Anti-inflammatory and anti-catabolic effects of TENDOACTIVE® on human tenocytes *in vitro*

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Summary. Tendons have a limited capacity for self-repair due to the low density and mitotic activity of tenocytes. Pro-inflammatory cytokines such as interleukin-1ß (IL-1ß) have been identified as the main initiators of tendinopathies, stimulating inflammation, apoptosis and extracellular matrix (ECM) degradation. The aim of this study was to evaluate the potential of Tendoactive®, a newly developed proprietary nutraceutical formulation that includes mucopolysaccharides, collagen and vitamin C, in an *in vitro* model of tendon inflammation. The effects of Tendoactive® were studied in primary cultures of human tenocytes treated with IL-1ß for up to 72 h. Expression of collagen type I, integrin ß1, cyclo-oxygenase-2 (COX-2), caspase-3 and matrix metalloproteinase-1 (MMP-1) was monitored by western blotting. The effects of Tendoactive® on the expression, phosphorylation and nuclear translocation of protein components of the NF-κB system were studied by western blotting and immunofluorescence respectively. Treatment of tenocytes with Tendoactive® suppressed IL-1ß-induced NF-κB activation and p65 nuclear translocation. These events correlated with down-regulation of NF-κB targets including COX-2, MMP-1 and activated caspase-3. Tendoactive® also reversed the IL-1ß-induced down-regulation of collagen type I and ß1-integrin receptor expression. These results indicate that Tendoactive® has nutraceutical potential as an anti-inflammatory agent for treating tendinopathy through suppression of NF-κB mediated IL-1ß catabolic signalling pathways in tenocytes.

Key words: Tendon, Tenocyte, Tendinopathy, NF-κB, IL-1ß, MMP, COX-2, Tendoactive®, Anti-inflammatory, Nutraceutical formulation

Introduction

Tendons are populated by highly specialized cells (tenocytes) embedded in a three dimensional network of extracellular matrix (ECM) consisting predominantly of longitudinally oriented collagen type I (>95%), other types of collagens (type III, V and VI), proteoglycans, elastin and fibronectin (Bernard-Beaubois et al., 1997; Scott et al., 1981). Tenocytes are responsible for the synthesis and maintenance of all tendon specific ECM-components that give tendon its biomechanical and biochemical properties and maintain its structure, the EMC also acts to regulate the differentiation of the cells (Schulze-Tanzil et al., 2004).

The interaction between cells and ECM-proteins is mediated largely by the ß1 subfamily of integrin receptors (Shakibaei et al., 1997; Shakibaei and Mobasheri, 2003). This interaction plays a crucial role in regulating several biological phenomena, including cell morphology, gene expression and cell survival. It also induces signal transduction pathways that change cell behaviour (Giancotti and Mainiero, 1994; Wary et al., 1996). In addition to their function as cell adhesion receptors, integrins play an important role as signalling receptors. Integrin-mediated adhesion can activate intracellular signalling proteins such as focal adhesion kinase (Clark and Brugge, 1995; Schaller et al., 1995) and mitogen-activated protein kinases (Chen et al., 1994; Shakibaei et al., 1999; Shakibaei et al., 2001). Furthermore, integrin-mediated adhesion regulates the organization of intracellular cytoskeleton proteins (Yamada and Miyamoto, 1995) and regulates gene

expression (Lin et al., 1994).

The production of ECM macromolecules (e.g. collagen type I) is altered in tenocytes from degenerated or injured tendons (Maffulli et al., 2000). However little is known about the signalling pathways involved in regulation of cell growth and apoptosis in tenocytes under normal conditions and in degenerative diseases of tendons (tendinopathies). However, signalling proteins in apoptotic pathways play an important role during the pathogenesis of degenerative disorders (Hashimoto et al., 1998).

Tendinopathies are a serious problem among athletes and workers (Corps et al., 2004) and lead to disability, lost productivity, reduced physical activity, and early retirement from sports or labor (Coleman et al., 2000; Rees et al., 2006). The pro-inflammatory cytokines (i.e. interleukin-1ß (IL-1ß) and tumor necrosis factor alpha (TNF-α)) are well known as mediators of degradation and apoptosis during degenerative joint diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) (Hashimoto et al., 1998; Blanco et al., 1999). Similarly, in tendon, IL-1ß and TNF-α up-regulate matrix degrading enzymes such as matrix metalloproteinases (MMPs) and pro-inflammatory mediators such as cyclooxygenase-2 (COX-2) leading to tendon matrix destruction and tendon inflammation. IL-1ß and TNF-α are also well known to activate the ubiquitous transcription factor NF-κB (Ding et al., 1998), which leads to further pro-inflammatory cytokine production and thus to further tendon degradation. However, the mechanism of tenocyte apoptosis during tendon disease has not been fully elucidated. Since several arthritis-associated catabolic signalling pathways lead to the induction of apoptosis, the inhibition of these signalling pathways in the apoptosis activated tenocytes seems to be a promising approach in tendinitis therapy.

Remodeling in tendon tissue plays an important role in responding to microtrauma from repetitive or injurious loading. This repair mechanism is mediated by resident tenocytes, which maintain a fine balance between tendon ECM production and degradation. MMPs are key regulators of ECM remodeling, and their synthesis, activation and secretion are altered during the process of tendon healing (Riley et al., 2002).

A variety of dietary phytochemicals and herbal remedies have been introduced for arthritis therapy including stinging nettle extract, catechins such as epigallocatechin, *trypterygium wilfordii* hook F extract, resveratrol and curcumin (Sylvester et al., 2001; Schulze-Tanzil et al., 2002; Singh et al., 2002; Shakibaei et al., 2007a,b). Some of these are already known to inhibit NF-κB in chondrocytes and other cell types in the synovial joint (Liacini et al., 2003; Riehemann et al., 1999; Sylvester et al., 2001). Moreover, it has been shown that extracellular matrix molecules exert anti-inflammatory effects on cells. Hyaluronan reduces free-radical formation, affords protection to the corneal endothelium, to the collagen-induced arthritis and exerts anti-inflammatory properties (Campo et al., 2003; Camillieri et al., 2004).

The purpose of this study was to investigate an *in vitro* model of tendinitis to evaluate the effects of Tendoactive® on IL-1ß signalling and determine whether Tendoactive® can suppress NF-κB-activation and NF-κB-regulated gene products.

Materials and Methods

Antibodies

Polyclonal anti-collagen type-I (AB749), monoclonal anti-ß1-integrin (MAB1977), proteoglycan (MAB2005) antibodies and alkaline phosphatase conjugated secondary sheep anti-mouse and sheep anti-rabbit antibodies for immunoblotting (AP303A, AP304A) were purchased from Chemicon international, Inc., (Temecula, CA, USA). Monoclonal anti-chondroitin sulfate (C8035) antibody was obtained from Sigma (Munich, Germany). Antibodies to ß-actin (A5316) were from Sigma (Munich, Germany). Antibodies raised against MMP-1 (MAB911) and polyclonal anti-active caspase-3 antibody were purchased from R&D Systems (Abingdon, UK). Cyclo-oxygenase-2 (160-112) antibody was obtained from Cayman Chemical (Ann Arbor, MI, USA). Antibodies to NF-κB p65 (Rel A) and phospho-specific pS529 (100-401-266) were obtained from Rockland laboratories (Biomol, Hamburg, Germany). The MTT assay was purchased from Sigma (Munich, Germany). Secondary antibodies for immunofluorescence were purchased from Dianova (Hamburg, Germany).

