Hypoxia inhibits primary cilia formation and reduces cell-mediated contraction in stress-deprived rat tail tendon fascicles

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Summary

Background: **Hypoxia, which is associated with chronic tendinopathy, has recently been shown to decrease the mechanosensitivity of some cells. Therefore, the purpose of this study was to determine the effect of hypoxia on the formation of elongated primary cilia (a mechanosensing organelle of tendon cells) *in vitro* and to determine the effect of hypoxia on cell-mediated contraction of stress-deprived rat tail tendon fascicles (RTTfs).**

Methods: **Tendon cells isolated from RTTfs were cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions for 24 hours. The cells were then stained for tubulin and the number of cells with elongated cilia counted. RTTfs from 1-month-old male Sprague-Dawley rats were also cultured under hypoxic and normoxic conditions for three days and tendon length measured daily.**

Results: **A significant (p=0.002) decrease in the percent of elongated cilia was found in cells maintained in hypoxic conditions (54.1%±12.2) when compared in normoxic conditions (71.7%±6.32). RTTfs in hypoxia showed a significant decrease in the amount of contraction compared to RTTfs in normoxia after two (p=0.007) and three (p=0.001) days.**

Conclusion**: The decreased incidence of elongated primary cilia in a hypoxic environment, as well as the decreased mechanoresponsiveness of tendon cells under these conditions may relate to the inability of some cases of chronic tendinopathy to respond to strain-based rehabilitation modalities (i.e. eccentric loading).**

KEY WORDS: cilia, contraction, hypoxia, mechanotransduction, tendinopathy.

Introduction

Various phases of hypoxic alterations of tenocytes have been found in ruptured tendons with degenerative tendinopathy[1]. Hypoxia-induced cell damage has been implicated as a potential mechanism in the progression of chronic tendinopathy[2-4] with higher levels of hypoxic degeneration indicative of non-reparative, end stage pathology[5]. Studies using human tenocytes have also demonstrated that a hypoxic environment, depending on the magnitude and duration of exposure, is capable of disturbing the balance between reparative and degenerative changes in the extracellular matrix[2, 6].

A critical mediator of the hypoxic response is the transcription factor hypoxic inducible factor 1α (HIF-1α)[7]. HIF-1α has been shown to negatively regulate skeletal mechanotransduction by decreasing the sensitivity of bone cells to mechanical signals[8]. The inability of some tendinopathy patients to respond to therapies designed to stimulate a mechanotransduction response (i.e. eccentric loading) may reflect a decrease in the mechanosensitivity of tendon cells secondary to a hypoxic environment[9].

Primary cilia are mechanosensitive organelles that can detect mechanical environmental changes[10] and are found in musculoskeletal tissue cell types, including tenocytes, osteocytes, and chondrocytes[10-14]. Passive cilia bending is required for mechanosensation of mechanical perturbations, with elongated cilia more sensitive to loading than shorter cilia[15]. A previous study on mesenchymal stem cells demonstrated a time dependent loss of elongated primary cilia in hypoxic conditions[7]. It is possible that tendon cells may also experience a hypoxia-induced loss of elongated cilia and subsequent loss of mechanosensitivity.

A hypoxic environment within tendons has been suggested to occur through mechanically induced collagen damage[16]. When collagen fibrils are damaged, or become lax, and lose the ability to bear load the tendon cells associated with the damaged collagen fibrils lose their cellular homeostatic tension[17,18]. The loss of cellular tensional homeostasis induces catabolic

processes associated with tendinopathy[17] and cilia elongation[19,20]. However, a cellular based contraction mechanism has been shown to recover tendon laxity and re-establish the cytoskeletal tensional homeostasis of these tendon cells[20]. This, in turn, allowed the tendon cells to recalibrate their catabolic gene expression and protein synthesis to its previous normal levels[20].

