Blockade of the sympathetic nervous system degrades ligament in a rat MCL model

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Dwyer, Kelley W., Paolo P. Provenzano, Peter Muir, Wilmot B. Valhu, and Ray Vanderby, Jr. Blockade of the sympathetic nervous system degrades ligament in a rat MCL model. J Appl Physiol 96: 711–718, 2004. First published October 3, 2003; 10.1152/japplphysiol.00307.2003.—We hypothesize that blockade of the sympathetic nervous system degrades ligament. We tested this hypothesis in a rat medial collateral ligament (MCL) model. Fifteen animals were treated for 10 days with the sympathetic chemotoxin guanethidine using osmotic pumps, whereas 15 control rats received pumps containing saline. A reduction in plasma concentrations of norepinephrine in the guanethidine rats indicated a significant decrease in sympathetic nerve activity. Vasoactive intestinal peptide and neuropeptide Y were decreased in MCLs from guanethidine animals, as quantified by radioimmunoassays. Tissue vascularity was substantially increased in guanethidine MCLs, whereas mechanical properties were significantly decreased. Proteases, such as matrix metalloproteinases (MMP) and cysteine proteases, play a major role in ligament degradation. The proteases MMP-13, cathepsin K, and tartrate-resistant acid phosphatase (TRAP) have collagenolytic activity and have been shown in rat ligament tissues. To determine whether the degradation seen in this study was due to protease activity, we determined the expression of these enzymes in control and treated MCLs. Real-time quantitative PCR revealed that guanethidine treatment increased expression of MMP-13 and cathepsin K mRNAs, although overall expression levels were decreased in MCLs from guanethidine animals. The expression of these enzymes is believed to elicit protease activity, which is essential for ligament homeostasis.

The peripheral nervous system (PNS) has three primary functions: 1) relaying sensorimotor information to and from the central nervous system; 2) controlling local blood flow; and 3) influencing inflammatory, proliferative, and reparative processes in injured tissue. In the PNS, neurogenic factors, such as neuropeptides and neurotransmitters, are chemical agents that mediate these functions (35). These factors can influence PNS-related actions by modulating immune cell responses, cytokine response, and local blood flow (5, 35). Although neurogenic factors are released from stimulated nerve endings, their actions are not restricted to the point of stimulation (10). For example, the autonomic neuropeptides vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) can have angiogenic, vasoregulatory, and cell proliferative actions hours to days after release from nerve endings (35, 44). In addition, Ackermann et al. (1) recently reported a role for peripheral neuropeptides in healing of tendon tissue. The above observations make it likely that these neurogenic factors play an important role in tissue homeostasis.

Clinically, chemical inhibition of the PNS is used to manage joint pain. Chemical blockade of the sympathetic nervous system is often accomplished through the administration of guanethidine. Guanethidine blocks the release and subsequent reuptake of norepinephrine (NE) (a major sympathetic neurotransmitter) in patients with osteoarthritis, rheumatoid arthritis, and reflex sympathetic dystrophy (12, 13, 24, 38, 40). This treatment, while effective in relieving pain, may have undesirable effects on connective tissues, because it alters the normal concentration of neurogenic factors.

Growing anatomic and physiological evidence suggest that the PNS is important to ligament and joint homeostasis. Ligaments, like many other connective tissues, are innervated with both unmyelinated afferent (sensory) and efferent (sympathetic) nerves (2). In normal medial collateral ligament (MCL) tissue, these nerves are often associated with blood vessels in the epiligament and contain neuropeptides, including substance P (SP), CGRP, NPY, and VIP (26). In addition to their association with blood vessels, efferent fibers can extend into deeper ligament matrix (2). These efferent nerve fibers in the MCL contain both autonomic (NPY and VIP) and sensory (SP and CGRP) neuropeptides that help regulate MCL blood flow (1, 2, 11, 26). Denervation of peripheral nerves leads to decreased healing of the MCL and promotes the onset of osteoarthritis (22, 34). Partial injury to the MCL can lead to increases in vascular volume, a factor that is largely controlled by ligament innervation (7). During periods of chronic overuse or disuse, homeostatic changes can be detrimental to the structural integrity of ligaments. Although the above evidence suggests that peripheral nerves play an essential role in ligament homeostasis, few studies exist that directly investigate this role.