Growth medium and chemicals

Growth medium (Ham's F-12/Dulbecco's modified Eagle's medium (50/50) containing 10% fetal calf serum (FCS), 25 μg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin, 2.5 μg/ml amphotericin B, essential amino acids and L-glutamine) was obtained from Seromed (Munich, Germany). Trypsin/EDTA (EC 3.4.21.4) were purchased from Sigma (Munich, Germany). Epon was obtained from Plano (Marburg, Germany). IL-1ß was obtained from Strathman Biotech GmbH (Hannover, Germany).

Preparation and Formulation of Tendoactive®

Tendoactive®, a nutraceutical formulation that contains mucopolysaccharides, hydrolyzed type I collagen and vitamin C was obtained from Bioiberica S.A. (Barcelona, Spain).

Tenocyte isolation and culture

Primary human tenocytes were isolated from human tendon (finger tendon of male middle-aged donor, age

between 35-55 years) obtained during tendon-rupture surgery with full informed consent and local ethics committee approval, as described in detail before by Schulze-Tanzil et al., 2004. Briefly, after 1-2 weeks, tenocytes continuously migrated from this explant and adhered to Petri dishes (Fig. 1). These cells (passage 2) were cultured at 0.1×10^6 cells/ml in Petri dishes in monolayer culture and on glass coverslips for a period of 24 h at 37°C with 5% CO_2.

Experimental design

Human tenocyte monolayer cultures were washed three times with serum-starved medium and were incubated for 1 hour with serum-starved medium (0.5% FCS). Serum-starved human tenocytes either were left untreated, were treated with 10 ng/ml IL-1ß alone for the indicated time periods, or co-treated with 10 ng/ml IL-1ß and 250 or 500 or 1000 μg/ml Tendoactive® for the indicated time periods, or were pre-treated with 250 or 500 or 1000 μg/ml Tendoactive® for 24 hours followed by co-treatment with 10 ng/ml IL-1ß and 250 or 500 or 1000 μg/ml Tendoactive® for 24 hours or for the indicated time periods.

For investigation of NF-κB translocation tenocyte cultures were treated either with 10 ng/ml IL-1ß or co-treated with 10 ng/ml IL-1ß and 250 or 500 or 1000 μg/ml Tendoactive® for 0, 10, 15, 30, 45 and 60 min and nuclear and cytoplasmic extracts were prepared. These experiments were performed in triplicate and the results are provided as mean values from three independent experiments.

Tenocyte viability and proliferation assay

The effects of Tendoactive® on the cytotoxic effects of IL-1ß were examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay kit from Sigma) uptake method as previously described (Csaki et al., 2009). Briefly, for the cell proliferation assay, 5000 human tenocytes per well were cultured for 24 h in a 96-well-plate to allow for attachment of cells. They were pre-treated with 250, 500 or 1000 μg/ml Tendoactive® for 24 hours and then co-treated with 10 ng/ml IL-1ß or cells treated with Tendoactive® alone (positive control), or with 10 ng/ml IL-1ß alone (negative control) or medium only (control) was added and the cells were incubated for 0, 6, 12, 24, 36 and 48 hours at 37°C before being subjected to cell proliferation test. For evaluation, the medium was removed and 100 μl of fresh medium and 10 μl of MTT solution (5 mg/ml PBS, sterile) were added to each well and incubated for 4 hours at 37°C/5% CO_2. Subsequently, 100 μl of MTT solubilization solution was added and the plates incubated until the cells were bleached. The transmission signal was determined at 570 nm using a micro plate reader (Bio-Rad, Munich, Germany). A sample without cell loading was used to establish baseline values. The

assay was performed in triplicate and the results are provided as mean values with standard deviations from three independent experiments.

Transmission electron microscopy (TEM)

Tenocytes were fixed 1 h with Karnovsky fixative and post-fixed in 1% OsO_4 solution (0.1 M phosphate buffer). The cell pellets were rinsed and dehydrated in an ascending alcohol series before being embedded in Epon and cut on a Reichert-Jung Ultracut E (Germany). Ultrathin sections were contrasted with 2% uranyl acetate/lead citrate. A transmission electron microscope (TEM 10, Zeiss, Jena, Germany) was used to examine the cultures.

Electron microscopic evaluation of mitochondrial changes or apoptotic cells

Serum-starved tenocytes were exposed to 10 ng/ml IL-1ß alone for 24, 36 and 48 hours or pre-treated with Tendoactive® (250, 500 or 1000 μg/ml) for 24 hours and then co-treated with IL-1ß (10 ng/ml) for an additional 24 and 48 hours. Ultra-thin sections of the samples were prepared and evaluated with an electron microscope (TEM 10, Zeiss, Jena, Germany). For statistical analysis, the number of cells with mitochondrial changes or apoptotic cell death was determined by scoring 100 cells from 30 different microscopic fields.

Immunofluorescence microscopy

The effect of Tendoactive® on the nuclear translocation of p65 was examined by an immuno-cytochemical method as previously described in detail (Shakibaei et al., 2007a). Briefly: Tenocytes were seeded on glass coverslips and incubated for 24 h. The cells were then rinsed three times and pre-incubated for 1 h with serum-starved medium before stimulated with 10 ng/ml IL-1ß or 250 μg/ml Tendoactive® alone or co-treated with 10 ng/ml IL-1ß and 250 μg/ml Tendoactive® for 30 min in serum-starved (0.5 % FCS) medium. Glass coverslips with tenocyte monolayers were rinsed three-times in Hanks solution before methanol fixation for 10 min at ambient temperature (AT), and rinsing with PBS. Cell and nuclear membranes of tenocytes were permeabilized by treatment with 0.1 % Triton X-100 for 1 min on ice. Cells were overlaid with protease-free bovine serum albumin (BSA) for 10 min at AT, rinsed with PBS and incubated with primary antibodies (p65, phospho-p65, 1:30 in PBS) in a humid chamber overnight at 4°C. They were gently washed several times with PBS before incubation with secondary antibody (goat-anti-rabbit immunoglobulin conjugated with FITC [GAR-FITC], diluted 1:50 in PBS) for 1h at AT. Cells were finally washed three-times with PBS, covered with fluoromount mountant, and examined under a light microscope (Axiophot 100,

Zeiss, Germany).

