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Cyclo-oxygenase-2 mediated prostaglandin release regulates blood flow in connective tissue during mechanical loading in humans

H. Langberg, R. Boushel *, D. Skovgaard, N. Risum and M. Kjær

*Sports Medicine Research Unit, Department of Rheumatology, Copenhagen University Hospital at Bispebjerg, DK-2400 Copenhagen NV, Denmark and * Department of Exercise Physiology, Concordia University, Montreal, Canada*

Mechanical loading is known to increase connective tissue blood flow of human tendons and to cause local release of vasodilatory substances. The present study investigated the importance of prostaglandins (PG) formed by cyclo-oxygenase isoforms (COX-1 and 2) for the exercise-related increase in blood flow in connective tissue. Healthy individuals ($n = 24$, age: 23–31 years) underwent 30 min of intermittent, isometric, plantarflexion with both calf muscles either without ($n = 6$, Control, C) or with blockade of PG formation, either COX-2 specific ($n = 10$, Celecoxib 2 x 100 mg day^{-1} for 3 days prior to the experiment) or COX unspecific ($n = 8$, indomethacin 100 mg (12 and 1 h pre-experiment) and acetyl salicylic acid 500 mg day^{-1} for 3 days pre-experiment). Prostaglandin E_2 (PGE_2) concentration was determined by microdialysis and blood flow by ^{133}Xe washout. In C, interstitial PGE_2 rose from (0.8 ± 0.2 (rest) to 1.4 ± 0.5 ng ml^{-1} (exercise), $P < 0.05$), whereas during unspecific COX inhibition, tissue PGE_2 was completely inhibited at rest and during exercise. COX-2 specific blockade did not inhibit tissue PGE_2 at rest, but totally abolished the exercise induced increase. Blood flow was similar in the three groups at rest ($P > 0.05$), whereas the increase in flow with exercise was reduced by 35 and 43 % with COX-2 specific blockade (3.2 ± 0.7 to 6.1 ± 1.5 ml (100 g tissue)$^{-1}$ min^{-1} or COX unspecific blockade (3.0 ± 0.8 to 7.6 ± 1.6), respectively, compared to C (2.7 ± 0.8 to 10.2 ± 2.0)($P < 0.05$). The findings indicate that COX-2 specific mechanisms are responsible for the exercise-induced increase in prostaglandin synthesis, and that increase in tissue prostaglandin plays an important role for blood flow in peritendinous connective tissue during physical loading *in vivo*.

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Corresponding author H. Langberg: Sports Medicine Research Unit, Department of Rheumatology, Copenhagen University Hospital at Bispebjerg, DK-2400 Copenhagen NV, Denmark. Email: hl02@bbh.hosp.dk

Eicosanoids such as prostaglandins and thromboxanes are important mediators of several biological responses such as inflammation, platelet activation and tissue perfusion (Ostrom *et al.* 2001; Ouellet *et al.* 2001), and formation of these is dependent on the presence of cyclo-oxygenase (COX; O'Banion, 1999). Isoforms of COX expressed in several cell types are considered to be either constitutive (COX-1) or predominantly inducible e.g. by inflammation (COX-2), although some overlap and especially redundancy has been demonstrated (Vane *et al.* 1994, 1998; Ballou *et al.* 2000). The use of selective COX-2 inhibitors has been suggested to result in an increased vascular tone (McAdam *et al.* 1999; Catella-Lawson *et al.* 1999; Muscara *et al.* 2000). Thus, prostanoids may play a major role for blood flow regulation in various local regions of the musculo-skeletal system that is subjected to mechanical loading in humans. Despite being clearly expressed in both peripheral and central tissues (Samad *et al.* 2001), as well as evident in circulating blood, cyclo-oxygenase mediated formation of prostaglandins and resultant tissue concentrations has been difficult to assess *in vivo* in humans during stressful stimuli.