Ligament homeostasis and remodeling is a complex process involving cell responses, cell-matrix interactions, and regulation through chemical mediators. In soft tissues, such as ligament, the process of remodeling and degradation is regulated by the production of proteases from fibroblasts (9). Of the many proteases that are produced by cells, two classes have gained attention for their ability to degrade collagen: cysteine proteases and matrix metalloproteinases (MMPs). Within these classes, a number of proteases have been isolated in rat MCL. MMP-13 has been thought to play a role in type I collagen degradation in the rat and...
has been found in a number of tissues, including uterus, cornea, bone (31), meniscus, synovium, anterior cruciate ligament, and MCL (19). Of the cysteine proteases, cathepsin K and an associated lysosomal protease, tartrate-resistant acid phosphatase (TRAP), also play a role in type I collagen degradation. TRAP, although not a cysteine protease (but is associated with many cysteine proteases), has the ability to degrade type I collagen (16) and was found in canine ligamentous tissue (27). Cathepsin K, a predominant protease in bone remodeling, has also been found in rat MCL. There is increasing evidence that cathepsin K plays a major role in MCL remodeling (27). Cathepsin K is a potent collagenase and, as such, has a substantial influence on ligament degradation.

The hypothesis of this study is that blockade of the sympathetic nervous system degrades ligament. To test this hypothesis, changes in ligament mechanical properties, ligament vascularity, the presence of neuropeptides, and levels of proteolytic enzymes were examined in a rat MCL model. Results have importance for a number of reasons. First, because guanethidine is used in pain management, it is essential to understand how it affects joint connective tissue. Second, numerous studies use tissue engineering approaches to regenerate connective tissues but do not consider the role of peripheral nerves or neuropeptides. Last, ligament grafting is common in orthopedic surgery.

METHODS

Surgical procedure. This study was approved by the Institutional Animal Use and Care Committee and meets the National Institutes of Health guidelines for animal welfare. Thirty female Wistar rats (207.4 ± 13 g) were divided into two groups: 1) guanethidine treatment and 2) saline control. For all groups, anesthesia (isoflurane 0.5–3%) was initiated in an induction chamber and maintained with a face mask. Into the treatment group (n = 15 animals), guanethidine (40 mg·kg⁻¹·day⁻¹) was infused via a subcutaneously implanted mini-osmotic pump (Alza). Pumps were placed into a subcutaneous pocket created between the scapulae. Into the control group (n = 15 animals), a similar volume of sterile saline was infused via the same route. Three-dimensional reconstructions were made from individual confocal images by stacking the sequentially scanned images. Images were reviewed with a computer and saved digitally.

RIA. Four MCLs were used for quantification of neuropeptide concentrations via RIA. MCLs were homogenized by using a mortar and pestle. Homogenate was used to complete standard SP, CGRP, NPY, and VIP RIA protocols (Phoenix Pharmaceuticals, Belmont, CA). Briefly, samples were mixed with an antibody specific to one of the neuropeptides and then incubated with a 125I-traced peptide. The radioactivity of each sample was measured by using a gamma counter. Standards for each neuropeptide were included in the kit, and MCL neuropeptide concentration was determined from a standards curve.

Organ culture. To assess the effect of guanethidine directly on extracellular matrix (ECM), MCLs were cultured. Following biopsy, pigs were sacrificed, and the MCL was employed. Bilateral MCLs (n = 6), including intact femoral and tibial segments, were aseptically harvested from untreated rats. The left knee was used as a control tissue, whereas the right knee was cultured with guanethidine.