A reduction in the mechanosensitivity of tendon cells exposed to hypoxic conditions could lead to a diminished cell-based contraction response of tendons and return to normal tensional homeostasis. Therefore, the purpose of the current study was to determine the effect of hypoxia on the formation of primary cilia, a mechanosensing organelle of tendon cells, *in vitro* and to determine the effect of hypoxia on cell-mediated contraction of stress-deprived rat tail tendon fascicles (RTTfs). We hypothesize that hypoxia will decrease the number of tendon cells expressing elongated primary cilia *in vitro*. In addition, we hypothesize that hypoxia will decrease the normal cell-induced tendon contraction that occurs with the loss of cytoskeletal tension.

Materials and methods

Institutional animal care and use approval was obtained prior to this study. This study was conducted ethically and in accordance with the international standards described by Padulo et al. in 2016[21].

The effect of hypoxia on elongated cilia formation

To determine the impact of hypoxia on the presence of elongated cilia, tendon cells were isolated from RTTfs of adult male Sprague-Dawley rats and cultured to the 3rd-4th passage. Each well of a 6-well tissue culture plate contained one cover glass on which tendon cells were cultured (37° F, 10% CO_2) in supplemented Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) as previously described[22] in the presence of normoxia (21% O_2, 69% N_2) or hypoxia (1% O_2, 89% N_2) for 24 hours (40,000 cells/well; n=7 plates/condition). After 24 hours, the media was removed and new media containing both Tubulin Tracker™ Green (250 nM), a cellular tubulin stain and Hoechst 33342 (5μg/ml), a nuclear stain (Thermo Fisher Scientific) was added to each well. The plates were incubated in the dark at 37° C, in their respective environmental oxygen conditions for 15 minutes. After incubation, the stain was removed and each cover-glass was rinsed twice with DMEM. Each cover-glass was mounted using ProLong® Gold (Thermo Fisher Scientific) and the cells were visualized using a Zeiss Axioplan2 microscope at 63x magnification. The presence or absence of elongated cilia were counted microscopically on 200 cells for each cover-glass for a total of 1200 cells per 6-well plate and 8400 cells per condition. The percent of cells with elongated cilia present on each cover-glass were averaged per plate. To determine if a significant difference in the presence of elongated cilia occurred between hypoxia and normoxia a paired t-test was performed with significance $p \leq 0.05$. All results are shown as mean ± standard deviation.

The effect of hypoxia on cell-based tendon contraction

To investigate the effect of hypoxia on cell-mediated tendon contraction RTTfs were removed from the tails of euthanized 1-month-old male Sprague-Dawley rats (n=6) and suspended vertically inside 15 ml conical centrifuge tubes containing supplemented DMEM[20]. RTTfs were cultured under either normoxic (21% oxygen) or hypoxic (1% oxygen) conditions for a total of 20 RTTfs/condition/rat for three days. Each RTTf was photographed daily to document length changes.

To determine if the low oxygen conditions were causing irreversible cell changes (i.e. cell death), following three days of hypoxia exposure, 10 of the RTTfs in hypoxia were moved into normoxia for an additional three days. On day six all RTTfs were photographed to document length changes due to the change in environmental oxygen conditions. RTTf contraction lengths were measured from calibrated photographs using Image-J software[23]. Measurements were standardized to a fixed scale present in each photo to account for any magnification effects. Tendon length was expressed as a percentage of day 0 length and results from differing environmental oxygen conditions were compared using multiple paired t-tests with a Bonferroni correction.

Results

The effect of hypoxia on elongated cilia formation

A significant ($p=0.002$) average decrease of 18.2%±9.34 was found in the number of elongated cilia present in those cells maintained in hypoxic conditions (54.1%±12.2) compared to cells in normoxic conditions (71.7% ± 6.32) (Fig. 1).

