Inflamm-Aging and Arachadonic Acid Metabolite Differences with Stage of Tendon Disease

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Abstract

The contribution of inflammation to the pathogenesis of tendinopathy and high prevalence of re-injury is not well established, although recent evidence suggests involvement of prostaglandins. We investigated the roles of prostaglandins and inflammation-resolving mediators in naturally occurring equine tendon injury with disease stage and age. Levels of prostaglandins E2 (PGE2), F2α (PGF2α), lipoxin A4 (LXA4) and its receptor FPR2/ALX were analysed in extracts of normal, sub-acute and chronic injured tendons. To assess whether potential changes were associated with altered PGE2 metabolism, microsomal prostaglandin E synthase-1 (mPGES-1), prostaglandin dehydrogenase (PGDH), COX-2 and EP4 receptor expression were investigated. The ability of tendons to resolve inflammation was determined by assessing FPR2/ALX expression in natural injury and IL-1β stimulated tendon explants. Alterations in the profile of lipid mediators during sub-acute injury included low PGE2 and elevated LXA4 levels compared to normal and chronic injuries. In contrast, PGF2α levels remained unchanged and were three-fold lower than PGE2. The synthetic capacity of PGE2 as measured by the ratio of mPGES-1:PGDH was elevated in sub-acute injury, suggesting aberrations in tendon prostaglandin metabolism, whilst COX-2 and EP4 receptor were unchanged. Paradoxically low tendon PGE2 levels in early injury may be attributed to increased local clearance via PGDH or the class switching of lipid mediators from the prostaglandin to the lipoxin axis. PGE2 is therefore implicated in the development of tendon inflammation and its ensuing resolution. Whilst there was no relationship between age and tendon LXA4 levels, there was an age-associated decline in FPR2/ALX receptor expression with concurrent increased PGE2 levels in injury. Furthermore, uninjured tendon explants from younger (<10 years) treated with IL-1β responded by increasing FPR2/ALX suggesting aged individuals exhibit a reduced capacity to resolve inflammation via FPR2/ALX, which may present a potential mechanism for development of chronic tendinopathy and re-injury.

Introduction

Tendinopathy of the human Achilles and the functionally equivalent equine superficial digital flexor tendon (SDFT) are significant causes of morbidity in athletic individuals [1,2]. Repetitive mechanical loading during exercise is cited as a major causative factor [3,4] with high risk of re-injury [5] due to the inferior mechanical properties of the poorly organised fibrous tissue following healing [6]. The importance of inflammation in tendinopathy is highly debated with the aetiology often cited as a degenerative mechanism [7,8]. However, this inference is influenced by analyses of injured human tendons that are often only available for examination at surgery, usually some time after the initial injury, by which time acute phase events are lost and chronic disease is well established.

The horse presents an attractive large animal model for the study of the equivalent human injury due to the shared characteristics of aging phenotypes [9,10] and elastic energy storing function common to the weight-bearing tendons of both species [11,12]. Equine tendons present a more readily attainable source than the human counterpart, permitting targeted investigation of disease throughout the injury phases as well as normal (uninjured) tendons of a wide age range for comparison. Furthermore, similar to the human injuries, tendon repair processes are frequently clinically classified into three phases in naturally occurring equine injury; the acute phase occurs immediately after the initial trauma lasting only a few days, followed by sub-acute (3–6 weeks) and chronic injury phases (>3 months after injury) [13]. The tensile region of the equine SDFT is most susceptible to overstrain injury [14,15]. Injured tendons are enlarged compared to normal and exhibit a haemorrhagic granular central core during early stage injury. The histological appearance of injured equine SDFTs are shown in Fig. 1, illustrating increased cellularity soon after injury compared to...
normal tendons. During healing, the damaged tissue is remodelled and a fibrogenic scar repair forms and the highly organised arrangement of collagen fascicles are not restored (Fig. 1c) and [16], predisposing to re-injury due to diminished mechanical strength. The effects of age, exercise and mechanical loading are inextricably linked and are potentially synergistic factors in the development of tendinopathy. The frequency of tendon injury in sprint horses has been shown to increase with age from 6% in 2 year olds to 16% in horses aged 5 years and over [17]. Similarly, an increased incidence of Achilles tendon rupture has also been reported in middle aged athletes or aged non-athletic persons [18,19]. Hence the effects of ageing and cumulative micro-damage can further exacerbate the risk of re-injury in diseased tendons.