During and after harvest, tissues were rinsed in sterile PBS containing penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). Bone blocks were trimmed to minimize the amount of bone marrow, which may alter the culture environment. Tissues were then cultured in DMEM supplemented with 10% fetal bovine serum, nonessential amino acids (0.1 mM), l-glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). Guanethidine or sterile PBS was added to media for the treated and control tissues, respectively. The concentration of guanethidine in media was selected to be the same as the concentration of guanethidine present in the blood during treatment (described above) using normative rat data for blood volume (5 ml blood/100 g body wt). Guanethidine was prepared and dissolved in sterile PBS and added to the media; controls received equal volumes of sterile PBS. The cultures were maintained in an incubator at 37°C and 5% CO₂. Media were changed every 48 h, at which time tissues were rinsed with sterile PBS to reduce drug carryover into the fresh media containing the treatment drug or an equivalent volume of carrier. Organ culture was maintained for 10 days, after which time the MCLs were tested mechanically in the same manner as the in vivo treatment group (described below).

Tissue mechanics. Immediately after death, animal hindlimbs for biomechanical testing (n = 8 control, n = 8 treated MCLs) were disarticulated at the hip joint and stored at −80°C until testing. Storage by freezing does not significantly affect the biomechanical properties of ligament (42). On the day of testing, hindlimbs were thawed at room temperature, and tissue harvest and testing were performed using methods similar to those previously published (30). Each MCL was exposed by carefully dissecting extraneous tissue. The MCLs, including intact femoral and tibial bone sections, were excised for ex vivo testing, with care taken not to disturb the ligament insertion sites. Tissues were kept hydrated in Hanks’ physiological solution during testing. Ligament thickness and width were measured optically, and MCL area was estimated with ellipsometric geometry. Each femur-MCL-tibia complex was then placed in a custom-designed tissue bath system. For strain measurements, optical markers were placed onto the ligament tissue near the insertion sites. A small preload of 0.1 N was applied to the ligaments to obtain a uniform starting point. The ligaments were then preconditioned to 1% strain.

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for 10 cycles. After preconditioning, ligaments were pulled to failure at a rate of ~10%/s.

Tissue displacement was obtained by using video dimensional analysis. The initial ligament length ($L_0$) was taken to be the length at preload. The change in distance between optical markers was calculated by analyzing the change in coordinate center of each marker from stored digital frames by using a custom macro for NIH Image. Engineering strain ($\varepsilon$) was measured from displacement data as the change in length between the stretched length ($L$) and $L_0$, divided by the $L_0$:

$$\varepsilon = \frac{L - L_0}{L_0} \quad (1)$$

Force was measured and displayed on the video screen in synchrony with the displacement. Engineering stress ($\sigma$) was calculated as the force ($F$) divided by the initial area ($A_o$):

$$\sigma = \frac{F}{A_o} \quad (2)$$

Stress-strain curves were created and fit with the microstructural model presented by Hurschler and coworkers (21). Elastic moduli were obtained from this model.

The mechanical properties that were examined in this study were maximum force, ultimate strain, strain at failure, elastic modulus, and area. Statistical analysis was performed by using an unpaired $t$-test ($P = 0.05$) to analyze differences between control and treatment groups for each mechanical property.

**Comparison of wet and dry weights.** Ligament hydration was evaluated by comparing hydrated and dehydrated weights. For conformity, ligaments were hydrated for 2 h in PBS, excess PBS was removed, and the tissues were then weighed immediately. Ligaments were weighed on a digital balance (Mettler-Toledo, Columbus, OH) to an accuracy of 0.00001 g. Each tissue was then dehydrated on glass coverslips at 60°C for 24 h and weighed a second time.

The difference between wet and dry weights, normalized to the dry weight, was compared, and group means were evaluated statistically by using a paired $t$-test ($\alpha = 0.05$).

**Evaluation of MCL vascularity.** To investigate tissue vascularity, the femoral arteries of six rats (3 treated, 3 controls) were cannulated. The proximal artery was tied off by using a 4.0 nylon suture. Into the artery, 4-µm red fluorescent microspheres ($2 \times 10^7$ spheres suspended in 5% albumin solution) were injected as a 3-ml bolus. Five minutes after injection, the rats were killed. Ligaments were harvested and fixed overnight in 4% formalin. Tissue was imaged with confocal microscopy to detect the presence of fluorescent microspheres. Ligaments were viewed in sagittal planes of focus, and scans were made through the ligament thickness. Stacking the sequentially scanned images created three-dimensional reconstructions. Images were then viewed with a computer and saved digitally.