The effect of hypoxia on cell-based tendon contraction

After 24 hours of incubation, RTTfs maintained under normoxic or hypoxic conditions demonstrated a similar ($p=0.084$), albeit small (8%±2 normoxia, 7%±2 hypoxia) decrease in their length. By day two, RTTfs under normoxic conditions showed a significant increase in the amount of contraction when compared to RTTfs under hypoxic conditions (85%±5 normoxia, 91%±2 hypoxia, $p=0.007$) (Figs. 2, 3). After three days, RTTfs under normoxic conditions were significantly shorter than RTTfs cultured under hypoxic conditions (56%±13 normoxia, 85%±5 hypoxia, $p=0.001$). Transferring RTTfs to normoxic conditions after three days of exposure to hypoxia resulted in significantly more contraction at day six than those RTTfs that re-

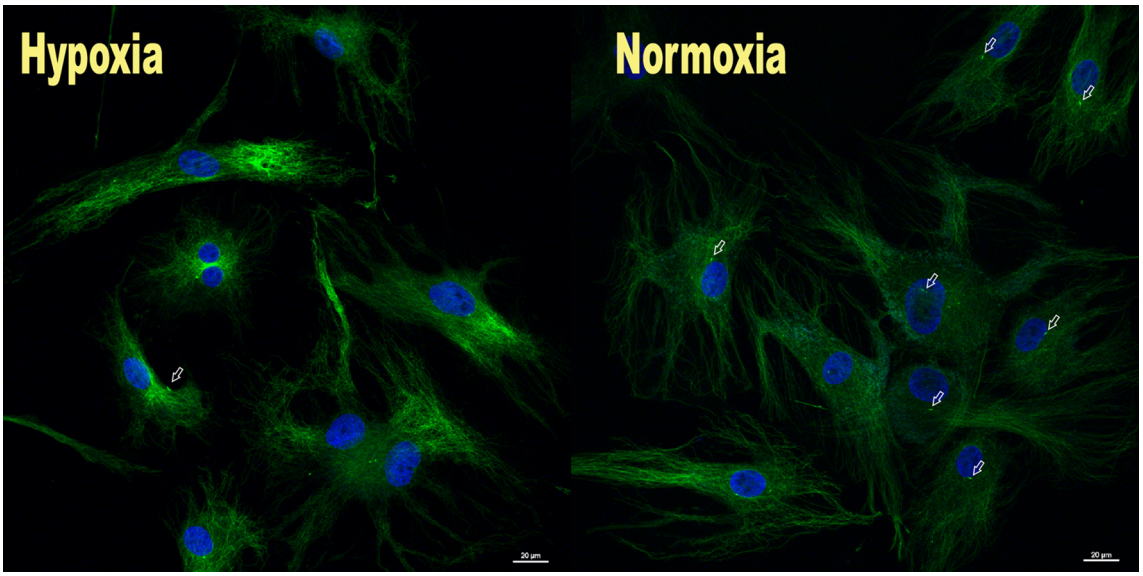

Figure 1. Photomicrographs showing the number of elongated cilia (white arrows) significantly decreased in cells maintained in hypoxia (left) compared to cells in normoxia (right).

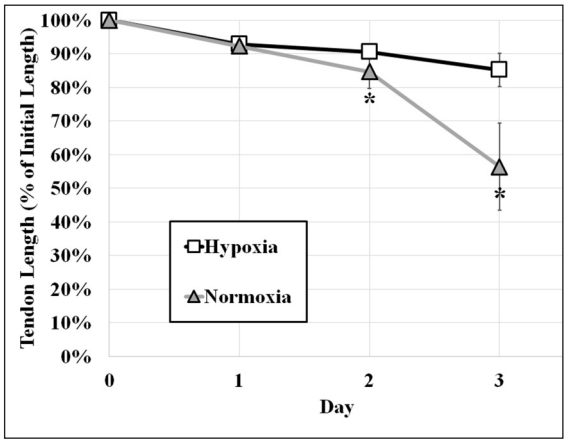

Figure 2. Graph showing tendons in hypoxia experienced a significant decrease in their amount of contraction (change in % of initial length) compared to those in normoxia at day 2 and day 3.
* Normoxia *vs* hypoxia. p<0.05