The contribution of inflammation to the development of tendinopathy is not fully elucidated and there is a paucity of data reporting inflammatory processes, particularly during the early stages of injury. However, several studies support the involvement of prostaglandins such as prostaglandin E₂ (PGE₂) in the development of tendinopathy via inflammatory processes [20–22]. Indeed, prostaglandin lipid mediators are synthesised in response to tissue insult or injury and contribute to pain and inflammation in many connective tissues within the body [23]. PGE₂ levels are reported to increase in the peri-tendinous space of the Achilles of healthy exercising human subjects [24] and in murine patellar and Achilles tendons following treadmill exercise [25], suggesting exercise can also induce tendon inflammation. These observations are supported by in vitro experiments whereby tendon fibroblasts in culture release PGE₂ in response to repetitive cyclic strain [26–28]. Furthermore, prostaglandins regulate MMP production, partly via an IL-1β mediated mechanism in catabolism of cartilage, periodontal ligament [29,30] and tendon [20,22] contributing to degradation of the extracellular matrix (ECM). However the involvement of other prostaglandins such as those of the D series and their cyclopentanone metabolites to the development of tendinopathy are not known.

The receptors mediating prostaglandin effects are also cited as contributors to the pathogenesis of tendon injuries. A series of four EP receptor subtypes are responsible for the downstream effects of PGE₂. The EP₄ receptor is reported to mediate the IL-1β induced catabolic metabolism via the p38 MAPK pathway in human tendon fibroblasts, implicating its role in the development of tendinopathy [31]. Regulation of mPGES-1 and PGDH enzymes controlling prostaglandin synthesis and the clearance mechanisms associated with degradation have been described for burn related injuries and sepsis in human patients [32]. However, little is currently known about prostaglandin metabolism in flexor tendons that have sustained a natural injury, nor the effect of injury stage and age.

In addition to prostaglandins, other products of the arachidonic acid pathway exert important roles in regulating inflammation. Lipoxin A₄ (LXA₄) is a specialised pro-resolving mediator that selectively signals through the FPR2/ALX receptor providing endogenous stop signals for inflammation [33,34]. The ability to resolve inflammation after injury or sepsis is well documented for other body tissues [33,35,36], although knowledge of the anticipated roles of specialised pro-resolving mediators such as lipoxins is limited for tendon injuries. We recently described significantly increased expression of FPR2/ALX in sub-acute injured equine tendons [16]; however expression appeared to be of insufficient duration and magnitude to suppress inflammation, which may potentiate development of chronic disease and fibrotic repair.

Taking all these observation together, it is likely that additional factors play a role in repair-processes during tendon injury. A reduced ability to respond to inflammation may be a contributing factor influencing the reduced efficacy of tendon repair. Inflamm-ageing is a component of immunosenescence which is an age associated decline in immune function, whereby the major cell types of the immune system exhibit age-related changes, resulting in a diminished ability to cope with inflammation [37]. Although tendon pathology and incidence of injury are known to increase in aged individuals [18,38], the effect of age on the ability to resolve tendon inflammation and the contribution of immunosenescence to the development of disease are not understood. The aims of this study were to assess the temporal and differential alterations in prostaglandin and resolving lipid mediators in normal and naturally injured equine tendons throughout the stages of healing and to determine the effect of age and injury stage on the regulation of prostaglandin metabolism. We hypothesised that the production of PGE₂ increases with age in injured flexor tendons and that pro-resolving lipid mediators are activated during the early injury phase. We report altered PGE₂ metabolism and elevated LXA₄ levels occur during the early stage of tendon disease, and reduced expression of the inflammation resolving receptor FPR2/ALX with increasing age, which has implications for sustaining chronic injury.

Figure 1. Typical microscopic appearance of normal and injured equine flexor tendons. Longitudinal histology sections stained with Haematoxylin and Eosin showing: (A) normal superficial digital flexor tendon (SDFT) from a 6 year old horse showing regular arrangement of parallel collagen fibrils. Scale bar = 100 μm. (B) Sub-acutely injured SDFT 3 weeks post injury from a 4 year old horse showing marked cellular infiltration (black arrows). Scale bar = 100 μm. (C) Chronic injured SDFT >3 months post injury from a 12 year old horse showing increased cellularity and poor organisation of collagen fibrils compared to (A). Scale bar = 100 μm.
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Results