**Histological and immunohistochemical evaluation of proteases.** Immediately after death, eight MCLs (from 8 treated, 8 control rats) were excised and dissected in toto from the tibial and femoral insertions. Ligaments were immediately fixed in Zamboni’s fixative for 3 days at 4°C and then stored overnight in a solution of 30% sucrose and fixative. Specimens were flash frozen, sectioned (6 µm), and mounted on slides.

Immunohistochemical labels were applied by using a diaminobenzidine immunohistochemistry kit for rodent tissue (Innogenex, San Ramon, CA). Slides were labeled for MMP-13 (collagenase 3), MMP-1, and cathepsin K after the manufacturer’s instructions. Histological evaluation of TRAP was performed following previously published methods (3, 15). Briefly, slides were incubated for 2 h in equal parts of two buffer solutions: naphthol AS-BI phosphate buffer and hexazonium pararosaniline. Slides were evaluated by using light microscopy.

**Real-time quantitative PCR.** Primer sets for cathepsin K, rat collagenase (MMP-13), TRAP, and GAPDH have been developed and tested for specificity in our laboratory. The primer sets were either derived from previously published reports [rat collagenase (25, 39)], or designed from sequences available from GenBank (GAPDH, cathepsin K, and TRAP: GenBank accession numbers are AF106860, AF101036, and M76110, respectively) using the PrimerSelect module of the LaserGene 5.01 software (DNASTar, Madison, WI). Primer sequences are as follows: MMP-13: forward: 5’-AAA GAA CAT CGT GAC TTC TAC C-3’, reverse: 5’-ACT GIA TTC CTT GAA CGT C-3’, length: 283 bp; cathepsin K: forward: 5’-TGC CAG CGT GAT AAT GTG AAC C-3’, reverse: 5’-ATG GCC TGG CTG GCT TGA ATC T-3’, length: 205 bp; TRAP: forward: 5’-CGC CAG ACC GTG GCA GAT TAT G-3’, reverse: 5’-AAC AGT GCC ACG GTG ATG TTC G-3’, length: 297 bp; and GAPDH: forward: 5’-GAC TGT GGA TGG CCC CTC TG-3’, reverse: 5’-GCG CTG CTT CAC CAC CTT CT-3’, length: 239 bp.

For total RNA isolation, the tissues ($n = 4$ control, $n = 4$ treated) were rinsed, after sterile harvesting, in DNase-, RNase-, and protease-free PBS to remove blood or other contaminants from the tissue and placed in RNA later solution (Ambion, Austin, TX). Tissues were pooled to ensure adequate measurable RNA yield. RNA isolation was performed by using methods similar to those utilized by Reno and coworkers (32), which combine the TRIzol method with the column fractionalization steps of the RNeasy Total RNA kit (Qiagen, Valencia, CA). Methods primarily followed the manufacturer’s instructions for the respective steps. For tissue homogenization, MCLs were frozen with liquid nitrogen, placed in a liquid nitrogen-cooled Braun Micro-Dismembrator vessel (B. Braun Biotech International, Allentown, PA), and reduced to powder. After total RNA isolation, yield and purity of RNA were quantified by spectrophotometric measurement at 260, 325 nm (UltraSpec 3000 Spectrophotometer, Pharmacia Biotech, Cambridge, UK). RNA was reverse transcribed into cDNA (20 µl total volume) by combining a mixture of 5 µl total RNA, 1 µl oligo(dT)$_{12-18}$ (250 ng/µl), and 5 µl RNase free water, with 9 µl of First Strand Synthesis buffer (Life Technologies) containing 40 units of RNase inhibitor (Ambion), 500 µM dNTP mix, 10 mM dithiothreitol, and 200 units of Superscript II reverse transcriptase.