Muscular activity is associated with marked increases in blood flow of the contracting extremity, and it has been demonstrated that this includes not only contracting muscle but also the flow through adjacent tendon-related connective tissue, which rises up to 7-fold with exercise (Langberg *et al.* 1998; Boushel *et al.* 2000). Whereas in skeletal muscle tissue a significant vasodilatory role of prostanoids during exercise is debatable, other factors may play a larger role (Wilson & Kapoor, 1993; Davy *et al.* 1993; Duffy *et al.* 1998). The regulation of blood flow in tendon-related connective tissue with exercise remains unexplained and the role of prostaglandins in relation to this has never been studied. Interestingly, formation of inflammatory mediators presents a major problem in overuse injuries of the locomotor system, and aims to reduce prostanoid levels pharmacologically with non-steroidal anti-inflammatory drugs (NSAIDs) is widely used in clinical practice (Kurumbail *et al.* 1996).

The present study evaluated the importance of prostaglandin for connective tissue blood flow during physical stress. This was done by using differential blockade of cyclo-oxygenase in human subjects exercising with their calf muscles, and by determining interstitial concentrations of prostaglandin E_2 in the peritendinous tissue of the Achilles tendon together with measurements of tissue blood flow using ^{133}Xe-washout technique. It is hypothesized that blockade of cyclo-oxygenase activity will decrease tissue blood flow both at rest and during exercise.

METHODS

Subjects and medication

Twenty-four healthy young males (age: 23–31 years (range)) participated after informed written consent in this study was given and approved by the Ethical Committee of Copenhagen (KF 11-043/02) and performed in accordance with the Declaration of Helsinki. Subjects were randomised to three groups either receiving placebo (control, $n = 6$), unspecific blockade of cyclo-oxygenase by indomethacin (100 mg at 12 and 1 h prior to the experiment) and acetylsalicylic acid (500 mg per day, taken for 3 days prior to experiment; COX-1 and COX-2, $n = 8$) or receiving a selective COX-2 inhibitor Celebra (Celecoxib, Pfizer Inc. Groton, USA; Gierse *et al.* 1999; 100 mg twice daily for 3 days prior to the experiment; COX-2, $n = 10$). Subjects were blinded to the type of medication they received and were carefully instructed to take the medication as prescribed. Prior to the study the subjects were questioned to ensure that prescriptions were followed. None of the individuals took any other medicine regularly, and all were free from diseases and complaints of the musculo-skeletal system. Specifically, none of the subjects had signs of any overuse injuries in their muscles or tendons, nor did any have a previous history of Achilles tendon symptoms. The three experimental groups resembled each other with regard to age, anthropometric data and level of physical activity. All subjects were non-smokers.

In vivo microdialysis

A microdialysis catheter (CMA, Solna, Sweden) under ultrasonography guidance was placed adjacent to the anterior border of the Achilles tendon (Fig. 1) as previously described with details in Langberg *et al.* 1999*c*,*d*. The microdialysis catheter (CMA 60; CMA/Microdialysis AB; 20 kDa molecular cut off, 0.5 mm outer diameter; length 30 mm) was perfused with Ringer–acetate solution and radioactive labelled with [15-^{3}H(N)]PGE_2 (specific activity 3.7 GBq (mmol)$^{-1}$; NEN, Boston, MA, USA) at 2 μl min^{-1} using a high-precision syringe pump (CMA 100, Carnegie Medicine, Solna, Sweden). Units of 30 μl were sampled at the outlet of the microdialysis catheter for determination of PGE_2 concentrations.