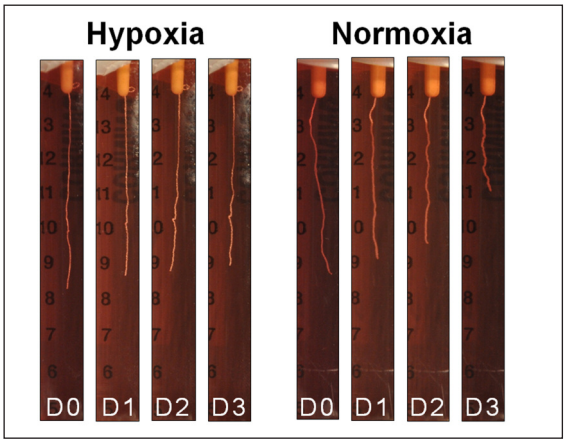

Figure 3. Photographic series showing a noticeable decrease in the contraction of tendons at days 2-3 (D2-D3) when placed in hypoxia (left) compared to normoxia (right).

mained in a hypoxic environment (12%±5 normoxia, 34%±7 hypoxia, p=0.008) (Fig. 4). This increase in contraction rate following exposure to normoxic conditions confirms that the decrease in tendon contraction seen under hypoxic conditions was not due to cell death.

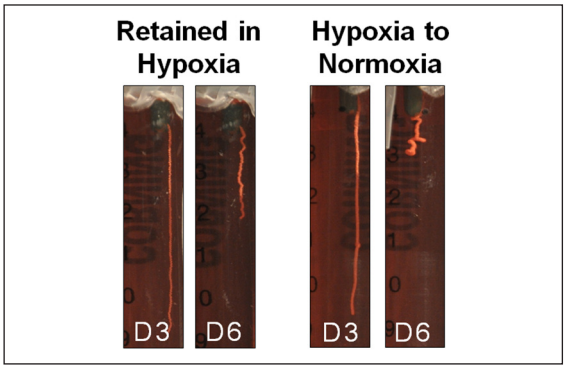

Figure 4. Tendons that were transferred from hypoxic to normoxic conditions showed a significant increase in contraction compared to tendons that were retained in hypoxia.

Discussion

In the current study, cells maintained in hypoxic conditions were found to have a significant decrease in the number of elongated cilia present when compared to cells in normoxic conditions (Fig. 1). Two recent studies have also investigated the role of hypox-

ia on cilia prevalence[7, 24]. In one study, renal epithelial cells exposed to a chemically induced hypoxic environment simulated using cobalt chloride ($CoCl_2$) resulted in an increase in the length of primary cilia, but with no change in percentage of cells with a cilium (~82%) compared to normoxia (~85%)[24]. However, this method of using $CoCl_2$ to mimic the conditions of hypoxia may not have the same cellular effect as regulating the hypoxic enviroment through controlled oxygen levels[25].

The results of the current study are similar to a previous study that also induced hypoxia through controlled oxygen levels in murine bone marrow-derived mesenchymal stem cells (MSC)[7]. A significant decrease in the number of cells with elongated cilia was observed with hypoxia (~20%) compared to normoxia (~70%)[7]. However, results from the MSC study showed a greater decrease in the percentages of cells expressing elongated cilia in hypoxic conditions (~20%) compared to the current study (54%)[7]. The reason for this discrepancy is unclear and may be due to the use of different cell types.

Cilia are believed to play an important role in maintaining tissue homeostasis[14]. The membrane of the cilia contains several cilia-specific receptors such as ion channels and signaling molecules that serve to receive signals from the environment in order to produce an appropriate response[15]. Primary cilia have been shown to transmit signals to the cytoskeleton and other cellular organelles that regulate the cells' mechanoresponse in a manner dependent on the cilia length[26]. The elongation of the primary cilia has been shown to increase the mechanosensitivity of cells to signals and has been found as a biomarker of alterations in cellular homeostasis[19,26,27]. In the current study, cells under hypoxic conditions revealed a significantly lower percentage of elongated cilia than those seen in normoxia. These results suggest that with a lower percentage of elongated cilia that are perceptive to mechanotransduction signals, a decreased cellular response to alterations in mechanical loading will occur with hypoxia.

