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Physical exercise can influence local levels of matrix metalloproteinases and their inhibitors in tendon-related connective tissue

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Koskinen, S. O. A., K. M. Heinemeier, J. L. Olesen, H. Langberg, and M. Kjaer. Physical exercise can influence local levels of matrix metalloproteinases and their inhibitors in tendon-related connective tissue. J Appl Physiol 96: 861–864, 2004. First published September 23, 2003; 10.1152/japplphysiol.00489.2003.—Microdialysis studies indicate that mechanical loading of human tendon tissue during exercise or training can affect local synthesis and degradation of type I collagen. Degradation of collagen and other extracellular matrix proteins is controlled by an interplay between matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). However, it is unknown whether local levels of MMPs and TIMPs are affected by tendon loading in humans in vivo. In the present experiment, six healthy young men performed 1 h of uphill (3%) treadmill running. Dialysate was collected from microdialysis probes (placed in the peritendinous tissue immediately anterior to the Achilles tendon) before, immediately after, 1 day after, and 3 days after an exercise bout. MMP-2 and MMP-9 were measured in dialysate by gelatin zymography, and amounts were quantified by densitometry in relation to total protein in the dialysate. MMP-2 and MMP-9 were analyzed by reverse gelatin zymography and semiquantitated visually. Pro-MMP-9 increased markedly after exercise and remained high for 3 days after exercise. Pro-MMP-2 dropped from the basal level immediately after exercise and remained low 1 day after exercise but was slightly elevated 3 days after exercise. The MMP-2 inhibitory activity of TIMP-1 was clearly elevated 1 and 3 days after exercise, and the MMP-2 inhibitory activity of TIMP-2 rose 1 day after loading. The present findings demonstrate enhanced interstitial amounts of MMPs and TIMPs after exercise in the human peritendinous tissue in vivo, and the magnitude and time pattern of these changes may well indicate that MMPs and TIMPs are playing a role in extracellular matrix adaptation to exercise in tendon tissue.

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MATERIALS AND METHODS

ACUTE EXERCISE AND PHYSICAL training have been demonstrated to increase type I collagen synthesis in peritendinous connective tissue of the Achilles tendon in humans, as determined from changes in interstitial concentrations of carboxy-terminal propeptide of type I collagen with the microdialysis technique (4, 8, 10). Furthermore, physical training induced a transient increase in interstitial concentration of type I collagen degradation product, carboxy-terminal cross-linked telopeptide of type I collagen, also indicating a rise in type I collagen degradation with exercise (8). Degradation of collagen, as well as a great number of other extracellular matrix compounds, is initiated by matrix metalloproteinases (MMPs). It is most likely that MMPs play an important role in extracellular matrix turnover of collagen-rich tissues such as tendons. However, it is unknown whether MMPs are involved in the stimulation of type I collagen turnover in human tendon tissue with mechanical loading. Gelatinolytic activity in tendon extracts obtained postmortem from cadavers has been shown to include MMPs, especially MMP-2 (15), which is also one of candidates to be activated after exercise in tendons.

The activity of MMPs is inhibited by specific inhibitors called tissue inhibitors of metalloproteinase (TIMPs). Four members of the TIMP family have been characterized. TIMP-1 and TIMP-2 are capable of inhibiting the activities of all known MMPs. TIMP-2 is known to bind most effectively to MMP-2, whereas TIMP-1 has a high affinity to MMP-9 (2). To our knowledge, the effects of exercise on expression of TIMPs in tendons have not been studied earlier.

Inasmuch as repeated tendon tissue sampling, especially from the human Achilles tendon, is not advisable in humans, microdialysis has been proven to be a useful technique to indirectly determine local interstitial tissue concentrations of extracellular matrix compounds from the Achilles tendon (8). Repeated sampling from the same tissue is a notable advantage of the microdialysis technique.

To study the effect of mechanical loading, such as uphill running, which loads the Achilles tendon substantially, on extracellular matrix degradation, gelatinolytic activities of MMP-2 and MMP-9 were measured in the present study in dialysate obtained from the Achilles tendon. To our knowledge, this is the first attempt to measure local concentrations of MMP-2 and MMP-9 activities in interstitial space fluid of the Achilles tendon in humans in response to exercise. Furthermore, the inhibition of extracellular degradation by TIMP-1 and TIMP-2 was investigated.