Isolation of tenocyte nuclei

To determine the levels of protein expression in the nucleus, we prepared extracts as previously described in detail (Shakibaei et al., 2007a). Briefly: Tenocytes were trypsinized and washed twice in 1 ml ice cold PBS. The supernatant was carefully removed. Cell pellets were re-suspended in 400 μl hypotonic lysis buffer containing protease inhibitors and incubated on ice for 15 min. 12.5 μl of 10 % NP-40 were added and the cell suspension vigorously mixed for 15 seconds. The extracts were centrifuged for 1.5 min. The supernatants (cytoplasmic extracts) were frozen at -70°C. 25 μl ice cold nuclear extraction buffer was added to the pellets and incubated for 30 min with intermittent mixing. Extracts were centrifuged and the supernatant (nuclear extracts) transferred to pre-chilled tubes for storage at -70°C.

Western blot analysis

Tenocyte monolayers were washed three times with Hank's solution and whole cell proteins were extracted by incubation with lysis buffer (50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF) on ice for 30 min, and cell debris was removed by centrifugation. Supernatants were stored at -70°C until use. Total protein concentration of whole cell lysates, nuclear and cytoplasmic extracts was determined according to the bicinchoninic acid system (Uptima, Interchim, Montlucon, France) using BSA as a standard. After adjusting the equal amounts (50 μg of protein per lane) of total proteins were separated by SDS-PAGE (5, 7.5% gels) under reducing conditions. The separated proteins were transferred onto nitrocellulose membranes. Membranes were pre-incubated in blocking buffer (5% (w/v) skimmed milk powder in PBS/0.1% Tween 20) for 30 min, and incubated with primary antibodies (1 h, AT). Membranes were washed three times with blocking buffer, and incubated with alkaline phosphatase conjugated secondary antibodies for 30 min. They were finally washed three times in 0.1 M Tris pH 9.5 containing 0.05 M $MgCl_2$ and 0.1 M NaCl. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (p-toluidine salt; Pierce, Rockford, IL, USA) was used as substrates to reveal alkaline phosphatase conjugated specific antigen-antibody complexes.

Statistical analysis

The immunoblotting results were expressed as the means +/-SD of a representative experiment performed in triplicate. The means were compared using student's t-test assuming equal variances. P<0.05 was considered statistically significant.

Results

The goal of this study was to investigate the effects of Tendoactive® on the signalling pathway leading to inflammation and the activation of the transcription factor NF-κB signalling pathway and on NF-κB-regulated gene products in tenocytes during tendinopathy. To examine the effect of Tendoactive® on the NF-κB activation pathway, we used IL-1ß, because the pathway activated by this cytokine is relatively well understood.

Cell culture

Tenocytes cultures treated with 250, 500 and 1000 μg/ml Tendoactive® showed no signs of cytotoxic
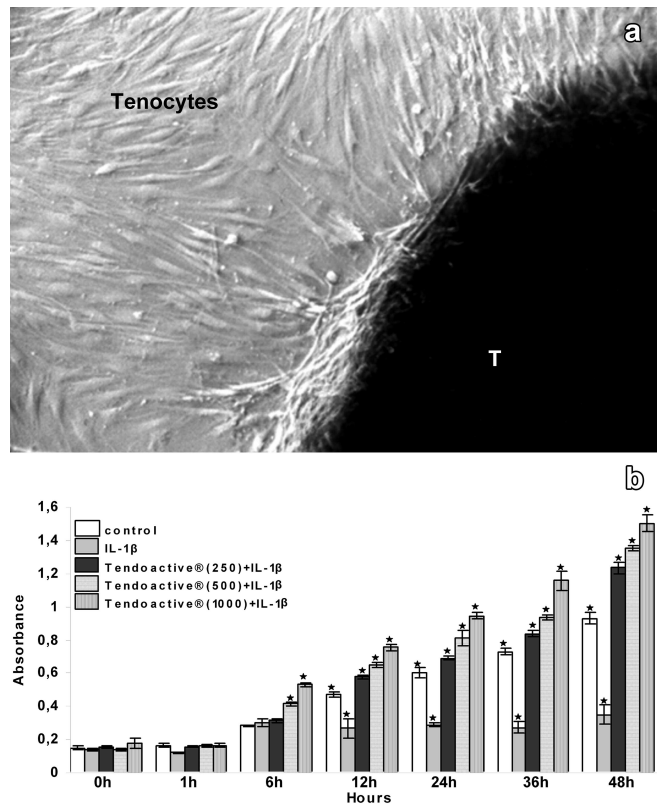