Quantitative PCR (QPCR) standards for the genes indicated above were prepared from purified PCR products of the target sequences. From spectrophotometric quantitation of the PCR products, the number of copies per microliter of each standard was calculated, and 10-fold serial dilutions (ranging from 1 x 10$^7$ to 10 copies/µl) were prepared. The housekeeping gene GAPDH was used as internal control. Real-time PCR was performed by using a Bio-Rad iCycler iQ real-time PCR system (Bio-Rad, Hercules, CA). All reactions were carried out in a total volume of 20 µl containing 1× Platinum QPCR Supermix (Life Technologies), 10 nM fluorescein, 200 nM forward primer, 200 nM reverse primer, 0.25× SYBR green, 5 µl template, and 3.95 µl diethyl pyrocarbonate-treated H$_2$O. PCR cycling consisted of initial denaturation at 95°C for 3 min and up to 50 cycles of 95°C denaturation for 15 s, 55 or 60°C annealing (depending on template) for 30 s, and 72°C extension for 30 s. During each cycle, optical data were collected during the annealing and extension steps. Each gene was run in triplicate. The copy numbers of the respective cDNAs in the samples were determined relative to the standard curves. The cDNA copy number of the target gene was normalized to the copy number of GAPDH.

**RESULTS**

**Surgical outcomes.** All guanethidine-treated rats developed ptosis (an indication of sympathetic inhibition) by the second day of treatment, and ptosis persisted throughout the 10-day period. No other gross changes were observed in vivo. No abnormalities in movement, body weight, or behavior were observed in either the treated or control groups. On tissue
harvest, no morphological differences were macroscopically observed between control and treated MCL tissue.

Neurotransmitter and neuropeptide concentrations. Plasma concentrations of NE in the treated group were significantly lower than those in control animals (0.1717 vs. 6.272 ng/ml; P < 0.04; Fig. 1), indicating significant inhibition of the sympathetic nervous system. Immunofluorescent labeling of neuropeptides revealed a disappearance of VIP from ligament tissue after guanethidine treatment, whereas NPY, SP, and CGRP remained unchanged (Fig. 2), indicating an impairment to sympathetic and parasympathetic neuropeptides but not neuropeptides from sensory nerves. RIA data confirmed the absence of VIP from guanethidine-treated MCLs compared with controls (0.28 vs. 164.42 pg/100 μl; P < 0.001) and showed greatly decreased concentrations of NPY in treated MCLs (1.65 vs. 14.67 pg/100 μl; P < 0.001). SP concentrations were also increased in guanethidine-treated MCLs (50.173 vs. 1.97 pg/100 μl; P = 0.03); however, CGRP concentration remained unchanged (3.36 vs. 1.58 pg/100 μl).

MCL mechanics. Results from organ culture experiments performed to examine the direct effect of guanethidine on ECM structural integrity revealed no changes in ultimate stress, area, or force. Ultimate stress values were nearly identical between contralateral ligaments [71.01 ± 19.20 MPa (control) vs. 70.29 ± 19.15 MPa (guanethidine); P = 0.96].

Ultimate stress in guanethidine-treated animals was significantly decreased (Fig. 3A; P = 0.0006), as was the strain at failure (Fig. 3B; P = 0.031) and elastic modulus (Fig. 3C; P = 0.0003) compared with MCLs from saline-receiving control animals. A significant increase in MCL area was also present in treated tissues. The mean ultimate tensile force also decreased in treated tissues, but it was not statistically significant (P = 0.76). This increase in area is due (at least in part) to an increase in fluid content in the ligament, as supported by a statistically significant increase in the wet weight (P = 0.042).

MCL vascularity. Analysis of tissue vascularity using fluorescent microspheres qualitatively revealed that guanethidine treatment increased vascularity compared with that in control animals (Fig. 4). Substantial and consistent differences in microsphere deposition were easily detectable between treated and control animals.