Class Switching of Lipid Mediators Occurs in Early Stage Tendon Injury

PGE\textsubscript{2} concentrations were reduced in extracts prepared from sub-acute injured tendons compared to normals and chronic injuries \((P<0.001)\) and \(P<0.05\) respectively \((\text{Fig. 2a})\). In contrast, PGF\textsubscript{2}\textalpha{} concentrations were similar in normal and injured tendons and were 3-fold less compared to PGE\textsubscript{2} \((\text{Fig. 2b})\). Furthermore, increased \((\sim 2\text{-fold})\) level of LX\textalpha{}\textsubscript{4} was found in sub-acute injury compared to normal and chronic injured tendons \((P<0.05; P<0.01\text{ respectively})\) \((\text{Fig. 2c})\), although no correlation was seen between tendon LX\textalpha{}\textsubscript{4} levels and age within each group. The relationship between PGE\textsubscript{2} levels with age in normal and injured tendons was also assessed in these samples. In normal tendons, there was a significant negative correlation between PGE\textsubscript{2} levels and horse age \((P<0.05, r^2 = 0.31)\) \((\text{Fig. 3a})\). In contrast, with injury there was a significant positive correlation between PGE\textsubscript{2} levels and increasing horse age \((P<0.05, r^2 = 0.3)\) \((\text{Fig. 3b})\), although when separated for injury stage, neither sub-acute nor chronic injuries were significant in isolation.

Regulation of Tendon PGE\textsubscript{2} Metabolism

As PGF\textsubscript{2}\textalpha{} levels were lower than PGE\textsubscript{2} and did not differ with injury stage, further analyses were focused towards PGE\textsubscript{2}. To assess whether the measured differences in PGE\textsubscript{2} were attributable to altered prostaglandin metabolism, we analysed gene expression of the key enzymes responsible for PGE\textsubscript{2} synthesis \((\text{COX-2, mPGES-1)}\) and degradation \((\text{PGDH})\) based on their roles in prostaglandin metabolism. Normalized mPGES-1 and PGDH expression did not change significantly between normal and injured tendons \((\text{data not shown})\). To assess the balance between PGE\textsubscript{2} synthesis and degradation, we analysed the ratio of the two key enzymes involved in PGE\textsubscript{2} metabolism mPGES-1 and PGDH at the different stages of injury. This comparison revealed a \(\sim 3\)-fold increase of mPGES-1:PGDH in sub-acute injury compared to normals \((P<0.001, r^2 = 0.77)\) \((\text{Fig. 6})\). Interestingly, its expression was lowest in chronic injuries which mostly occurred in the older animals. To test the hypothesis whether the predominance of chronic injuries with age was related to a diminished ability of tendons to resolve inflammation, FPR2/ALX expression was determined in explant cultures of normal tendons stimulated with 5 ng/ml IL-1\beta. FPR2/ALX expression could be upregulated by IL-1\beta in tendons derived from young horses \((<10\text{ years old})\) but its expression was significantly reduced in explants derived from horse’s \(\geq 10\text{ years of age (mean } \sim 10\text{-fold reduction; } P=0.01)\) \((\text{Fig. 7a})\). In contrast, FPR2/ALX expression was not detectable in the corresponding non-stimulated controls \((\text{Fig 7b})\). There was no correlation between media LX\textalpha{}\textsubscript{4} levels and age from tendon explants stimulated with IL-1\beta \((\text{data not shown})\).

LXA\textsubscript{4} Levels in Media after Combined Stimulation with IL-1\beta and PGE\textsubscript{2}

Stimulation of tendon explants with either IL-1\beta or a combination of IL-1\beta and PGE\textsubscript{2} enhanced LX\textalpha{}\textsubscript{4} release in media after 24 hours compared to non-stimulated controls \((P=0.005)\). Combined stimulation with IL-1\beta and 1.0 \(\mu\text{M PGE}\textsubscript{2}\

Figure 2. Levels of prostaglandins and lipoxin A\textsubscript{4} in extracts of normal and injured tendons. (A) Mean PGE\textsubscript{2} and (B) mean PGF\textsubscript{2}\textalpha{} levels in normal \((n=19)\); sub-acute \((3-6\text{ weeks post injury, } n = 6)\) and chronic injured \((>3\text{ months post injury, } n = 9)\) equine superficial digital flexor tendons. PGE\textsubscript{2} levels are significantly reduced in sub-acute injury compared to normal and chronic injuries. In contrast PGF\textsubscript{2}\textalpha{} levels are 3 fold lower than PGE\textsubscript{2} and do not change with injury. (C) Mean LX\textalpha{}\textsubscript{4} levels in normal \((n=8)\); sub-acute \((n = 7)\) and chronic \((n = 6)\) injured tendons, showing significantly increased levels in sub-acute injury compared to normal and chronic injuries. Error bars denote standard deviation. * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\).

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Prostaglandins and Lipoxins in Tendinopathy
induced higher levels of LXA₄ release compared to 0.01 μM PGE₂ and IL-1β (Fig. 8, \( P = 0.32 \)).

