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SKELETAL MUSCLE SECRETED FACTORS PREVENT GLUCOCORTICOID-INDUCED OSTEOCYTE APOPTOSIS THROUGH ACTIVATION OF B-CATENIN

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Abstract

It is a widely held belief that the sole effect of muscle on bone is through mechanical loading. However, as the two tissues are intimately associated, we hypothesized that muscle myokines may have positive effects on bone. We found that factors produced by muscle will protect osteocytes from undergoing cell death induced by dexamethasone (dex), a glucocorticoid known to induce osteocyte apoptosis thereby compromising their capacity to regulate bone remodeling. Both the trypan blue exclusion assay for cell death and nuclear fragmentation assay for apoptosis were used. MLO-Y4 osteocytes, primary osteocytes, and MC3T3 osteoblastic cells were protected against dex-induced apoptosis by C2C12 myotube conditioned media (MT-CM) or by CM from *ex vivo* electrically stimulated, intact extensor digitorum longus (EDL) or soleus muscle derived from 4 month-old mice. C2C12 MT-CM, but not undifferentiated myoblast CM prevented dex-induced cell apoptosis and was potent down to 0.1 % CM. The CM from EDL muscle electrically stimulated tetanically at 80 Hz was more potent (10 fold) in prevention of dex-induced osteocyte death than CM from soleus muscle stimulated at the same frequency or CM from EDL stimulated at 1 Hz. This suggests that electrical stimulation increases production of factors that preserve osteocyte viability and that type II fibers are greater producers than type I fibers. The muscle factor(s) appears to protect osteocytes from cell death through activation of the Wnt/β-catenin pathway, as MT-CM induces β-catenin nuclear translocation and β-catenin siRNA abrogated the positive effects of MT-CM on dex-induced apoptosis. We conclude that muscle cells naturally secrete factor(s) that preserve osteocyte viability.

Keywords: Bone; aging; apoptosis; osteocytes; glucocorticoid; β-catenin.

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Introduction

Glucocorticoid treatment is the major cause for secondary osteoporosis in industrialized societies (Grossman *et al.*, 2010; Lane and Lukert, 1998). The development and disease progression of secondary osteoporosis is associated with great morbidity and mortality as increased vertebral and non-vertebral fractures are strongly correlated with daily glucocorticoid dose (van Staa *et al*., 2000). Glucocorticoids are, however, to this date the universal treatment for a variety of inflammatory diseases. Several mechanisms by which glucocorticoid-induced osteoporosis affects bone have been described, such as prolongation of osteoclast life span resulting in increased bone resorption, in addition to apoptosis of both osteoblasts and osteocytes (Weinstein *et al.*, 1998; Weinstein *et al.*, 2000), and induction of osteocyte autophagy (Jia *et al.*, 2011; Xia *et al.,* 2010a) resulting in a net bone loss.

Glucocorticoids appear to compromise the function of the osteocyte network that regulates and maintains adult bone mass. Osteocytes represent the majority of cells found in bone, and in recent years, the osteocyte was acknowledged as a multifunctional cell (Bonewald, 2007a; Bonewald, 2007b; Klein-Nulend, 2008). Osteocytes arise from osteoblasts that become embedded in the bone matrix and terminally differentiate into stellar-shaped cells that are connected to one another *via* long slender processes building a network inside the mineralized bone matrix (Bonewald, 2011). It has been postulated that mechanical loading is sensed by osteocytes exposed to fluid flow shear stress. Osteocytes transmit the fluid flow signal to other osteocytes in the bone matrix *via* signaling molecules such as nitric oxide, Ca^{2+}, ATP and prostaglandin E_2 (PGE_2). PGE_2 is secreted very rapidly and in large amounts by osteocytes in response to fluid flow shear stress (Klein-Nulend *et al.*, 1995). We and others have postulated that the Wnt/β-catenin pathway is activated in response to the initial PGE_2 release from connexin 43 hemichannels (Cherian *et al.*, 2005) leading to β-catenin nuclear translocation and transcription of target genes (for review see Bonewald and Johnson, 2008).