Subjects and exercise protocol. Six healthy moderately trained men [mean 26 (range 25–28) yr of age and mean 77 (range 70–80) kg body wt] participated in the present study. All subjects gave their written informed consent, and the study was approved by the Ethical Committee of Copenhagen (KF 11-088/01). The exercise intervention consisted of a 10-min warm-up, during which the subjects ran on the treadmill at 10 km/h, followed by 1 h of 3% uphill treadmill running at 12 km/h. Participants fasted from 10 PM on days preceding experiment days. They were told not to engage in any type of exercise or training for 48 h before the day on which exercise was performed and for the entire length of the experiment.

Design of microdialysis catheters. Microdialysis catheters were constructed from a long inlet tube (0.76 mm ID), a section of permeable membrane (hollow plasma separator tubes, 4 mm diameter,

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3,000 kDa molecular mass cutoff; Asahi), and a short outlet tube. A piece of tubing (0.50 mm ID, 0.63 mm OD) was inserted at the border between the inlet and the membrane. A stainless steel wire was placed inside the catheter to increase mechanical stability. The catheters were sterilized using ethylene oxide (ABOTOX, Herlev, Denmark; Maersk Medical, Lyng, Denmark).

**Insertion of microdialysis catheters.** During each microdialysis run, two microdialysis catheters were placed under ultrasound guidance diagonally in the peritendinous space of both Achilles tendons, with an angle of 45° to the tendon, leaving the outlet of the catheter at the medial side of the tendon. A high-precision syringe pump (model CMA/100, Carnegie, Solna, Sweden) was used to perfuse the catheters at a rate of 5 μl/min with Ringer-acetate containing 1H-labeled human type IV collagen (130 kDa; NEN, Boston, MA) with a perfusate activity of 5.6 kBq/ml. This internal standard was used to ensure a high and similar recovery through the microdialysis catheters. Microdialysis catheters were placed at new sites at each insertion to minimize the effect of trauma on results.

**Collection of microdialysis samples.** Microdialysate samples were collected before and immediately after the subjects ran as well as 1 and 3 days after exercise. Experiments started at 8 AM on all days. Microdialysis was performed for a 3-h period before exercise and a 6-h period after exercise. Subjects were placed in a supine position at room temperature while microdialysis was performed. Microdialysis catheters were removed before the subjects ran, and new catheters were inserted thereafter. Microdialysis samples were collected at 30-min intervals for the 1st h and at 60-min intervals for the remaining periods. Additionally, microdialysis was performed for 4 h at 1 and 3 days after the subjects ran. The samples analyzed in the present study were taken 2 h after insertion of catheters. Samples were weighed to ensure a high and similar recovery through the microdialysis catheters. Microdialysis catheters were placed at new sites at each insertion to minimize the effect of trauma on results.

**Gelatin zymography.** Zymography was used to quantify gelatinase activities of pro-MMP-2 and pro-MMP-9 and carried out by minor modification of the methods of Kleiner and Stetler-Stevenson (5). SDS-11% polyacrylamide gels containing 1 mg/ml gelatin were overlaid with 4% stacking gels. Microdialysis samples were mixed 1:5 (vol/vol) with sample buffer consisting of 40 mM Tris, pH 6.8, 5% SDS, 20% glycerol, and 0.03% bromphenol blue without reducing agent or heating. An equal amount of total protein of each microdialysis sample was loaded into the wells of a gel, and electrophoresis was carried out at 80 V until the dye front had reached the bottom of the gel. Gels were removed from the glass plates and incubated for 30 min in a solution containing 2% Tween 80 and 50 mM Tris, pH 7.5, to remove SDS from gels. The incubation was continued for 30 min with solution containing 2% Tween 80, 50 mM Tris, pH 7.5, 5 mM CaCl₂, and 1 μM ZnCl₂. The gels were then incubated at 37°C for 18 h in a solution containing 50 mM Tris, pH 7.5, 5 mM CaCl₂, and 1 μM ZnCl₂. Gelatinase activity was revealed by negative staining with Coomassie brilliant blue. Purified pro-MMP-2 and pro-MMP-9 (gelatinase zymography standards; Chemicon, Temecula, CA) were used for identification of enzyme activity. The degree of digestion was determined by densitometry (Personal Densitometry SI, Molecular Dynamics, Sunnyvale, CA). The values of integrated optical density were used as results.