Fig. 1. a. A piece of human tendon (T) obtained during tendon surgery, was cultured in growth medium for several days. After a few days tenocytes migrated from this explant culture forming a monolayer. x 20. **b.** Effect of Tendoactive® and IL-1ß, on the viability and proliferation of primary tenocytes *in vitro*. Serum-starved human tenocytes (0.1×10^4 cells/ml) were pre-treated with 250, 500 or 1000 μg/ml Tendoactive® for 24 hours and then co-treated with 10 ng/ml IL-1ß or cells treated with 10 ng/ml IL-1ß alone, or with Tendoactive® (250 μg/ml) alone or left untreated and the cells were incubated for indicated time. Cell viability was examined by MTT assay. Spectrophotometric measurement of the cell viability was used as a function of mitochondrial activity. This assay was performed in triplicate and the results are provided as mean values with standard deviations from three independent experiments.
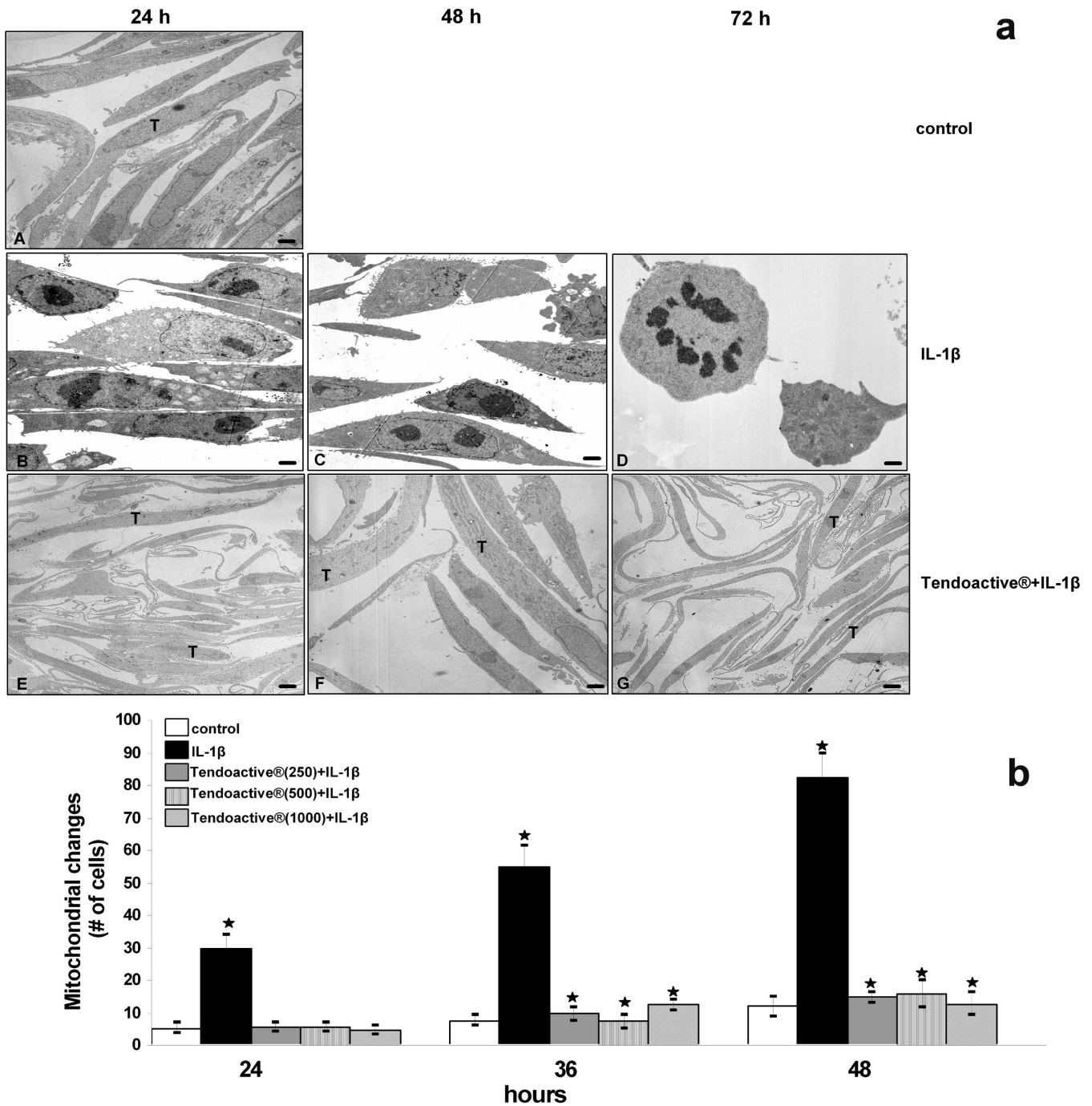
Fig. 2. a. Effect of Tendoactive® on IL-1ß-induced apoptosis in tenocytes *in vitro*. Serum-starved human tenocytes were exposed to 10ng/ml IL-1ß alone for 0, 24, 48 hours or pre-treated with Tendoactive® (250, 500 or 1000 μg/ml) for 24 hours and then co-treated with IL-1ß (10 ng/ml) for an additional 24 and 48 hours and evaluated with a transmission electron microscope. Tenocytes, treated with 10 ng/ml IL-1ß exhibited characteristic features of degeneration: annular chromatin condensation at the nuclear envelope and swelling of mitochondria and rough ER in a time-dependent manner (**B-D**). Tenocytes that were pre-treated with Tendoactive® and then co-treated with IL-1ß (**E-G**) showed less severe cell degeneration at the ultrastructural level. In control cultures no adverse ultrastructural changes were observed (**A**). A-G: x 5000; Bar: 1 μm. **b.** Effect of Tendoactive® on IL-1ß-induced mitochondrial changes and apoptosis in tenocytes in vitro. Serum-starved human tenocytes were exposed to 10ng/ml IL-1ß alone for 0, 24, 48 hours or pre-treated with Tendoactive® (250, 500 or 1000 μg/ml) for 24 hours and then co-treated with IL-1ß (10 ng/ml) for an additional 24, 36 and 48 hours. The number of cells with mitochondrial changes and apoptotic features was determined by scoring 100 cells from 30 different microscopic fields in ultrathin sections evaluated with an electron microscope (Zeiss EM 10). This assay was performed in triplicate and the results are provided as mean values with standard deviations from three independent experiments. Values were compared to the control and statistically significant values with p<0.05 were designated by an asterisk (*).

effects or any negative effects on the viability of cells, they exhibited a spindle-shaped, fibroblast-like appearance as determined by the phase-contrast light microscopy (data not shown) and by electron microscopy at the ultrastructural level. The tenocytes treated with IL-1ß (10 ng/ml) became more flattened and polygonal compared to the control cells, which maintained their fibroblast-like appearance during the
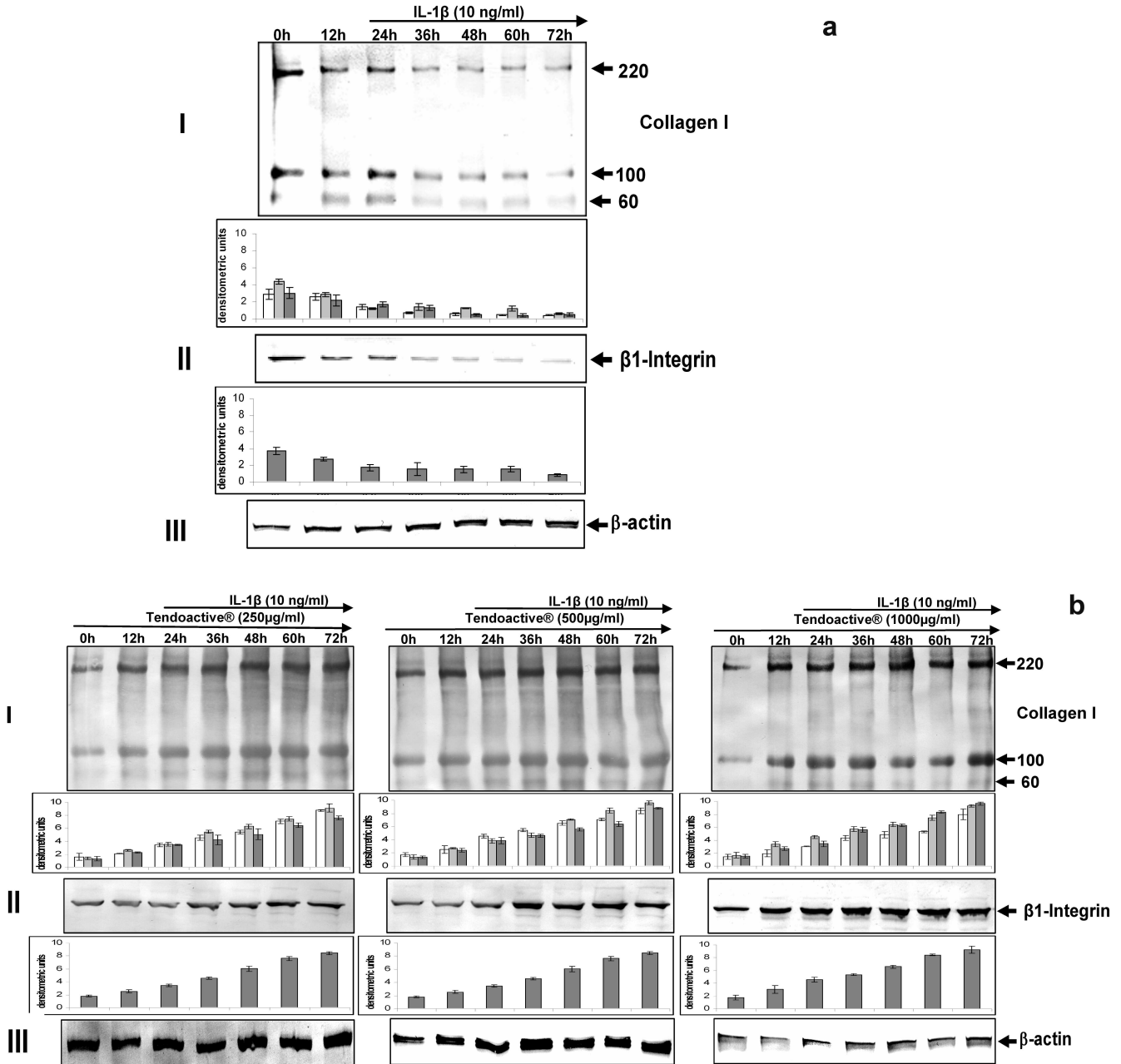