**Discussion**

Prostaglandins such as PGE₂ are produced by tenocytes and other fibroblasts in response to injury and after stimulation with pro-inflammatory cytokines [21,22], initiating MMP mediated catabolism of tendon ECM [40]. Although seemingly destructive to the local tissue architecture, this process facilitates clearance of cellular debris and debridement of the affected ECM as described for wound healing in other connective tissues. Prostaglandins may also exert beneficial regulatory actions in healthy tissues maintaining normal physiologic processes such as local bone remodelling [41] and modification of renal blood flow [42]. Furthermore, their presence following injury signals the onset of lipoxin mediated resolution processes, such that the duration and magnitude of the inflammatory response can be regulated to restrict the degree of tissue damage [33]. Thus prostaglandins can be said to possess ‘double-edged sword’ properties in terms of their dichotomous roles in wound healing processes. The extent to which these properties play a role in tendinopathies with injury and repair stage remains unclear.

In the current study, PGF₂α levels were unchanged with injury and were substantially lower than PGE₂ levels in normal tendons. This may imply differential regulation of these prostaglandins in tendon, with PGF₂α less susceptible to changes with injury suggesting PGE₂ is the main prostaglandin operative in tendon injury. PGE₂ levels were found to decrease with aging in normal tendons. This could be a consequence of the reduction in tendon cellularity with increasing age [43,44] leading to a decreasing tendon prostaglandin synthetic capacity. Alternatively, it may be related to a lack of PGE₂ synthesising pro-inflammatory macrophages as we have described previously for uninjured tendons [16]. The relationship between age and the pattern of PGE₂ levels was difficult to determine in injured tendons because of the confounding issue that sub-acute injury predominated in younger horses compared to chronic injury, which occurred with greater frequency in older individuals. However, the positive correlation between increasing tendon PGE₂ levels with age in injured horses could be attributable to a greater PGE₂ synthetic capacity both by increased tendon fibroblast cellularity and infiltration of pro-inflammatory macrophages into injured regions of tendon [16]. This was supported by the increase in mPGES-1:PGDH ratio in sub-acute injury which suggests an interplay between PGE₂ synthesis and degradation could lead to an increased synthetic
Regulation of prostaglandin metabolism is not well documented for normal and pathologic tendons, although the majority of circulating prostaglandins are degraded in the pulmonary vasculature via PGDH [32]. However, tissue levels of PGE2 are fine-tuned by locally produced PGDH [46] and the net balance between synthesis and degradation may be a mechanism for controlling the action of PGE2 at this earlier phase of injury. However as macrophages are known to release PGE2 and tendon injury has been shown to be associated with activation and recruitment of these cells [16], they represent an important source of PGE2 during tendon injury.

PGDH is a cytoplasmic enzyme whose replacement is dependent upon de novo protein synthesis at the level of translation rather than that of transcription, due to the prolonged half life of PGDH mRNA [47]. Thus, PGDH mRNA is present in low abundance as a stable moiety, presumably as a mechanism for rapid and precise control of enzyme activity. Although these data suggest PGE2 levels should be increased due to elevated mPGES-1 mRNA, the observed reduction in PGE2 levels in sub-acute injury compared to normal and chronic injuries could be explained by the increased PGDH protein levels in sub-acute injury. This suggests a secondary (cellular) clearance mechanism for PGE2 degradation whereby local PGE2 levels can be regulated [46]. The increased vascularity of tendon that occurs after recent injury may also be a contributing factor to the paradoxical lower levels of PGE2 after injury [48], as the increased vascular perfusion is likely to facilitate efficient systemic prostaglandin clearance. In addition to vascular clearance, the lower levels of PGE2 in sub-acute tendon injury could also be attributable to lipid substrate re-routing towards the resolving pathways that are activated during inflammation. These recently discovered pathways demonstrate critical roles in the switching of lipid mediators from the prostaglandin to the lipoxin axis, returning injured tissues to their previous state [49] by depleting PGE2 levels due to reduced arachidonic acid substrate availability. Indeed, the current study shows significantly increased LXA4 levels in sub-acute injury compared to normal and chronic injured tendons, suggesting pro-resolving processes are active during the early stage of tendon injury. The alterations in the profile of lipid mediators during this time include low PGE2 and elevated LXA4 levels compared to normal and chronic injuries and suggest lipid mediator class switching is active in the early phase of tendon injury. We propose this class switching represents an endogenous protective mechanism to limit the degree of damage to tendon ECM and preserve tissue integrity. This concept is supported by the findings from this study, demonstrating combined stimulation of normal tendon explants with 5 nM 1-IL-1β and 0.01 μM or 1.0 μM PGE2 induced LXA4 release, with greater production with the higher dose of PGE2. It has been previously shown in an identical experimental system that addition of 1.0 μM PGE2 to normal tendon explants induced maximal LXA4 release after 72 hours in tissue culture [16]. These observations suggest that PGE2 may exert anti-catabolic effects on capacity in the tissue. Furthermore, activated macrophages from aged humans and mice are reported to produce more PGE2 than macrophages from younger individuals [45] which may contribute to the greater frequency of tendon injury in older individuals through sustained activation of proteolytic action on the ECM.

