Fatigue Loading Of Tendon In Vivo Upregulates The Pro-Apoptotic Marker BNIP3: Expression Is Mediated By HIF-1α And Repressed By CITED2

Introduction: Growing evidence suggests that mechanical overload from repeated stress may contribute significantly to tendinopathy. Repetitive stress causes pathological changes including extracellular matrix degradation and loss of tenocytes, producing a weak tendon susceptible to tearing [1]. Yet how cell death and matrix degradation are related is not well understood. Tenocyte loss was suggested to result from apoptosis, which is common in degenerative diseases. However, experimental evidence that load-induced apoptosis leads to tendinopathy is limited. Moreover, little is known about the regulatory pathways that could lead to apoptosis in mechanically overloaded tendons. In the present study, we characterized the expression of putative regulators of tenocyte apoptosis in rat patellar tendons subjected to in vivo low-cycle (high strain) fatigue loading [2]. Specifically, we measured expression of BCL2 adenovirus E1B 19kDa interacting protein 3 (BNIP3), a pro-apoptotic protein of the BCL2 family [3], as well as Hypoxia Inducible Factor 1α (HIF-1α). HIF-1α was reported to mediate BNIP3 expression due to hypoxic stress [4]. CITED2 is a transcriptional suppressor of certain matrix-degrading proteases that we had previously shown to be upregulated by low level fatigue loading in tendon [5]. Moreover, since CITED2 was proposed to repress HIF-1α transactivation activity by competing with it for limited amounts of the cofactor p300 [6], we assessed these interrelationships and their effects on BNIP3 expression in cultured tenocytes as well.

Materials and Methods: In vivo Fatigue Loading Following IACUC-approved protocols, left patellar tendons were surgically exposed in adult female Sprague-Dawley rats and cyclically loaded to reach strain levels of 0.6%, 1.7% and 3.5% over basal, corresponding to low, moderate and severe damage loading. Sham-operated and contralateral (non-operated) tendons were used as controls. Tendon samples were collected 1 hr after loading and immediately frozen in liquid nitrogen until analysis.

Cell culture Confluent cultures of a rat tenocyte cell line we established were cultured in DMEM/10% FBS, loaded with intermittent hydrostatic pressure (IHP) from 0 to 7.5 MPa for 1h, or alternatively transfected with cDNA or siRNA. Target gene expression was assayed either by RT-PCR or immunoprecipitation followed by Western blot.

RNA isolation and RT-PCR Total RNA from tissue or cultured cells (Qiagen RNeasy), was reverse transcribed (SuperScript II, Oligo (dT) primer) and analyzed by semi-quantitative and real-time PCR.

Statistics All experiments were conducted at least three times. Results are expressed as mean ± SD Data were analyzed by one-way ANOVA with Tukey’s test for post hoc analysis. Significance was set at p < 0.05.

Results: Expression of BNIP3 and HIF-1α in normal and fatigue loaded patellar tendon Unloaded rat patellar tendons expressed low levels of BNIP3 and HIF-1α, both of which rose with loading to approximately 2-fold over basal at 7.5% strain. In contrast, CITED2 expression increased nearly 5-fold, but only at 0.6% strain; CITED2 was below basal levels at 1.7% and 3.5% strain (Fig 1). Mechanical loading upregulates BNIP3 and HIF-1α expression in vitro Consistent with in vivo findings, expression of BNIP3 and HIF-1α in cultured tenocytes were low and rose with loading from 0 to 7.5 MPa IHP (Fig 2A). Both increases were blocked by siHIF1α, indicating that BNIP3 expression was dependent on HIF-1α. Moreover, HIF1α must be able to interact with the CH1 domain of p300 in order to regulate BNIP3, since transfection of wild type p300 cDNA up-regulated BNIP3 even in the absence of loading; however, transfection p300 with its CH1 domain deleted (unable to bind HIF-1α) did not (Fig 2B).

CITED2 regulates BNIP3 via interaction with p300 Overexpression of wild type CITED2 in cultured tenocytes abolished HIF-1α-induced BNIP3 expression (Fig 3A), while decreasing CITED2 and decreasing HIF-1α present in complexes with p300 (Fig 3B). In contrast, transfection of truncated CITED2 (aa1-160), unable to bind the CH1 domain of p300 had no effect either on BNIP3 expression or on the interaction of p300 with HIF1α (Fig 3B).

Discussion: The combined in vivo and in vitro findings in this study strongly suggest that low-cycle fatigue loading of tendons in vivo activates a BNIP3-mediated apoptotic pathway, and that this BNIP3 response is dependent on the stress-induced regulator HIF-1α. The data also identify a mechanism for BNIP3 regulation by both HIF-1α and CITED2, based on competition of these factors for binding to p300.

Increased expression of both BNIP3 and HIF-1α with increased in vivo loading reflected the progressive loss of tendon mechanical properties with loading that we previously demonstrated in this model [7], and suggests a direct relationship between tenocyte apoptosis and tissue damage induced by low-cycle (high stress) fatigue [8]. Interestingly, however, CITED2 (which suppresses expression of matrix-degrading enzymes MMP-1 and MMP-13 in this system [5]) did not follow this expression pattern. Moreover, elevated CITED2 levels, while capable of suppressing BNIP3 expression in vitro, did not produce an expected drop in BNIP3 in vivo. This apparent discordance between tenocyte apoptosis (indicated by BNIP3) and tissue degradation (indicated by MMP expression) in this model of tendon damage suggests that these two processes are subject to distinct regulatory pathways, may involve different subpopulations of tenocytes and likely contribute uniquely to the development of tendinopathy.


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