Fig. 3. Effects of Tendoactive® on IL-1ß-induced down-regulation of extracellular matrix and signalling proteins in human tenocytes. Serum-starved human tenocytes (0.1x10^6 cells/ml) were treated with 250 or 500 or 1000 μg/ml Tendoactive® for 24 h before co-treatment with 10 ng/ml IL-1ß and 250 or 500 or 1000 μg/ml Tendoactive® for 12, 24, 36, 48 h (**b**). To examine the cytokine induced alterations in the last 48 h, we performed additional time-course experiments exclusively with IL-1ß (without Tendoactive®) (**a**). Results of western blot analysis revealed down-regulation of collagen type I (**a, I**) and ß1-integrin (**a, II**) in tenocytes by IL-1ß. Co-treatment of tenocytes pre-incubated with Tendoactive® and IL-1ß suppressed the IL-1ß-induced inhibition of collagen type I and ß1-integrin (**b, I, II**). In untreated control cultures, expression of collagen type I and ß1-integrin were equally strong in tenocytes (**a/b, I-II**). Expression of ß-actin was not affected by IL-1ß and Tendoactive® (**a/b, III**). Data shown are representative of three independent experiments.

experiment at each observation time.

Effects of Tendoactive® and IL-1ß on human tenocyte viability and proliferation

Because IL-1ß inhibits proliferation and induces cell death, in this study we evaluated the effects of different concentrations of Tendoactive® (250, 500 and 1000 μg/ml) on the IL-1ß-induced tenocytes. Human tenocytes were cultured in a 96-well plate and pre-treated with Tendoactive® for 24 hours and co-treated with IL-1ß or treated only with IL-1ß or only with Tendoactive® (250 μg/ml) or left untreated for the indicated times. Proliferation and viability experiments performed with MTT-test demonstrated that the tenocytes cultivated only in the presence of IL-1ß were significantly lower compared to those of tenocytes treated with Tendoactive® or Tendoactive® and IL-1ß or cells that were left untreated (Fig. 1b). Tenocytes showed an increased proliferation rate when treated only with Tendoactive® (250 μg/ml) or co-treated to Tendoactive® concentration 250, 500 and 1000 μg/ml. Differences in cell numbers between control and Tendoactive®-treated cells were statistically significant ($p<0.05$) after 24 and 48 h. The results showed a marked dose- as well as time-dependent increase in proliferation and viability of tenocytes pre-treated with all three concentrations of Tendoactive®. Co-treatment of human tenocytes with 10 ng/ml IL-1ß and with Tendoactive® (250 μg/ml) simultaneously produced similar results such as those described above (data not shown).

Tendoactive® inhibits IL-1ß-induced degenerative features and apoptosis in human tenocytes in monolayer cultures

Tenocytes in control monolayer cultures exhibited a typical fibroblast-like morphology with small cytoplasmic processes, large (mostly euchromatic) nuclei with distinct nucleoli and a well-structured cytoplasm (Fig. 2a: A). Treatment of tenocyte cultures with 10 ng/ml IL-1ß for 24 hours resulted in degenerative changes, such as the appearance of multiple vacuoles, swelling of mitochondria and rough ER and degeneration of other cellular organelles (Fig. 2a: B). Longer incubation periods with IL-1ß (36 and 48 hours) resulted in more severe cellular degeneration. These included areas of condensed heterochromatin in the cell nuclei and multiple and autophagic cytoplasmic vacuoles. The flattened monolayer tenocytes became increasingly spherical, lost their microvilli-like processes and became apoptotic (Fig. 2a: C-D). Tenocyte cultures that were pre-treated with Tendoactive® (250, 500 or 1000 μg/ml) for 24 hours and then co-treated with IL-1ß (10 ng/ml) for an additional 24 and 48 hours did not only show less severe cellular degeneration on the ultrastructural level but further, in the presence of Tendoactive®, 250 μg/ml (Fig. 2a: E-G), 500 and 1000 μg/ml (data not shown) the morphological degenerative features and mitochondrial swelling and apoptosis of the

tenocytes nearly disappeared. The tenocytes regained a fibroblast-like shape and numerous microvilli-like cytoplasmic processes.

Statistical evaluation of the ultrastructural data clearly highlighted changes in the number of tenocytes with mitochondrial changes before and after IL-1ß treatment. Co-treatment with three different concentrations of the Tendoactive® (250, 500 or 1000 μg/ml) clearly decreased the number of cells with mitochondrial changes (Fig. 2b).

Down-regulation of collagen type I and ß1-integrin expression through IL-1ß on tenocytes is blocked and revoked by Tendoactive®

Serum-starved human tenocytes, were stimulated with 250 or 500 or 1000 μg/ml Tendoactive® for 24 h before co-treatment with 10 ng/ml IL-1ß and 250 or 500 or 1000μg/ml Tendoactive® for 12, 24, 36, 48 h (Fig. 3b). To examine the cytokine induced alterations in the last 48 h, we performed additional time-course experiments exclusively with IL-1ß (without Tendoactive®) (Fig, 3a). Tenocytes, stimulated with IL-
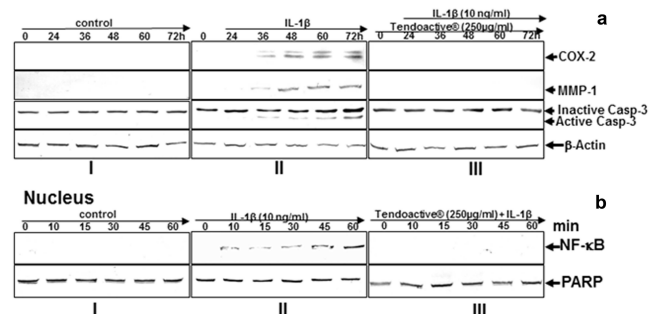