Figure 5. Representative Western blots illustrating expression of PGDH and β-actin in normal, sub-acute and chronic SDFT extracts. Monomeric (30 kDa) and dimeric (60 kDa) bands are shown for PGDH and a 42 kDa band for β-actin. Samples were loaded on a volume basis and the ratio of PGDH normalised to β-actin was calculated for each sample using band densitometric analysis. Graph shows densitometric analysis of western blots for PGDH in protein extracts prepared from normal (n = 7) sub-acute (n = 5) and chronic injured SDFTs (n = 8). The densitometric values were normalized to levels of β-actin expressed in each sample. There was a significant increase in PGDH in sub-acute injured tendon extracts compared to normals but this was not significantly different in the chronic injury group. *P < 0.05, **P < 0.01. Mean values are shown, error bars denote standard deviation. doi:10.1371/journal.pone.0048978.g005

Figure 6. FPR2/ALX protein expression in natural tendon injury. The relationship between FPR2/ALX levels with age is shown in injured flexor tendons (n = 10). Horse age ranged between 4 and 16 years (mean 11 ± 4 years). FPR2/ALX expression was significantly reduced with increasing age (P = 0.0008, r² = 0.77). Overlapping points are present for tendons derived from more than one 15 and 16 year old horses. doi:10.1371/journal.pone.0048978.g006
tendon ECM via the induction of pro-resolving LXA4 and switching of lipid mediators from the prostaglandin to the lipoxin axis. Furthermore, in the setting of a pro-inflammatory environment, the presence of higher levels of PGE2 may exert an autoregulatory feedback effect on IL-1 activity in order to modulate the inflammatory reaction [50]. Although the cell types responsible for lipid mediator class switching have not been identified in inflamed tendons, we hypothesise that the interaction between resident tendon cells and infiltrating pro-inflammatory macrophages during early stage injury initiates activation of pro-resolving processes. LXA4 levels were reduced during the chronic injury phase where the tendon does not return to normal structure and function. As LXA4 is a key determinant of pro-resolving processes [51] it is therefore plausible that incomplete resolution sustains a low level of inflammation, perpetuating chronic disease. Although the present study did not measure the multiple enzymes that synthesise the components of prostaglandin and lipoxin pathways, it is hypothesised that control of class switching involves the regulation of some of these enzymes.

The lipoxin A4 receptor FPR2/ALX is reported to have a pivotal role in controlling the duration and magnitude of the inflammatory response, providing endogenous stop signals for inflammation [33,34]. Despite the anticipated importance of specialised pro-resolving mediators such as LXA4 in healing, these resolving pathways are not widely studied in injured tendons. We recently identified significantly increased expression of FPR2/ALX by tenocytes in early equine tendon injury [16] and studies in other inflamed connective tissues have emphasised the importance of resolution processes for regulating inflammation, including inhibition of leukocyte recruitment and modification of vascular permeability [33]. The current study provides novel data illustrating levels of FPR2/ALX are markedly diminished in the tendons of aged injured individuals. Because these mediators are essential for controlling the inflammatory cascade, this suggests an age-related deterioration of tendons to mount a counter-response to inflammation via FPR2/ALX. A component of immunosenescence is ‘inflamm-aging’ whereby aged individuals exhibit diminished ability to modulate inflammation [37,52]. Studies in humans and rodents report an age related decline in cutaneous wound repair [45,53], suggesting age contributes to dysregulated tissue repair. Our data of reduced FPR2/ALX in tendon explants from horses aged 10 years and above showed a diminished capacity to express FPR2/ALX compared to individuals less than 10 years of age. Interestingly, tenodermal cells from older horses also had a reduced response to IL-1β induced PGE2 production compared to younger horses.