The interstitial concentrations (C_i) were calculated using the internal reference calibration method (Scheller & Kolb, 1991). Three microlitres of perfusate were added to 3 ml of liquid

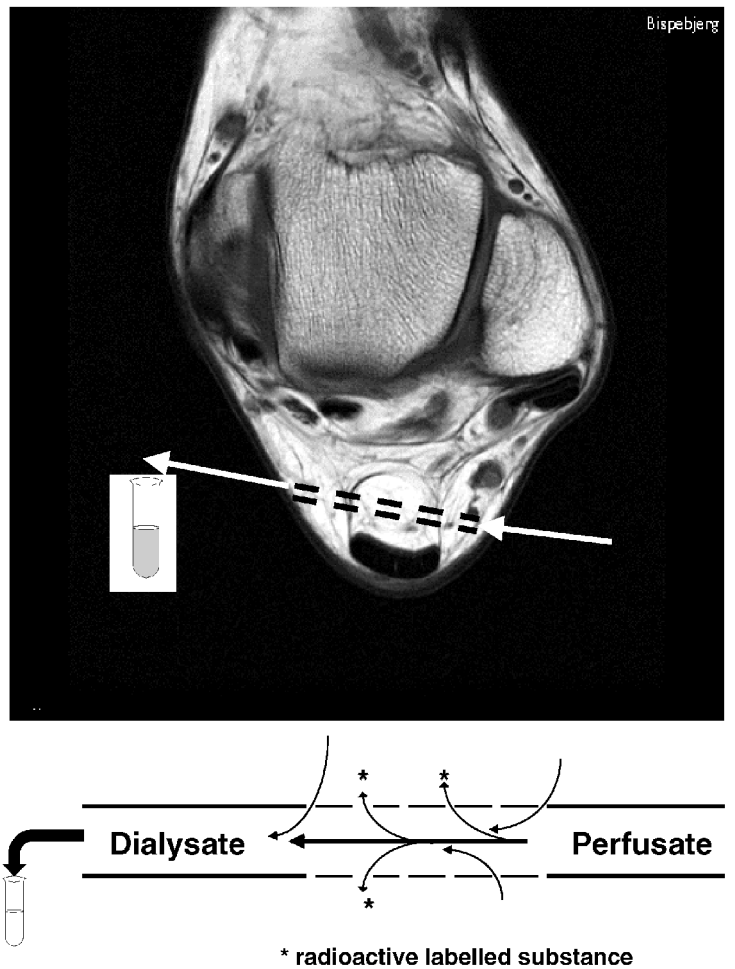

Figure 1. Microdialysis in human peritendinous connective tissue

Illustration of the positioning of the microdialysis catheter in the peritendinous Achilles region as depicted on a magnetic resonance cross section image at the malleoli ankle level. Below, a schematic representation of microdialysis tubes used to determine interstitial concentrations of prostaglandin E_2.

scintillation medium (Ultima Gold, Packard, Gronningen, Netherlands) and measured in a β -counter (Wallac 1409, Wallac, Turko, Finland). The relative recovery (*RR*) was calculated for each microcatheter as: $(C_p - C_d)/C_p$, where C_p is disintegration min^{-1} in the perfusate and C_d is disintegration min^{-1} in the dialysate. It is assumed that *RR* from interstitial fluid to perfusate of unlabelled procollagen molecule equals relative loss from perfusate to interstitial fluid of labelled collagen molecule.

Blood flow

Peritendinous blood flow was determined by administration of ^{133}Xe (in isotonic saline, ~10 MBq ml^{-1}, 0.1 ml) injected directly ventrally to the Achilles tendon. Great care was taken not to inject any gas bubbles. The injection was made with a fine needle (outer diameter 0.4 mm) from the medial side at a depth of 1–2 cm. The depot was placed 5 cm proximal to the upper medial portion of the Achilles tendon insertion on the calcaneus on both right and left side. The needle was withdrawn from the tissue 0.5 min after the injection had been given to ensure that no leak appeared. The ^{133}Xe washout was measured via portable scintillation detectors strapped to the skin above the ^{133}Xe depots. The detectors were connected to a multichannel analyser system (Oakfield Instruments, Oxford, UK). The initial counting rate was ~1.5×10^3 s^{-1}. Counts were collected in 30 s periods.