In the present study, the freely contracting RTTFs maintained in a hypoxic environment showed a significance decrease in the amount of contraction compared to tendons cultured in normoxic conditions at both two and three days (Figs. 2, 3). Cell mediated contraction may contribute to the recovery of tendon laxity caused by injury[17], surgical manipulation[28], or repetitive exercise[29]. Alterations in the normal, residual tension of the extracellular matrix in tendons, such as laxity, disrupt the cell tensional homeostasis and have been demonstrated to result in an up regulation of catabolic gene expression and protein synthesis in tendon cells[17]. Prolonged catabolic degradation leads to degeneration of the material properties of the tendon[18]. Cell mediated contraction in tendons that are initially lax but fixed between two points has been shown to regain cellular homeostatic tension and inhibit collagenase protein synthesis[20]. Thus a hypoxic induced decrease in tendon cell-mediated contraction may delay or inhibit the ability of the tendon to regain cellular homeostasis and thus prolong the catabolic degradation that may lead to tissue degeneration.

In the current study, tendons that were exposed to hypoxia for three days and then moved to normoxia for an additional three days demonstrated significantly more contraction at day six than those that remained in hypoxia for the full six days (Fig. 4). The increase in contraction with the return to normoxic conditions suggests that this decrease in mechanoresponsiveness to hypoxia was not due to cell death, but rather to the ability of the cell to respond to its decreased oxygen environment. The ability of cells to return to a normal contraction response within three days following three days of exposure to hypoxia also suggests that in the short term the diminished cellular response to hypoxia is reversible.

As a limitation of the current study, hypoxia was viewed as an isolated event rather than a sequence of events leading up to the development of a hypoxic environment in the tissue. A potential event leading to a hypoxic environment is damage to the extracellular matrices and surrounding vasculature, consequently diminishing the supply of nutrients to the tissue[4]. The current study utilizes an *in vitro* and an *in situ* model system at different time points to analyze the role of hypoxia on cellular mechanoresponsiveness. The decrease in elongated cilia prevalence that is shown to occur *in vitro* at 24 hours is assumed to occur *in situ* and remain in effect over time. Although previous studies analyzed the prevalence and/or length of cilia *in situ*[11], the current study utilized tendon cells in monolayer to examine the effect of hypoxia on elongated cilia prevalence. This allowed for a greater numbers of cells (8400 cells/condition) to be examined than in previous *in situ* studies (90 cells/condition)[11]. Furthermore, previous research in mesenchymal stem cells demonstrated a continued decrease in the prevalence of elongated cilia after 2 days in hypoxia[7]. Therefore, although it appears from previous research that the decrease in elongated cilia prevalence with hypoxia would occur both *in situ* and over time, this requires further demonstration. The current study also focuses primarily on the alterations occurring to the cells primary cilia as a source of mechanosensation. However, other cellular changes take place in a hypoxic environment that may play a role in mechanotransduction such as a reduced cellular generation of ATP, failure of energy dependent systems within the cell such as ion pumps, depletion of glycogen stores, lowered pH of the intracellular environment, and a reduction in the synthesis of proteins[30]. Although the current study only investigated a representative mechanosensory pathway and cellular response to hypoxia, additional cellular responses may also play a role in the mechanosensation of cells under a hypoxic environment.

Primary cilia are important mechanosensing organelles in tendon cells and are thought to play a key role in maintaining tendon cell homeostasis[27]. The decreased incidence of elongated primary cilia in a hypoxic environment, as well as the decreased

mechanoresponsiveness of tendon cells under these conditions may relate to the inability of some cases of chronic tendinopathy to respond to strain-based rehabilitation modalities (i.e. eccentric loading).

Conflicts of interests

The Authors declare that they have no competing interests.

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