that the main production seems to occur in the blood vessel walls, more specifically in the endothelium where SP mRNA was detected to a high degree. These findings, to a certain extent, contradict earlier studies on man, showing that tenocytes seem to be an important source of locally produced SP in human Achilles tendinosis. However, also in the human studies, the immunohistochemical methods were found to be too insensitive to detect intracellular SP in tenocytes, whereas SP mRNA was detected in the human tenocytes by in situ hybridisation, a discrepancy not unexpected, since it is previously reported that, on a whole, SP of non-neuronal cells is expressed in low quantities, in contrast to SP of neuronal cells. All the same, even SP mRNA was only occasionally noticed in tenocytes of the rabbit tissue in the present study, suggesting a variation between species in this regard. It is, none the less, frequently described that there can be a production of SP in the endothelium of blood vessel walls, and also cultured endothelial cells, both bovine and human, have been shown to contain/release SP, thus supporting the findings of the present study.

Aside from locally produced SP, discussed above, neuronal sources of this neuropeptide must of course also be considered. Indeed, as for human tendons the paratendinous tissue of the rabbits contained some thin SP-positive fascicles, albeit only occasionally. No SP mRNA was detected in nerve structures, which is to be expected as the production is known to take place in the neuronal cell body; SP then being transported to the terminal nerve endings.

It should finally be pointed out that another tendinosis model, affecting the flexor digitorum tendons, has shown SP-immunoreactivity in additional cell types of tendons, such as tenocytes and peritendon mast cells and macrophages.

The findings of this study of reactions for SP in the smooth muscle cells of the blood vessel walls were inconsistent, as positive reactions were clearly observed on protein level but not for the mRNA, suggesting presence of SP in those cells without it being produced there. This observable fact can be explained by the SP-induced trafficking of NK-1 R. It is known that SP induces an internalisation of NK-1 R, creating a SP/NK-1 R complex, where SP is intact in the early endosomes but slowly degrades in the perinuclear vesicles while NK-1 R are recycled to the cell surface. Bearing this in mind, the positive immunoreactions in the smooth muscle cells could be explained by a paracrine loop; the protein being produced by the endothelial cells with effects not only in the endothelial layer but also on the smooth muscle cells, since the latter were found to express NK-1 R as well. It is also previously shown that SP can be detected in the smooth muscle cells of blood vessel walls, and that SP can have autocrine/paracrine effects in the smooth muscle layer of the vessels.

The phenomenon of SP production in non-neuronal cells has also been reported for other tissues, such as the keratocytes in the cornea of the eye. It should, however, also be pointed out that some studies have reported an increase of SP-positive nerve fibres in Achilles tendinosis; a phenomenon that was not seen in the present study, nor in our previous studies on human tendinosis.

In our earlier study on this animal model for tendon overuse, we demonstrated that tendinosis-like tissue changes in this material also occur to the same degree in the contralateral, unex-
ercised, Achilles tendon as on the exercised side. Also on the resting side, these changes were noted only after a minimum of three weeks of exercise. The present study shows, accordingly, that the endogenous local SP-production does not differ between the tendons of the different legs, suggesting that SP is released in elevated amounts also in the resting tendon as a response to exercise in the contralateral tendon. It should here, however, be pointed out that the increased SP-production that was noted for the unexercised legs of the experimental groups, as compared to the controls, was not statistically significant (p=0.14). A bilateral SP-response to exercise has previously been described in a rat model for tendinopathy, in which SP-immunoreactivity increased in the contralateral side in response to repetitive strain, although this increase was accredited to the use of the control limb as a postural support. Furthermore, experimentally induced monoarthritis in the knee joint triggers not only ipsilateral but also contralateral expression of SP in the dorsal horn of the spinal cord. It has also been shown in rats that SP in the spinal cord dorsal horns increases following high repetitive movements. For human Achilles tendinosis, bilateral symptoms are common, making the findings of the present study also clinically relevant. It is tempting to speculate, that the increase of contralateral local SP production in the tendon tissue, as well as the tendinosis-like tissue changes that follow, are elicited by neuronal signalling pathways.

In summary, this study confirms previous observations from research on man that there is a production of SP in the Achilles tendon, and furthermore verifies that this production is increased when the tissue undergoes development to tendinosis. The SP elevation, being noticeable after only one week of exercise, precedes the tendinosis-like tissue changes, the latter not occurring until a minimum of three weeks of exercise, making it tempting to speculate of a causal connection between SP and the development of the tissue changes (hypercellularity and angiogenesis). Earlier evidence of trophic and angiogenic effects of SP, as well as findings in the present study of SP receptors on tenocytes and blood vessels, support this. Nevertheless, further experimental studies, involving exogenously administered SP, on this animal model will have to determine if SP actually induces and/or accelerates these changes in rabbit tendon tissue undergoing tendinosis. Finally, this study brings about further interesting signs of bilateral effects in tendinosis.

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Note added in proof

While the present paper was in press, another paper based on studies on the present tendinopathy model was accepted for publication (ref. 38). In that study, it was shown that exogenously administered SP accelerates hypercellularity and angiogenesis in the rabbit tendon tissue in response to overuse.

References


L.J. Backman et al.: Intratendinous SP increases with loading