Calculations of blood flow

From the clearance rate of ^{133}Xe (Fig. 2) it is possible to calculate the blood flow (b.f.) in millilitres per 100 g tissue per minute, when the tissue–blood blood partition coefficient λ (being 5–10 μC g $tissue^{-1}$/μC ml $blood^{-1}$ in adipose tissue) is known (Kety, 1951):

$$\text{b. f.} = -100\,\lambda\kappa,$$

where κ (ml (100 g tissue)$^{-1}$ min^{-1}) is the elimination rate constant for the mono-exponential washout of ^{133}Xe (Lassen *et al.* 1964).

Previous studies have excluded any influence of lymph drainage on peritendinous blood flow during exercise (Langberg *et al.* 1999*a*).

Experimental protocol

After insertion of the microdialysis catheters and positioning of the xenon depots the subjects rested 120 min before starting the experiment to minimize the influence of the insertion procedure (Langberg *et al.* 1999*c*). Following this pre-period the subjects rested for additional 90 min during which the resting blood flow and resting tissue concentration was measured (Langberg *et al.* 1999*a*). The resting period was followed by an exercising period. During exercise the subjects were seated in a specially constructed experimental set up (Fig. 3), with the trunk perpendicular to the seat and the knees extended as previously described (Langberg *et al.* 1999*a*). The study was terminated by a recovery phase of 60 min rest. All subjects performed 30 min of intermittent static plantar flexion exercise (1.5 s contraction, 1.5 s relaxation in time with a metronome) at a torque output of 800 ± 50 N m per contraction (equivalent to individual body weight). The load imitated the workload of the triceps muscles during normal walking. Visual feedback ensured a constant force output during contraction.

Immunohistochemistry

Blood samples were all added to indomethacin (10 μg ml^{-1}, Sigma) and 4.5 mM EDTA, centrifuged at 2000 *g* for 10 min, and the plasma used for analysis.

Prostaglandin E_2 concentrations both in plasma and in microdialysate were analysed using a commercially available PGE_2 radioimmuno-assay kit (RIA KIT catalogue number NEK-020; NEN Research Products, Du Pont, Boston, USA). Samples or standards, together with ^{125}I-PGE_2 as the tracer, were incubated with rabbit anti-PGE_2 antibodies overnight at 4 °C. The samples were precipitated by polyethylene glycol, centrifuged, decanted and the quantity of radioactivity in the pellet was determined in a gamma counter.

Statistics

All data are means ± S.E.M. or the range. Mann-Whitney's or Wilcoxon's non-parametric summed rank tests were used to test differences between groups or within groups, respectively. $P < 0.05$ (two-tailed test) was considered significant.