Proteolytic enzymes. The MCLs from the guanethidine-treated animals labeled positively for cathepsin K and TRAP (Fig. 5). Cathepsin K labels were observed near the peripheral portions of the ECM and within the ligament matrix. TRAP staining was seen throughout the ligament, with the heaviest staining seen in the ligament matrix. No ligaments (except the positive control slides) had positive staining for MMP-1 or MMP-13 (results not shown). Real-time QPCR produced consistent results and showed increases in cathepsin K, MMP-13, and TRAP (Fig. 6). Increases in the expression of cathepsin K (38.04 ± 8.08 vs. 31.97 ± 9.40 copies/1,000 copies GAPDH) and MMP-13 (6.91 ± 3.1 vs. 31.97 ± 9.40 copies/1,000 copies GAPDH) were seen in the treated tissues compared with control tissues. In both the untreated and treated tissues, the overall expression of TRAP was very small.

DISCUSSION

PNS inhibition degrades ligament. Results of this study support the hypothesis that inhibition of the sympathetic nerves...
degrades ligament. Data reveal that guanethidine does not directly affect structural integrity (via tissue culture experiments) but that chemically induced sympathectomy does. Reduced plasma concentrations of NE as well as ptosis in the treated group indicate that guanethidine was effective in blocking the sympathetic nervous system. Although histological ligament sections only showed changes in VIP immunoreactivity, RIA data from the guanethidine-treated rats showed decreases in both NPY and VIP, indicating that levels of autonomic neuropeptides were altered. SP concentrations were also increased in guanethidine-treated MCLs, indicating that blockade of efferent nerve fibers may alter concentrations of afferent neuropeptides as well. In vivo administration of guanethidine decreased ultimate stress, decreased elastic modulus, decreased strain at failure, and increased cross-sectional area. The mean ultimate force decreased after 10 days of in vivo guanethidine treatment, but the change was not statistically significant. The increased area is likely the result of increased tissue hydration, as exhibited by a significant increase in the MCL wet weight. Increased hydration may be due to changes in vessel permeability (as mediated by neuropeptides). Alternatively, changes in ECM components, such as proteoglycans, which are known to affect tissue water content as well as stabilize collagen ECM organization, could account for the increased hydration. Strain at failure was significantly altered, implying some alterations to ECM organization and integrity. Further studies are necessary to elucidate details of the changes and the mechanisms.

**PNS influences MCL physiology.** Tissue vascularity was significantly higher in MCLs from guanethidine-treated animals, which is consistent with previous reports (6, 26, 35). The increased vascularity was most likely the result of altered concentrations of neurotransmitters and neuropeptides. Our results in rats agree with rabbit experiments (26) in which the authors report that altering levels of neuropeptides and NE alters MCL blood flow. Others (7) report that partial tears to both the MCL and anterior cruciate ligament increase ligament blood volume, which also may be mediated by the PNS.

Guanethidine-treated rats increased their expression of proteolytic enzymes (MMP-13 and cathepsin K) within MCL tissue. Expression of TRAP was low in control and treated MCLs. Histology confirmed the presence of cathepsin K and showed the presence of TRAP in guanethidine-treated MCLs. The presence of these enzymes may have altered the MCL mechanical properties in the treated animals. Remodeling and

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**Fig. 3.** After 10 days of treatment, mechanical properties of rat MCLs were measured in control and guanethidine-treated rats. A: guanethidine-treated rats had significantly lower ultimate stress compared with controls \((P = 0.0006)\). B: guanethidine-treated rats had significantly lower strain \((P = 0.031)\) compared with controls, which may indicate that the ligament matrix organization was disrupted after sympathectomy. C: guanethidine-treated rats also had significantly lower elastic moduli compared with controls \((P = 0.0003)\). Values are means \(\pm\) SD.