**Reverse gelatin zymography.** Reverse zymography is an electrophoresis technique in which substrate (gelatin) and protease (MMP-2) are incorporated directly into acrylamide gels. After the gels are stained, the inhibitory activity of TIMPs results in dark blue areas, where TIMPs have inhibited the gelatin-degrading activity of MMP-2. Inhibitory activities of TIMP-1 and TIMP-2 were analyzed with reverse zymography as described by Oliver et al. (13). SDS-12% polyacrylamide gels were prepared with 3 mg/ml gelatin and 35 mg/ml pro-MMP-2 (Oncogene Research Products, San Diego, CA). A standard stacking gel of 4% was used. Microdialysis samples were treated as described above (see Gelatin zymography). After electrophoresis, gels were removed from the glass plates and incubated on a rotary shaker for 3 h in 100 ml of 2.5% Triton X-100. The Triton X-100 solution was decanted and replaced with 100 ml of solution containing 50 mM Tris, pH 7.5, 5 mM CaCl₂, and 1 mM ZnCl₂, and the gels were incubated at 37°C for 20 h. Gels were stained with Coomassie brilliant blue. TIMP inhibitory activity resulted in dark blue bands on a clear background. Purified TIMP-1 and TIMP-2 (Oncogene Research Products) were run as standards. Because of the low intensity of the bands in reverse zymography gels, MMP-2 inhibitory activity of TIMP-1 and TIMP-2 was only estimated visually. The intensities of the bands were divided into four categories: invisible, faint, visible, and intense, which represent numerical values 0, 1, 2, and 3, respectively.

**ELISA for lactoferrin.** Lactoferrin was measured by ELISA as described by Faur schou et al. (1). Neutrophils express lactoferrin during maturation, and lactoferrin is used as a marker for specific granules.

**Statistics.** Variability of the data is expressed as means ± SD. The statistical significance of the results was determined by nonparametric Wilcoxon’s test for two related samples. Differences were considered statistically significant at P < 0.05. A nonparametric equivalent of variance analysis, Friedman’s test, showed statistically significant differences over time in all parameters studied.

**RESULTS**

Gelatinolytic activity of pro-MMP-2 decreased immediately after the subjects ran. At 1 day after exercise, the activity was still lower than before exercise, whereas it was significantly elevated 3 days after exercise (Fig. 1). Gelatinolytic activity of pro-MMP-9 increased immediately after running and clearly remained elevated 3 days after exercise (Fig. 1). The increase in gelatinolytic activities of pro-MMP-9 after exercise was remarkably high, and the amount of substrate in the gel was not enough to show the magnitude of postexercise samples. Therefore, the comparison was not made between individual postex-

![Fig. 1. A: gelatin zymography gels used to quantify activities of matrix metalloproteinase (MMP)-2 and MMP-9. Time series of microdialysis samples before and immediately after uphill running as well as 1 and 3 days after exercise are shown. Purified pro-MMP-2 and pro-MMP-9 were used to identify the bands. B: gelatinolytic activities of pro-MMP-2 and pro-MMP-9. Values (means ± SD) were calculated relative to before exercise. *P < 0.05.](jap.physiology.org/doi/fig/10.1152/jappl.00549.2003)
exercise time points, but all postexercise levels were significantly higher than the preexercise level. Active forms of MMP-2 and MMP-9 were below the limit of detection.

MMP-2 inhibitory activity of TIMP-1 and TIMP-2 was estimated visually because of the low intensity of the bands in reverse zymography gels (Fig. 2). MMP-2 inhibitory activity of TIMP-1 was clearly elevated 1 and 3 days after exercise, whereas a slight increase was observed 1 day after exercise in MMP-2 inhibitory activity of TIMP-2 (Table 1).

Lactoferrin, a marker for specific granules of neutrophils, increased immediately after running and was clearly elevated 3 days after exercise. Lactoferrin concentration before the exercise was $31.1 \pm 29.5$ ng/mg total protein. An 11- to 12-fold increase in lactoferrin concentration was observed immediately after exercise ($355.8 \pm 129.4$ ng/mg total protein) and 1 day after exercise ($374.3 \pm 174.5$ ng/mg total protein), whereas 3 days after exercise the increase was 5-fold ($143.3 \pm 32.0$ ng/mg total protein).