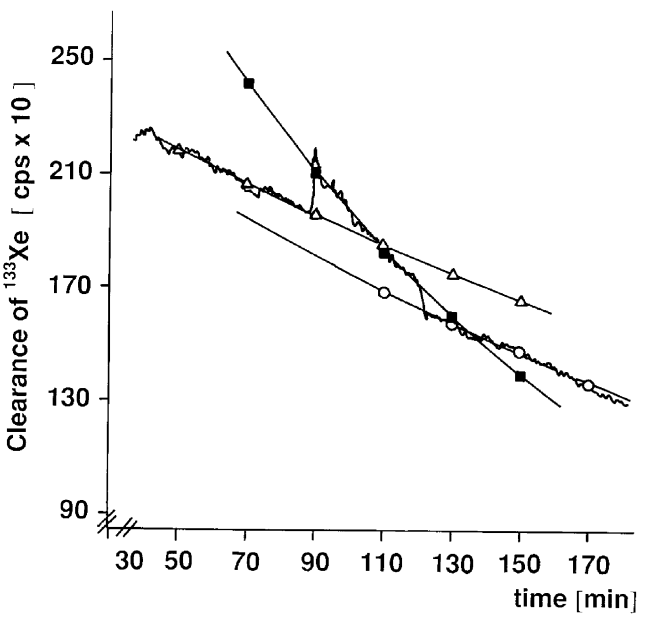

Figure 2. An example of the ^{133}Xe clearance curve for one subject

The clearance rate was measured during a resting period (0–90 min). The resting period was followed by a series of intermittent isometric exercises of the calf muscles (contraction 1.5 s–rest 1.5 s; 90–120 min). The study was terminated by a recovery phase of rest (120–180 min). For determining blood flow elimination rate the constant κ for the three mono-exponential curves fitting the various ^{133}Xe washout curves were used. The various elimination-rate constants used were: rest (△) $\kappa = -4.4 \times 10^{-5}$ ($R = -0.98$); exercise (□) $\kappa = -11.6 \times 10^{-5}$ ($R = -0.99$); and recovery (○) $\kappa = -6.4 \times 10^{-5}$ ($R = -0.99$) respectively. Reprinted with permission from *Clinical Physiology* (Langberg *et al.* 1999*a*).

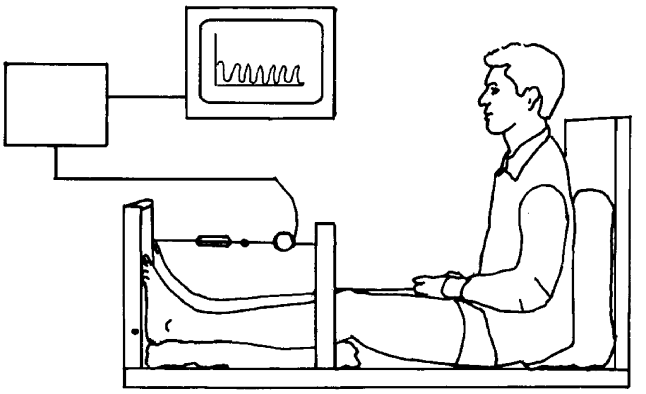

Figure 3. A schematical drawing of the experimental set up

The subject is seated with the trunk perpendicular to the seat, the knee extended and both feet positioned on the vertical sheet with the axis of the sheet and the axis of plantar–dorsal flexion in the ankle joint aligned. The torque moment developed by m. triceps surae of both legs in the plantar direction is registered by a precalibrated (range: 0–2000 N) strain gauge (lever arm: 28 cm). The torque is amplified by a costume-build instrumental AC-amplifier and displayed on-line to the subject. Reprinted with permission from *Clinical Physiology* (Langberg *et al.* 1999*a*).

RESULTS

It was shown that the relative recovery for PGE_2 was 44 ± 5 % at rest and 47 ± 5 during muscle contraction, and these results were used for determination of interstitial concentrations of PGE_2 in peritendinous connective tissue using the internal reference calibration method (Scheller & Kolb, 1991).

In the control group, the interstitial tissue PGE_2 concentration rose in response to exercise (0.8 ± 0.2 (rest) to 1.4 ± 0.5 ng ml^{-1} (exercise), $P < 0.05$; Fig. 4), whereas during unspecific cyclo-oxygenase inhibition (COX-1 and COX-2) tissue PGE_2 was inhibited by 96 % and no increase in PGE_2 was observed during exercise (0.03 ± 0.01 (rest) and 0.03 ± 0.01 ng ml^{-1} (exercise; Fig. 4)). COX-2 specific blockade did not inhibit tissue PGE_2 at rest, but totally abolished the exercise-induced increase (0.8 ± 0.1 (rest) to 0.7 ± 0.2 ng ml^{-1} (exercise; Fig. 4)).