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**Fig. 4.** After 10 days of treatment, red fluorescent-labeled microspheres were injected into the femoral artery. Yellow arrows locate some of the microspheres in each image. Guanethidine-treated rats had a qualitatively higher presence of microspheres located within their MCLs. This result indicates increased vascularity in the MCL after chemical sympathectomy with guanethidine.
degradation of connective tissues depend greatly on the action of proteolytic enzymes, including MMPs and cysteine proteinases (such as cathepsins). MMPs have been implicated in remodeling the ECM of a number of orthopedic tissues, including ligament, cartilage, and bone (18, 19, 28, 37, 41). MMP-13 has enzymatic specificity for type I collagen and, therefore, may contribute to matrix degradation. Cysteine proteinases also degrade collagen as well as other ECM components. Cathepsin K plays a substantial role in bone remodeling and also has specificity for type I collagen (4, 8, 14, 23, 33, 36, 43). Cathepsin K has been found in synovial fibroblasts and multinucleated giant cells, suggesting a role for the enzyme in rheumatoid arthritis and in response to calcified tendonitis in human rotator cuff tendon (20, 29). TRAP, which may play a collagenolytic role in calcified tissue, is also present in canine cruciate ligaments after a rupture (16, 27). TRAP is capable of generating reactive oxygen species that can target and cleave type I collagen (16). Hence, the presence of TRAP and cathepsin K in guanethidine-treated ligaments suggests that the collagenolytic activity of these enzymes contributes to the observed changes in mechanical behavior and morphology. The association herein between collagenolytic enzymes and the PNS suggests that neurogenic factors are important in chronic ligament and tendon problems, such as disuse or overuse (16, 27).

Limitations. Limitations of this study must be considered when interpreting the results. First, although guanethidine has been shown to inhibit sympathetic nerves, its specific effect on neuropeptides has not been quantified. A decrease in NPY and VIP was apparent in this study, but many other neuropeptides require further study. It should be noted that VIP is localized to a number of nerve fiber types, including sympathetic and parasympathetic fibers. Therefore, guanethidine affects autonomic nerves, but not necessarily sympathetic nerves exclusively. We have observed biomechanical and catabolic changes after chemical sympathectomy but have only correlated them with NPY and VIP. The specific mechanisms of the observed...
effects are not identified. Second, we present only a correlation between the presence of collagenolytic enzymes in guanethidine-treated specimens and altered mechanical properties. Whereas these enzymes are capable of degrading ECM proteins, further studies are needed to show their relative magnitude and time course for ECM catabolism. Third, the rat model is limited in its ability to predict human response to clinically relevant alterations to a local sympathetic nervous system. Fourth, a dose-response study for guanethidine has not been examined. Clinically relevant doses must be evaluated before predictions can be made regarding its effect in human patients. Fifth, we used female animals in our study. Female animal models have been used consistently in literature (17, 22); however, it does not preclude the possibility of confounding hormonal effects. Hart et al. (17) have examined the effect of reproductive hormones on MCL mechanical properties. In a study using female rabbits, this group showed that pregnant rabbits had increased ligament laxity but had no change in failure load, strength, stiffness, or viscoelastic properties in the MCL compared with controls (17). Last, only one time point (10 days) was examined herein. Longer times are necessary to show the extent to which local changes in blood flow, hydration, mechanics, and collagenolytic enzymes affect connective tissues. We expect that the MCL changes observed herein will become more pathological with time. However, further study is required.

Significance. This study suggests a significant regulatory role for the sympathetic nervous system in the homeostasis of ligaments. Although guanethidine is used clinically to treat pain associated with orthopedic diseases, the effects of this drug on connective tissues have not been previously shown. This study suggests that the administration of guanethidine can be detrimental to the ligament structure. Additionally, this study strongly suggests that sympathetic peripheral nerves influence ligament homeostasis by altering MCL vascularity and levels of neuropeptides, while mediating levels of degradative enzymes. Changes of the ECM mediated by neurogenic factors have clinical relevance. Ligament and tendon grafting is common. Grafting studies primarily focus on the repair of damaged matrix but do not consider regeneration of peripheral nerves to help regulate tissue healing and remodeling. The PNS appears to have an important regulatory role in normal ligaments. A fundamental understanding of this process could provide novel ways to improve clinical outcomes with tissue grafts or with tissue-engineered materials.

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