**Figure 7. FPR2/ALX protein expression in tendon explants in vitro.** (A) FPR2/ALX protein expression is shown for IL-1β stimulated macroscopically normal tendon explants derived from horses < 10 years of age (n = 5) or ≥ 10 years of age (n = 8). There was significantly greater FPR2/ALX expression by tenocytes in IL-1β stimulated explants from horses less than 10 years of age compared to older horses (P = 0.01). Data represent average FPR2/ALX expression whereby 2 replicates were analysed per horse and median values are shown. (B) Panel of representative 2-dimensional confocal images illustrating FPR2/ALX expression in tendon explants from a 4 year old horse showing non-stimulated control (left) compared to stimulation with 5 ng/ml IL-1β (right). FPR2/ALX expression was not detectable in non-stimulated controls. Immunopositive staining is green, with Hoechst nuclear counter stain in blue. Scale bar = 25 μm.

**Figure 8. Mean LXA4 levels 24 hours after stimulation with pro-inflammatory mediators.** Explants were derived from macroscopically normal tendons from 3 horses aged between 9–14 years of age and stimulated with 5 ng/ml IL-1β or combined stimulation with low (0.01 μM) or high (1.0 μM) doses of PGE2 with 5 ng/ml IL-1β compared to non-stimulated controls. LXA4 release was increased in all stimulated samples compared to respective controls (P = 0.005). Treatment with IL-1β induced greater LXA4 production compared to controls (P = 0.011). Combined stimulation with high dose PGE2 enhanced LXA4 release compared to low dose PGE2 (P = 0.032). Error bars represent standard deviation. * P < 0.05.

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young horses (Fig. S1). Taken together these data suggest aged individuals are less capable of mounting a protective response to tendon inflammation by this mechanism compared to younger individuals. Immunosenescence may therefore represent a potential mechanism for tendon re-injury via this pathway and in the development of chronic injury and explain, at least in part, the high frequency of tendon injuries sustained by aged individuals [17,18]. We hypothesise that "inflamm-aging" is an important component in the complex aetiology of tendinopathy, to which additional contributing factors such as load-induced proteolytic matrix disruption [9] and lower tendon metabolic activity with age [54] are superimposed. Furthermore, as we did not observe any relationship between LXA4 levels with age in samples of natural disease or in vitro, it appears that it is the ability to generate the FPR2/ALX receptor that is impeded by age and not synthesis of the LXA4 ligand, diminishing the ability to mediate downstream processes culminating in a deficit to resolve tendon inflammation. From the findings in the present study, we conclude that PGE2 is implicated in the development of tendon inflammation and its ensuing resolution. Temporal analysis suggests class switching of lipid mediators occurs in early stage injury, driving synthesis of LXA4. However, ageing individuals exhibit a reduced capacity to resolve tendon inflammation via the FPR2/ALX receptor, which may present a mechanism for the development of chronic tendinopathy. Improved understanding of injury pathogenesis throughout the phases of tendon repair will facilitate identification of novel therapeutic targets to modulate or prevent disease progression, such as the use of FPR2/ALX agonists.

Materials and Methods

Ethics Statement

Ethical approval for the collection of post mortem equine tendons from an abattoir or local equine veterinary referral hospital for this study was sought and approved from the Ethics and Welfare Committee at the Royal Veterinary College (URN 2011 1117).

Classification of Injury Stage

Equine forelimbs from Thoroughbred or Thoroughbred cross breed horses aged between 2–20 years were obtained from an abattoir or local equine referral hospital with known history of injury and the tensile region of the SDFT harvested within 4 hours of death. Tendons were grouped as sub-acute injured (3–6 weeks post injury, n = 6, mean age 9 ± 5 years) or chronically injured (>3 months post injury, n = 9, mean age 13 ± 4 years), as shown before [16]. Tendon injuries were aged based on historical information obtained from either the owner or referring veterinary surgeon prior to euthanasia of the horse. Tendons were classified as normal based on their macroscopic post mortem appearance which included lack of visible signs of swelling of the tendon body and a consistent pattern of fascicles on transverse sections (n = 19, mean age 8 ± 3 years). The typical microscopic appearance of normal, sub-acute and chronic injured tendons are shown in Fig. 1.

Determination of Prostaglandin Levels in Tendons

After harvest, samples were stored at −80°C and assayed within 6 months. Tendon extracts were prepared as described by Zhang and Wang [53]. Briefly, approximately 1 g of the central portion of the tensile region of the SDFT was cut into 5 mm³ cubes. Tissue was placed in 5 ml of homogenization buffer (0.1 M Phosphate Buffered Saline (PBS) (PAA, UK) pH 7.4, containing 1 mM EDTA and 10 μM Indomethacin) and homogenized using a dismembranator (Retsch MM2000, Germany). Acetone (2 ml) was added and the sample vortexed and allowed to stand at room temperature for 5 minutes. Tissue was pelleted by centrifugation at 16,000×g and the supernatant harvested. Acetone was removed by evaporation at room temperature for 10 hours and the residual supernatant used for determination of PGE2 and PGF2α measurement via radioimmunoassay as described by Cheng et al. [56].