Blood flow was similar in the three groups at rest (3.2 ± 0.7 (COX-2), 3.0 ± 0.8 (COX-1 and -2), and 2.7 ± 0.8 ml (100 g tissue)$^{-1}$ min^{-1} (C), $P > 0.05$; Fig. 5), whereas during exercise the increase in flow was significantly reduced ($P < 0.05$) in the COX-2 (to 6.1 ± 1.5 ml (100 g tissue)$^{-1}$ min^{-1}) and COX unspecific inhibited group (to 7.6 ± 1.6 ml (100 g tissue)$^{-1}$ min^{-1}), respectively, compared with control (to 10.2 ± 2.0 ml (100 g tissue)$^{-1}$ min^{-1}) (Fig. 5).

DISCUSSION

The present study demonstrates several findings. First, it is possible to detect tissue concentrations of prostaglandins in humans both during rest and exercise in humans (Fig. 4). Second, it is shown that a blockade of cyclo-oxygenase inhibits the exercise-induced increase in prostaglandin synthesis, and that a cyclo-oxygenase-2 specific mechanism is responsible for this inhibition (Fig. 4). Third, and maybe most importantly, the present study shows that the cyclo-oxygenase-2 mediated inhibition of tissue prostaglandin synthesis during exercise plays an important role for the increased tissue blood flow in peritendinous connective tissue during muscular contractions and physical loading of human tendons *in vivo* (Fig. 5).

The findings of the present study suggest a differentiated role of the vasodilatory agents in the human peritendinous area, depending on whether the tissue is at rest or metabolic stressed as during muscular activity (Boushel *et al.* 2000) by demonstrating a separate and pronounced role of prostaglandins in exercise vasodilatation of

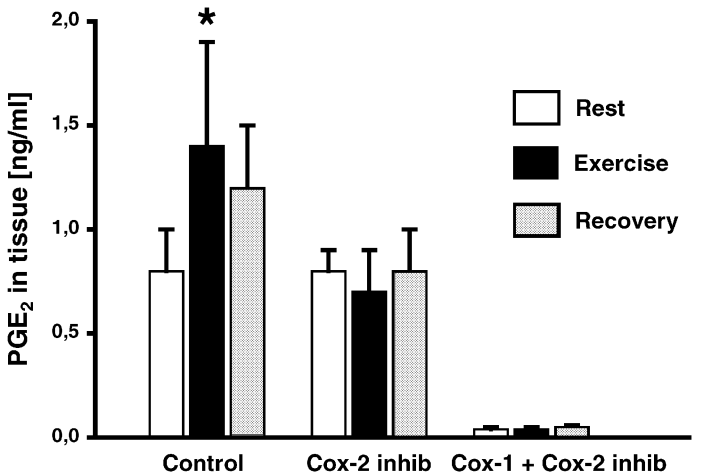

Figure 4. Tissue prostaglandin concentrations around the human Achilles tendon, and the effect of cyclo-oxygenase blockade at rest and during physical activity

Interstitial tissue concentrations of prostaglandin E_2 (PGE_2) in human connective tissue around the Achilles tendon was determined using microdialysis *in vivo*. Intermittent, isometric plantar flexion was performed for 30 min by healthy males, either without (Control) or with blockade of cyclo-oxygenase 1 and 2 (indomethacin and acetyl salicylic acid) (COX-1and COX-2) or cyclo-oxygenase-2 (Celecoxib) (COX-2). * $P < 0.05$ *vs.* resting values.