Fig. 4. a. Effects of Tendoactive® on IL-1ß-induced up-regulation of pro-inflammatory enzymes in human tenocytes. Serum-starved human tenocytes were pre-treated with Tendoactive® (250 or 500 or 1000 μg/ml) for 24h before being co-treated 10 ng/ml IL-1ß for 12, 24, 36 and 48h (III). To examine the cytokine-induced alterations in the last 48 h, we performed additional time-course experiments exclusively with IL-1ß (II) or left the cultures untreated (II). The whole cell extracts were prepared and analysed by western blot analysis using specific antibodies against COX-2, MMP-1 and active caspase-3. IL-1ß-stimulation leads to an increase in synthesis of COX-2, MMP-1 and active caspase-3 (II). However, COX-2, MMP-1- and active caspase-3-up-regulation was blocked in tenocytes pre-incubated with Tendoactive® (250 μg/ml) and then co-treated with IL-1ß (10 ng/ml) (III) as revealed by western blot analysis. In untreated control cultures, expression of COX-2, MMP-1 and caspase-3 were not seen in tenocytes (I). Expression of the housekeeping gene ß-actin was not affected by treatment with IL-1ß and / or Tendoactive® (I, II, III). Data shown are representative of three independent experiments. **b.** Tendoactive® inhibits IL-1ß-induced phosphorylation and translocation of p65 in nuclear extracts of tenocytes. Western blot analysis with IL-1ß-treated tenocytes nuclear extracts. Serum-starved tenocytes (0.1 x 106 cells/ml) were treated with 10 ng/ml IL-1ß for 0, 10, 15, 30, 45, 60 min alone and/or cultures were pre-treated first with Tendoactive® (250 μg/ml) for 4h alone and then were co-treated with 10 ng/ml IL-1ß, for 0, 10, 15, 30, 45, 60 min and the nuclear extracts were prepared and analysed by western blot analysis using specific antibodies against phospho-specific p65 and PARP (control).

1ß, alone showed down-regulation of synthesis of collagen type I (Fig. 3a, I) and of ß1-integrin (CD 29) (Fig. 3a, II) in a time-dependent manner in the final 48h. Co-treatment of tenocytes with Tendoactive® and IL-1ß resulted not only in an inhibition of cytokine-induced effects on collagen type I- and ß1-integrin expression, but further in the presence of all three different concentrations of Tendoactive® (each one) collagen type I- and ß1-integrin production augmented significantly, reaching amounts of healthy, primary tenocytes (Figs. 3b, I-II). Expression of the housekeeping protein ß-actin remained unaffected in tenocytes exposed to Tendoactive® (Fig. 3a, 3b, III).

Densitometric analysis of these results showed that collagen type I- and ß1-integrin-expression increased in tenocytes co-treated with Tendoactive® (each concentration, 250, 500 and 1000 μg/ml) and IL-1ß compared to IL-1ß-treated tenocytes.

Tendoactive® inhibits IL-1ß-induced NF-κB-dependent pro-inflammatory and matrix degradation gene products in tenocytes

We investigated whether Tendoactive® can modulate IL-1ß-induced NF-κB-regulated gene products involved in inflammation and degradation processes in tenocytes. Serum-starved human tenocytes were pre-treated with Tendoactive® (250 or 500 or 1000 μg/ml) for 24h before being co-treated with 10 ng/ml IL-1ß for 12, 24, 48h, or left untreated and the whole cell extracts were prepared and analysed by western blot analysis using specific antibodies against COX-2, MMP-1 and caspase-3 (Fig. 4a). In response to 10 ng/ml IL-1ß alone tenocytes showed up-regulation of expression of COX-2, MMP-1 and caspase-3 in a time dependent manner (Fig. 4a, II). Co-treatment of Tenocytes with Tendoactive®, 250 μg/ml (Fig. 4a, III) or 500 or 1000 μg/ml (data not shown) and IL-1ß (10 ng/ml) resulted in an inhibition of cytokine-induced effects on COX-2, MMP-1 and caspase-3. Expression of the housekeeping protein ß-actin remained unaffected.

Effects of Tendoactive® on IL-1ß-induced phosphorylation of p65 in the nucleus

Translocation of NF-κB to the nucleus is necessary for regulation of inflammatory gene expression by NF-κB. The translocation of activated NF-κB is preceded by phosphorylation of the p65 subunit of NF-κB (Kumar et al., 2004). To test this in human tenocytes, protein extracts of serum-starved cells were treated with 10 ng/ml IL-1ß for 0, 10, 15, 30, 45, 60 min alone and other cultures were pre-treated first with Tendoactive® (250 or 500 or 1000 μg/ml) for 4h alone and then were co-treated with 10 ng/ml IL-1ß for 0, 10, 15, 30, 45, 60 min and the nuclear extracts were prepared and analysed by western blot analysis using specific antibodies against phosphorylated p65 NF-κB-subunit (Fig. 4b).
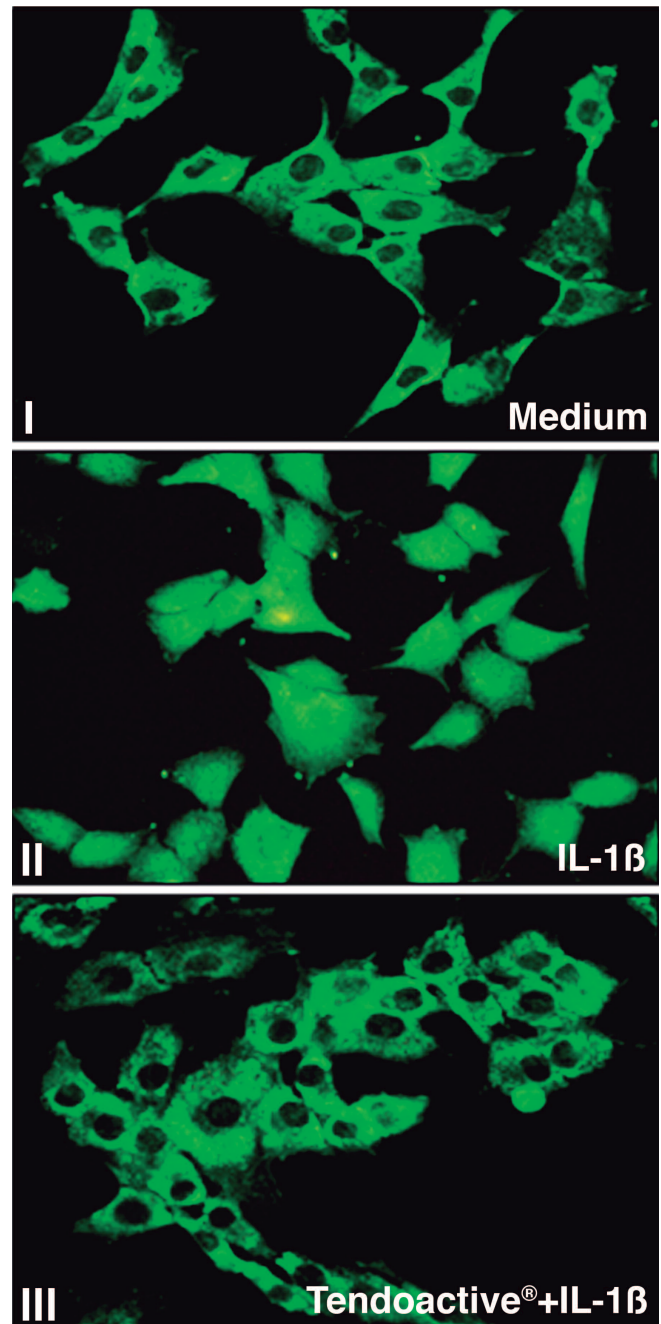
Treatment of human tenocytes with 10 ng/ml IL-1ß