Measurement of Lipoxin A4 in Tendon Extracts and Tissue Culture Media

LXA4 levels were determined as an indicator of resolution of inflammation in the supernatant prepared earlier to determine prostaglandin levels in extracts of normal (n = 8), sub-acute (n = 7) and chronic injured SDFTs (n = 6) and in samples of tissue culture media from cytokine stimulated tendon explants as described later. LXA4 was separated from the supernatant used to determine prostaglandin levels by passage through Bond Elut C18 columns (Agilent Technologies, USA) followed by elution with methyl formate. Samples were evaporated to dryness in a stream of nitrogen and the resulting residues used to determine LXA4 levels by ELISA (Neogen Corp, USA) according to the manufacturer’s instructions. LXA4 levels in culture media samples [see section on tendon explant culture below] were determined in an identical manner. The ELISA kit is specific for LXA4 showing minimal cross-reactivity [LXA4 100%, Lipoxin B4 1.0%, 15-hydroxyeicosatetraenoic acid (HETE) 0.1%, 5-HETE <0.1% and 12 HETE <0.1% as determined by the supplier].

Quantitative RT-PCR Analysis of PGE2 Synthesis and Degradation Enzymes

RNA was extracted from 200 mg of tendon tissue (normal n = 6, sub-acute, n = 8 and chronic injury n = 6) as described by Young et al. using the RNeasy kit (Qiagen, UK) [44]. RNA (22 μl) was used for cDNA synthesis using random primers (Promega, UK) and Superscript II reverse transcriptase (Invitrogen, UK) as described by Young et al. Gene specific primers (Table 1) were used to prepare amplicons which were cloned into a pGEM®-T Easy Vector (Promega) and plasmid DNA was used to prepare standard curves as described previously for subsequent absolute copy number evaluation [57]. We investigated expression levels of COX-2, mPGES-1 (inducible terminal enzyme in PGE2 synthesis), PGDH and the PGE2 receptor EP1 which is reported to be implicated in the pathogenesis of tendinopathy [31]. Expression levels of GAPDH and 18S ribosomal RNA were used for normalization. Equine oligonucleotide sequences used for quantitative real-time PCR are shown in Table 1. For each gene of interest and housekeeping gene, 1 μg of cDNA and 1 μM of each forward and reverse primer were used per reaction (conducted in triplicate) and amplified using SYBR® Green JumpStartTM Taq ReadyMixTM (Sigma-Aldrich, UK) for quantitative PCR using an Opticon II DNA engine thermocycler (MJ Research, UK). Standard curves ranged from 1 × 10³ to 1 × 10⁷ copies (GAPDH) or 1 × 10³ to 1 × 10⁸ copies (18 S) such that absolute copy number could be calculated according to cycle threshold. PCR efficiency was tested in each experiment and confirmed to be approximately 2.0, indicating 100% amplification efficiency according to a previously described mathematical model [38].

Western Blot Analysis for mPGES-1 and PGDH

To assess if mRNA changes in PGE2 metabolism were reflected at the protein level, Western blotting was performed to assess mPGES-1 and PGDH protein expression in tendon extracts. Protein extracts of normal (n = 7), sub-acute (n = 5) and chronic...
injured tendons (n = 8) were prepared by extraction of 100 mg of finely diced tendon in 15 volumes of 4 M Guanidine hydrochloride with protease inhibitor cocktail III (Merck, Calbiochem®, UK) for 48 hours at room temperature. Insoluble material was separated by centrifugation (13,000 g, 20 mins) and proteins precipitated by the addition of 9 volumes (v/v) ethanol buffer (ethanol with 50 mM sodium acetate) and chilling to −80°C for 2 hours. Pellets were washed twice with ethanol buffer and dried pellets resuspended in Laemmli sample buffer (Bio-Rad, UK). Samples were reduced by addition of DTT to 0.1 M and heated to 75°C for 20 minutes [59], washed in PBS-T and mounted under 0.1% Sudan Black B (BDH, Poole, UK) in 70% alcohol. Protein pellets were cut on a cryostat (Bright, UK), mounted onto poly-L-lysine coated slides (VWR, UK) and allowed to dry for 2 hours at room temperature prior to immunofluorescent analysis as described below.