connective tissue (Figs 4 and 5). In support of this notion, it has recently been shown that IL-1β-induced COX-2, but not COX-1 formation in isolated human tendon fibroblasts, and that this was followed by PGE_2 synthesis (Tsuzaki *et al.* 2003). Somewhat in contrast, the regulation of vasodilation in skeletal muscle at rest, as well as during exercise, has previously proved redundancy with regards to the interplay between PGE_2, nitric oxide (NO) and endothelial-derived hyperpolarising factor (EDHF; Pohl *et al.* 2000). Despite the fact that some studies have been able to identify a separate role for prostaglandin in the regulation of flow in muscle in the resting state (Duffy *et al.* 1998), it has been questionable as to whether there is an effect of prostaglandin-synthesis blockade on skeletal muscle blood flow in humans during exercise and reactive hyperaemia (Wilson & Kapoor, 1993; Davy *et al.* 1993; Engelke *et al.* 1996). This indicates that regulation of blood flow in tissue regions dominated by connective tissue in close proximity to the tendon differ from that of contracting skeletal muscle itself. In this respect, it is interesting that earlier studies have found that the site of formation of prostaglandins in skeletal muscle was not located within the muscle cell, but rather originated from connective tissue of fascia and mysial sheets as well as from the vascular endothelium, and also that tendon connective tissue was the major source for prostaglandin formation in mature tendon and/or skeletal muscle preparations (McLennan & Macdonald, 1991). This also fits with a previous demonstration of a local prostaglandin release from peritendinous tissue in response to prolonged running exercise (Langberg *et al.* 1999*d*). In that study it was shown, that the microdialysis technique in itself created a short lasting insertion trauma-related increase in tissue prostaglandin and thromboxane for 90–120 min, and therefore all determinations in the present study are always performed more than 2 h after catheter insertion (Langberg *et al.* 1999*c*).

Although the present findings indicate that prostanoid formation is important for an increase in tendon blood flow during exercise and that blockade of prostaglandin formation is associated with a ~40 % reduction of this increase, it remains clear that a large part of the blood flow increase during muscular contractions remains present even in the absence of changes in tissue prostaglandin concentrations. What factors are responsible for the remaining response can only be speculated upon, but NO and EDHF cannot be excluded. Such a hypothesis is supported by the finding of increased connective tissue concentrations of bradykinin in response to exercise in humans (Langberg *et al.* 2002). A purely mechanical phenomenon similar to the muscle pump pulling blood through the connective tendon region cannot be excluded from contributing to exercise-induced increase in blood flow, as it has been found that a pronounced negative interstitial fluid pressure in the Achilles peritendinous region occurs during exercise in parallel with increased regional blood flow (Langberg *et al.* 1999*b*).

The fact that COX-2 inhibition abolished the exercise-induced rise in prostaglandin, whereas unspecific cyclo-oxygenase resulted in an almost total inhibition of the entire prostaglandin formation (Fig. 4), fits very well with animal models in which prostaglandin production was induced by carrageenan-induced paw inflammation in rats (Anderson *et al.* 1996; Portanova *et al.* 1996; Zhang *et al.* 1997) or by a subcutaneous air pouch in mice (Vane *et al.* 1994). Local tissue concentrations cannot be directly compared between those models and the findings in the present study, but it is interesting to note that the relative changes from basal level with stimulation is of the same order of magnitude whether done with mechanical loading or with pharmacological intervention (Anderson *et al.* 1996; Portanova *et al.* 1996; Smith *et al.* 1998), and that indomethacin was able to block tissue concentrations of PGE_2 almost completely (Seibert *et al.* 1994; Anderson *et al.* 1996; Zhang *et al.* 1997).

The findings of this work suggest that cyclo-oxygenase-2 is a key contributor to the increase in connective tissue levels