Fig. 5. Tendoactive® inhibited IL-1ß-induced nuclear translocation of phospho p65 revealed by immunofluorescence microscopy. Tenocytes cultures either served as controls (I, not treated) or were treated with 10 ng/ml IL-1ß alone for 10 min or pre-treated first with Tendoactive® (250 μg/ml) for 1h alone and then were co-treated with 10 ng/ml IL-1ß for 10 min before indirect immunolabeling with anti-phospho p65 antibody and FITC-coupled secondary antibodies. In control cells anti-phospho p65 labelling was restricted to the cytoplasm (**I**). Cells treated with IL-1ß alone revealed nuclear translocation of phospho p65 (**II**) that was partly inhibited by co-treatment with Tendoactive® (**III**). Data shown are representative of three independent experiments. x 160

at different time points visibly revealed an increase in phospho p65 in a time-dependent manner in the nuclear extracts (Fig. 4b, II). The co-treatment of tenocytes with Tendoactive® 250 μg/ml (Fig. 4b, III) or 500 or 1000 μg/ml (data not shown) and IL-1ß abolished the IL-1ß dependent phosphorylation of p65 in a time-dependent manner in the nucleus. These results show quite clearly that Tendoactive® inhibits the IL-1ß-induced translocation of p65 to the nucleus. Synthesis of the PARP protein remained unaffected.

Tendoactive® inhibits nuclear-translocation of p65 revealed by IL-1ß

Immunofluorescence microscopy was employed to reveal translocation of phosphorylated p65 subunit of NF-κB from the tenocyte cytoplasm to the nucleus in response to NF-κB-activation by IL-1ß. Human tenocytes remained either unstimulated (Fig. 5I) or were stimulated with Tendoactive® alone (not shown) or with 10 ng/ml IL-1ß alone for 10 min (Fig. 5, II) or pre-treated first with Tendoactive® (250 or 500 or 1000 μg/ml) for 1h alone and then were co-treated with 10 ng/ml IL-1ß for 10 min (Fig. 5, III) before indirect immunolabeling with anti-pan and anti-phospho p65 antibody. FITC-coupled secondary antibodies were used. Pan p65 labelling was restricted to the cytoplasm in control and treated cultures (not shown). Control tenocytes and tenocytes treated with Tendoactive® alone (not shown) showed only cytoplasmic labeling of phospho p65 (Fig. 5, I). IL-1ß-stimulated cells revealed clear and intensive cytoplasmic and nuclear staining for phospho p65 (Fig. 5, II). Co-treatment of tenocytes with Tendoactive® 250 μg/ml (Fig. 5, III) or 500 or 1000μg/ml (data not shown) and IL-1ß Tendoactive® resulted in inhibition of nuclear transition of activated phospho p65 and decreased cytoplasmic staining for this protein and showing a decrease in activation of NF-κB (Fig. 5, III). These immunomorphological findings were consistent with the NF-κB inhibition observed by western blotting.

Discussion

The present study was undertaken to investigate the effects of Tendoactive®, a nutraceutical formulation that contains mucopolysaccharides, hydrolyzed type I collagen and vitamin C on IL-1ß-induced transcription factor Nuclear Factor-κB (NF-κB) signalling and apoptosis in tenocytes. The data presented in this paper provides convincing molecular evidence to support the hypothesis that Tendoactive® suppresses IL-1ß-induced apoptosis through down-regulation of NF-κB and NF-κB-regulated gene products in human tenocytes *in vitro*.

The results presented here lead to the following findings: (1) IL-1ß-induced suppression of tenocytes viability and proliferation is relieved by Tendoactive® co-treatment. (2) Incubation of tenocytes with IL-1ß results in morphological alterations, the main

observations were swollen mitochondria, dilated endoplasmic reticulum and apoptosis, these effects are abolished through co-treatment with Tendoactive®. (3) Tendoactive® inhibits the IL-1ß-mediated suppression of key extracellular matrix and signalling proteins in human tenocytes. (4) Tendoactive® is able to antagonize the IL-1ß-dependent up-regulation of NF-κB-dependent pro-inflammatory and matrix degrading gene products, MMP-1, COX-2 and caspase-3. (5) IL-1ß results in phosphorylation and nuclear translocation of the p65 NF-κB subunit. (6) The activation and translocation of p65 from cytoplasm to the nucleus could be demonstrated immunohistochemically and by western blot analysis following IL-1ß treatment of human tenocytes. (7) The suppression of NF-κB activation by Tendoactive® is accompanied by the inhibition of phospho p65 translocation to the tenocyte nucleus.

The viability and proliferation results confirmed that Tendoactive® inhibits IL-1ß-induced suppression of proliferation and viability in tenocytes. This is an important indicator of the significant positive, anabolic and cyto-stimulatory effects of Tendoactive®. Indeed, it has been suggested that the slow rate of tendon healing may be due to an insufficient number of activated cells involved in tendon regeneration (Marsolais et al., 2003). Further, compounds, like Tendoactive® could promote proliferation, migration and activation of cells in tendon to influence tendon healing and function.

It has already been reported that other anti-inflammatory compounds such as resveratrol significantly inhibit the cell membrane binding of inactive IL-1ß and IL-1ß protein in OCIM2 cells and chondrocytes (Estrov et al., 2003; Csaki et al., 2008).

These results indicate that the inhibitory effect of Tendoactive® on IL-1ß-induced suppression of cell proliferation and viability is mediated, at least in part, by blocking IL-1ß production in tenocytes. Indeed, it has been reported previously that the extracellular matrix compounds have anti-inflammatory effects. Hyaluronan reduces free-radical formation, exerts protection on the corneal endothelium, to the collagen-induced arthritis and exerts anti-inflammation properties. Furthermore, hyaluronan reduced the number of inflammatory cells and PGE2 concentration in tissue (Campo et al., 2003; Camillieri et al., 2004). Furthermore, it has been reported previously that glycosaminoglycans, collagen and vitamin C have positive effects on tenocyte survival, proliferation and healing (Yamamoto et al., 1995; Qin et al., 2005; Omeroglu et al., 2009).