**DISCUSSION**

A pronounced increase in interstitial pro-MMP-9 was observed immediately after 1 h of uphill running, and it was still elevated on the last observation point, i.e., 3 days after exercise. This prolonged increase in activity of pro-MMP-9 in microdialysate was somewhat surprising, because Riley et al. (15) showed that most gelatinolytic activity from tendon extracts comes from MMP-2. However, it has recently been shown that endothe
telial cells isolated from canine flexor digito
trum profundus tendon and tendon cells from surrounding parietal sheath also produce MMP-9 in response to type I collagen attachment (16). It is widely accepted in the literature that MMP-9 is synthesized by differentiating granulates in the bone marrow and stored in the granules of circulating neutrophils and released after neutrophil activation by inflammatory mediators (3). In the present study, the increase of lactoferrin, a marker for specific granules of neutrophils, paralleled that of MMP-9. Our laboratory’s previous results showed that pro-
and anti-inflammatory cytokine IL-6 concentrations in periten
dinous tissue were elevated up to 2 days after exercise (7) and that prostaglandin E2 concentration in peritendinous tissue increased, at least for some hours, in response to mechanical load (9). The results from the present study and from our previous studies suggest that MMP-9 has a role in a potential inflammation reaction in the peritendinous connective tissue of the human Achilles tendon induced by intensive exercise. It cannot be excluded that the observed rise in MMP-9 activity, to some extent, can be coupled to increased inflammatory tissue reaction caused by mechanical lesion of microdialysis probe implantation. In rat brains subjected to microdialysis, myeloperoxidase-stained neutrophils were observed at the site of catheter implantation, and increased myeloperoxidase expression was detected from tissue homogenates 48 h after catheter implantation (14). Therefore, insertion of a microdialysis catheter could contribute to the rise in MMP-9. However, because gelatinolytic activity of pro-MMP-9 before running was low compared with that after running, an effect of mechanical loading on increased pro-MMP-9 activities is favored, rather than insertion of microdialysis catheters.

Immediately after running, pro-MMP-2 decreased, and 1 day after exercise it was still lower than before exercise, but 3 days after exercise pro-MMP-2 increased. According to cell culture studies, mechanical stress stimulates cell orientation and cell migration (17). It has been shown that tendon cell migration is required for successful tendon healing (18) and that MMP-2 plays an important role in cell migration (12). Therefore, it was expected in the present study that cell migration would have been stimulated in Achilles tendons in response to uphill running. However, the increase in gelatinolytic activity of pro-MMP-2 was not observed until 3 days after exercise. It could be that the microdialysis technique does not reflect MMP-2 tissue levels very well, because at least part of MMP-2 is attached to membrane-anchored TIMPs (12).

The key role of TIMPs is inhibition of extracellular matrix degradation. A balance between MMPs and TIMPs is critical to prevent undesirable changes in extracellular matrix, e.g., accumulation of different extracellular matrix compounds, and to ensure a proper remodeling of extracellular matrix due to changes in environment such as increased mechanical loading. In the present study, MMP-2 inhibitory activity of TIMP-1 and TIMP-2 increased in response to uphill running. A pronounced increase in MMP-2 inhibitory activity of TIMP-1 was observed 1 and 3 days after the exercise but not immediately after running, when a pronounced increase in pro-MMP-9 was observed. The modest and transient rise in MMP-2 inhibitory activity of TIMP-2 would fit with the transient decrease in gelatinolytic activity of pro-MMP-2, which was followed by the increase in gelatinolytic activity of pro-MMP-2. However, it is possible that MMP-2 levels are also underestimated, because TIMP-2 takes part in MMP-2 activation at the cell surface (12).

With use of the microdialysis technique, it is often speculated whether microdialysate actually reflects circulating levels

![Fig. 2. Reverse gelatin zymography gels used to estimate MMP-2 inhibitory activities of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. For each microdialysis sample, 17 μl of total protein were loaded into the wells of a gel. Purified TIMP-1 and TIMP-2 were used to identify the bands.](image-url)
of measured compounds. Although circulating MMPs and TIMPs were not measured in the present study, it has previously been shown that exercise similar to that performed in the present study does not cause long-lasting increases in circulating levels of studied MMPs and TIMPs (6). However, it was shown that exercise caused a rapid increase in serum MMP-9 concentration immediately after running (6), which was probably due to an increase in the number of leukocytes in the circulation (11). Furthermore, it has been suggested that any MMP activity in the circulation is inhibited irreversibly by the broad-spectrum protease inhibitor a2-macroglobulin (19).

Thus the demonstration of MMPs and TIMPs in microdialysate from human tissue provides information on local interstitial tissue concentration relevant for extracellular matrix subjected to loading. In conclusion, the present findings indicate that physical exercise can influence local MMP and TIMP activities in peritendinous tissue of human Achilles tendon in vivo and suggest that these changes play a role in extracellular matrix adaptation to exercise in human tendon tissue.

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