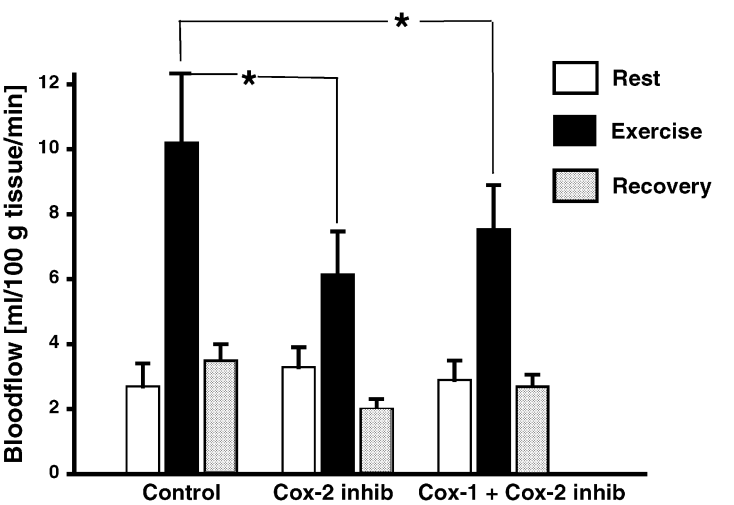

Figure 5. The effect of cyclo-oxygenase blockade on the connective tissue blood flow aound the human Achilles tendon at rest and during physical activity.

Blood flow in human connective tissue around the Achilles tendon determined using the ^{133}Xe wash out method. Intermittent, isometric plantar flexion was performed for 30 min by healthy males, either without (Control) or with blockade of cyclo-oxygenase 1+2 (indomethacin and acetylsalicylic acid) (COX-1and COX-2) or cyclo-oxygenase-2 (Celecoxib) (COX-2). *$P < 0.05$ *vs.* resting values.

of PGE_2 in response to muscular contraction. The blockade with COX-2 specific inhibitors that result in a lack of increase in tissue PGE_2 during exercise indicates that at least under physical stress, COX-2 acts as an inducible isoform. Anti-inflammatory medication (NSAID) is often considered the drug of choice in the treatment of chronic overused human tendons (Fredberg, 1997) although investigations on inflammatory markers within the chronic overused human Achilles and patella tendons have lacked the ability to show elevated levels of prostaglandins during rest (Alfredson *et al.* 1999, 2001). However, data obtained during exercise in the area around chronic overloaded human Achilles tendons have indicated that prostaglandin levels increase to a level significantly higher than around the contralateral healthy tendon with loading (H. Langberg, unpublished data). This could imply that the injured tendon represents a vulnerable structure that due to adherences in the peritendinous region (Abrahamsson *et al.* 1989) displays inflammatory reactions more easily upon loading. Whether such a rise in inflammation plays an important role either a stimulatory or a detrimental role in the tissue regeneration or in the nociceptive processes has not been widely addressed (Zhang *et al.* 1997). This (Mun-Bryce & Rosenberg, 1998) makes it difficult to judge whether inhibition of the elevation of prostaglandins by anti-inflammatory medication is of advantage or harm to the tendon. Cyclo-oxygenase unspecific NSAID, piroxicam, has been found to increase the strength of healing rat ligaments, but did not have any influence on the ultimate strength once the healing was completed (Dahners *et al.* 1988). Somewhat in contrast, recent studies on cyclo-oxygenase specific (COX-2) inhibitors (Celecoxib) indicated a small reduction in ligament strength early during healing, but no long-term results were provided (Elder *et al.* 2001). The positive effect of anti-inflammatory medication could also be a result of blockade of the inducible form of prostaglandins as found in the present study, thus reducing the hyper-vascularization that often accompanies an overloading of the tendon (Astrom & Westlin, 1994; Astrom & Rausing, 1995). A new study on the use of ultrasound guided sclerosis of neovessels in painful chronic Achilles tendinosis (Ohberg & Alfredson, 2002) supports the findings in the present study as it showed a good effect pointing towards hypervascularization as being part of the problem in chronic overused tendons. This would explain the rational behind NSAID blockade of PGE_2 release and decreasing the abnormal resting blood flow.

In conclusion, the present study demonstrated a cyclo-oxygenase-2 specific mechanism, inducible during mechanical tissue stress, being responsible for the increase in tissue synthesis of prostaglandin, and that increase in tissue prostaglandin plays a major role in the increased tissue blood flow in peritendinous connective tissue observed during muscular contractions and physical loading of human tendons. This observation may have implications for the mechanism by which cyclo-oxygenase-blocking drugs influence both healthy and diseased tendon tissue in humans.

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