Tendoactive® inhibits IL-1ß-induced tenocyte degenerative changes and cell death

Pro-inflammatory cytokines such as TNF-α and IL-1ß have been shown to mediate cartilage and tendon degradation and apoptosis in tenocytes and chondrocytes in degenerative joint diseases such as RA and OA in humans as well as in animals (Machner et al., 2003). These pro-inflammatory cytokines are produced by

activated synoviocytes, tenocytes, macrophages and chondrocytes (Fernandes et al., 2002). To test whether Tendoactive® might block IL-1ß-induced apoptosis we examined tenocytes by electron microscopy.

Tenocytes treated with IL-1ß showed significant morphological alterations including degenerative processes in cell organelles, swelling of the mitochondria, dilated rough endoplasmic reticulum and apoptosis. Co-treatment with Tendoactive® partially reversed the apoptotic effect of IL-1ß, suggesting that Tendoactive® partially inhibits the caspase activation pathway.

Tendoactive® inhibits IL-1ß-mediated extracellular matrix degradation and integrin down-regulation

Tendon consists mainly of densely packed, longitudinally arranged collagen bundles. Tenocytes align themselves in parallel with the collagen bundles that they produce and maintain (Riley, 2004). Approximately 95% of collagen in tendon tissue consists of collagen type I and other types of collagen (present in small amounts type III and type V), proteoglycans, elastin and fibronectin (Bernard-Beaubois et al., 1997; Rees et al., 2006). These tendon-specific matrix components provide the tensile strength, structure stabilization and biomechanical stability needed to transmit muscle force to bone (Birk et al., 1996).

In our *in vitro* model system of human tenocytes we have consistently observed a time-dependent decrease in the synthesis of tendon-specific extracellular matrix components such as collagen type I in response to IL-1ß. Treatment with Tendoactive® led to a recovery of collagen type I synthesis in IL-1ß-stimulated tenocytes. The ECM repair of tendon tissue is a complex process requiring specific response, tenocyte proliferation and matrix remodeling. Indeed, tenocyte proliferation and collagen synthesis are important cellular responses to tendon injury and have fundamental functions in the healing process because type I collagen is the major element responsible for structure stabilization and mechanical attributes of this tissue (Birk et al., 1996; Riley, 2004).

Interactions between the extracellular matrix and tenocytes are important for the proliferation, differentiation, and survival of the cells (Sendzik et al., 2005, 2009) since inhibition of cell-matrix interactions leads to tenocyte apoptosis (Sendzik et al., 2005, 2009; Mori et al., 2007). Cell-matrix interactions are primarily mediated via multi-functional ß1-integrins (Quaglino et al., 1997; Cao et al., 1999) which organize cell surface mechano-receptor complexes (Mobasheri et al., 2002) and function as signal transduction molecules (Albelda and Buck, 1990) stimulating MAPkinase pathways (Shakibaei et al., 1999, 2001).

In this study, down-regulation of ß1-integrins in response to IL-1ß stimulation was evident and Tendoactive®-treatment resulted in the recovery of these IL-1ß-repressed signalling proteins. The decreased expression of signalling proteins such as ß1-integrins may be a secondary effect of reduced cell-matrix interactions. Indeed, it has been reported that reduced cell-matrix interactions leads to inhibition of Erk1/2 signalling and stimulates the apoptotic pathway in tenocytes (Machner et al., 2003).

Tendoactive® antagonizes up-regulation of NF-κB regulated proteins by IL-1ß

The results presented in this study suggest that Tendoactive® may have therapeutic effects *in vivo*. Tendoactive® inhibited the expression of several important NF-κB regulated proteins.

Western blot analysis showed that Tendoactive® down-regulated the expression of MMPs, which are intimately linked with tendon matrix degradation in arthritis (McCawley and Matrisian, 2001; Sendzik et al., 2005). Indeed, several studies have reported that IL-1ß is a major inflammatory mediator in both human and animals, induces MMP-1, -3 and -13 in human tendon cells *in vitro* and *ex vivo* (Archambault et al., 2002; Tsuzaki et al., 2003). Furthermore, the up-regulation of COX-2 by IL-1ß, which is linked with tendon inflammation (Sun and Yokota, 2002; Tsuzaki et al., 2003) was significantly decreased by Tendoactive®.

Additionally, we investigated the effect of Tendoactive® on caspase-3, because caspase-3 is a key executioner of apoptosis (Cohen, 1997). We could also show that Tendoactive® clearly down regulated IL-1ß-induced caspase-3 in human tenocytes. The apoptotic pathways induced by IL-1ß in tenocytes remain to be clarified. The involvement of NF-κB in regulation of IL-1ß-induced pro-apoptotic changes would explain the anti-apoptotic capacity of Tendoactive®.

Tendoactive® blocks IL-1ß-induced phosphorylation and nuclear translocation of p65

Here we have shown, for the first time, that Tendoactive® inhibits the synthesis of MMP-1 and COX-2 in IL-ß1-stimulated tenocytes. We propose that this occurs via NF-κB inhibition, because the expression of these proteins is regulated by NF-κB as has been shown by other investigators (Yamamoto et al., 1995; Esteve et al., 2002). Indeed, cytokines are well known to activate the ubiquitous transcription factor NF-κB, which leads to further production and upregulation of pro-inflammatory cytokines and enzymes such as COX-2 and MMPs which in turn produce prostaglandins and degrade extracellular matrix macromolecules leading to cartilage degradation and further joint inflammation (Gowen et al., 1984).

In this study, increased phosphorylation of p65 in response to IL-ß1 was clearly demonstrated by western blot analysis of tenocyte nuclear extracts. Translocation of phosphorylated p65 to tenocyte nuclei was demonstrated and confirmed by immunofluorescence microscopy. The mechanism of NF-κB translocation has

not yet been elucidated, but may partly depend on phosphorylation of the p65 subunit. Immuno-fluorescence microscopy confirmed that Tendoactive® inhibits translocation of phosphorylated p65 to the nucleus. Indeed, it is well known that NF-κB plays a regulatory role during apoptosis in cells (Shishodia and Aggarwal, 2002). Interestingly, we have observed similar suppressive effects on MMP-, COX-2 and caspase-3 expression in chondrocytes by other phytochemicals (Csaki et al., 2008, 2009; Shakibaei et al., 2007a,b, 2008), and similar molecular mechanisms may operate in tendon.

Moreover, tendons are load-bearing tissues and respond to mechanical loads by changing their metabolism as well as structural and mechanical properties (Kjaer, 2004). These biological changes are largely brought about by tendon cells that are embedded in a tendon specific extracellular matrix. Indeed, it has been reported that mechanical stimulation (cell stretching) decreases inflammation-associated gene expression (COX-2 and MMP-1) and PGE2 production in tendon cells stimulated with IL-1ß (Yang et al., 2005).

In recent years, novel and modern therapeutic strategies have focused on specific inhibition of the NF-κB signalling pathway, especially its inappropriate activation; these approaches may prove to be important in the development of innovative arthritis treatments. These interesting *in vitro* effects of Tendoactive® potentially make it an interesting nutraceutical tendo-protective agent that may find future use as an adjunct to other anti-inflammatory drugs.

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