To investigate the influence of horse age on the ability of tendon cells to resolve inflammation via this mechanism, we assessed the effect of IL-1β on FPR2/ALX expression in an explant culture model. Explant tissues from normal tendons were grouped according to horse age as <10 (n = 5) or ≥10 years of age (n = 3). 0.4 cm³ explants (300 mg ± 30 mg) were incubated at 37°C and 5% CO₂ under humidified atmosphere in 3 ml of Dulbecco modified Eagle's medium (PAA, UK) containing 1% penicillin and streptomycin without foetal calf serum. Samples were stimulated with 5 ng/ml human recombinant IL-1β (Merck, Calbiochem®, UK) and non-stimulated (vehicle only) samples served as controls. At 72 hours after stimulation, explant tissues were embedded in OCT and snap frozen in chilled (−80°C) n-hexane, and cryosections cut as described above.

Subsequently, consecutive cryosections were blocked in 5% normal goat serum (Sigma-Aldrich) in PBS for 1 hour in a humid chamber and probed with a 1:100 diluted mouse monoclonal antibody to FPR2/ALX (Lipoxin A4 receptor, IgG1, AbCam, UK) and secondary goat anti-mouse IgG1 (Southern Biotech, USA) each for 2 hours at room temperature. To visualise nuclei, slides were incubated with 0.5 µmol−1 Hoechst 33342 (Invitrogen, UK) for 20 minutes and washed in PBS Tween-20® (PBS-T). To quench the background fluorescence, slides were incubated with 0.1% Sudan Black B (BDH, Poole, UK) in 70% ethanol for 20 minutes [59], washed in PBS-T and mounted under coverslips using a solution of 80% glycerol in 0.5 mM Tris buffer (pH 7.2). Slides were stored at 4°C in the dark until image acquisition. Cryosections of equine spleen were used as positive control tissue to validate suitability and dilution of antibodies for use on tendon sections. Negative controls consisted of spleen cryosections incubated with murine isotype matched primary control antibodies (Southern Biotech, USA) as previously reported [16]. To assess FPR2/ALX expression, images were recorded using a Leica SP5 confocal microscope (Leica Microsystems, UK) as reported elsewhere [16].

### Measurement of LXA₄ in Media from Explants Stimulated with IL-1β and PGE₂

Our previous work has shown that stimulation of normal tendon explants with 5 ng/ml IL-1β or 1.0 µM PGE₂ in vitro induced production of the pro-resolving lipid LXA₄, which binds to...
In the current study, LXA₄ levels in media were measured to ascertain if concurrent stimulation of macroscopically normal tendon explants with IL-1β (5 ng/ml⁻¹) and low (0.01 μM) or high dose (1.0 μM) PGE₂ induced a dose-dependent increase in LXA₄ levels. Tendon explants were derived from 3 horses aged between 9 and 14 years and LXA₄ levels determined 24 hours after stimulation with the combination of pro-inflammatory mediators.

### Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Normality was tested using a Kolmogorov-Smirnov test. One-way ANOVA with Tukey’s multiple comparison tests were performed to determine differences in PGE₂, LXA₄ and the ratio of PGDH to β-actin protein between normal, sub-acute and chronic injured tendons. Kruskal-Wallis tests were performed to compare gene expression of mPGES-1, PGDH, COX-2 and the EP₄ receptor normalized to housekeeping genes in normal, sub-acute and chronic injured tendons. A Mann Whitney test was used to detect differences in FPR2/ALX stimulated tendon explants in vitro from horses <10 or ≥ 10 years of age. Relationships between horse age and PGE₂ levels or FPR2/ALX expression in normal and injured tendons were assessed by linear correlation analysis. A linear mixed model using SPSS PASW Statistics 18 (SPSS Inc Illinois, USA) was used to analyse LXA₄ release from tendon explants stimulated with pro-inflammatory mediators to account for effects of horse and experimental condition. In all cases, the P value was considered significant if below 0.05.

### Supporting Information

#### Figure S1 Prostaglandin E₂ (PGE₂) production by tendon derived cells stimulated with IL-1β (5 ng/ml⁻¹) in vitro.

Tendon cells derived from 8 year old horses (n=3) had a reduced response to IL-1β induced PGE₂ production compared to 3 year old horses (n=3). Median values were shown with maximum and minimum range. (TIF)

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### Author Contributions

Conceived and designed the experiments: SGD JD DREA RKWS. Performed the experiments: SGD. Analyzed the data: SGD JD NJW RKKS. Contributed reagents/materials/analysis tools: SGD JD RKWS. Wrote the paper: SGD JD NJW DW DREA RKWS.

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