Increased levels of osteocyte apoptosis were first described in 1997 as a characteristic of pathological bone disease (Noble *et al.*, 1997) and a result of estrogen withdrawal (Tomkinson *et al.*, 1997). Osteocyte viability is essential for maintenance of bone mass and function. Either necrosis (Tatsumi *et al.*, 2007) or apoptosis (Kogianni *et al.*, 2008; Zhao *et al.*, 2002) leads to activation of osteoclasts, serving as a signal to initiate remodeling. Osteocyte cell death occurs in association with pathological conditions, such as osteoporosis and osteoarthritis (Weinstein *et al*., 2000), aging (Qiu *et al.*, 2002; Wong *et*

al., 1985), traumatic accidents (Noble, 2003), as well as in the prolonged glucocorticoid treatment of inflammatory diseases (O'Brien *et al.*, 2004), leaving empty lacunae that are frequently filled with mineral through a process called micropetrosis (Frost, 1960). Inhibitors of osteocyte cell death include estrogen (Tomkinson *et al*., 1997) and selective estrogen receptor modulators (Huber *et al.*, 2007), bisphosphonates (Plotkin *et al.*, 1999), calcitonin (Plotkin *et al*., 1999), CD40 ligand (Ahuja *et al.*, 2003) and calbindin-D28k (Liu *et al.*, 2004).

Recently, we have linked mechanical loading-induced release of prostaglandin to osteocyte survival (Kitase *et al.*, 2010). As muscle is necessary for bone healing and bone viability and is known to secrete 'myokines' that exert important regulatory effects on other organs (Chien and Karsenty, 2005; Pedersen, 2011), we proposed that skeletal muscle may produce factors that protect osteocytes against cell death. Here, we show that skeletal muscle produces anti-apoptotic activity that protects osteocytes against glucocorticoid-induced cell death and this protective effect is mediated through the activation of β-catenin.

Materials and Methods

Materials

Materials included 8 % paraformaldehyde from Alfa Aesar (Ward Hill, MA, USA); αMEM with phenol red, DMEM high glucose w/ phenol red, Hanks balanced salt solution (HBSS) 1x solution, penicillin-streptomycin (P/S) 10,000 U/mL each and trypsin-EDTA 1x solution from Mediatech Inc (Manassas, VA, USA); αMEM without phenol red, calf serum (CS) and fetal bovine serum (FBS), Cy-3 donkey anti-mouse and Alexa 488 phalloidin, Oligofectamin and OptiMEM from Invitrogen (Carlsbad, CA, USA); β-catenin siRNA and negative-control Risc-free siRNA from Ambion (Austin, TX, USA); BCA Protein Assay Kit, 10 %-Precise Protein Gels, SuperSignal West Pico Chemiluminescent Substrate, Tris-Glycine Buffer, Tris-HEPES SDS Running Buffer from Thermo Scientific (Rockford, IL, USA); bovine serum albumin (BSA), collagenase type IA from *Clostridium histolyticum*, DAPI, dexamethasone, EDTA and trypan blue 0.4 % solution from Sigma-Aldrich (St Louis, MO, USA); nitrocellulose membrane from Bio-Rad (Hercules, CA, USA); rat tail collagen type I from BD Biosciences (Bedfort, MA, USA); mouse anti-active β-catenin from Millipore (Billerica, MA, USA); and rabbit anti-β-catenin from Cell Signaling (Danvers, MA, USA).

Cell cultures

MLO-Y4 murine osteocytic cells were cultured as previously described (Kato *et al.*, 1997). Briefly, cells were seeded onto type I collagen-coated plates and cultured in αMEM + 2.5 % FBS + 2.5 % CS (100 U/mL P/S). Cells were maintained at 40-70 % confluence throughout the culture period. For experiments cells from passages 29-35 were used, plated at 6,500 cells/cm^2 cell density in 24 well plates and cultured overnight.

C2C12 murine multi-potent cells were cultured as previously described (Cai *et al.*, 2009; Shen *et al.*, 2009; Zhao *et al.*, 2008) in DMEM/high glucose +10 % FBS (100 U/mL P/S) and were maintained at 40-70 % cell density. Under these conditions, myoblasts proliferate, but do not differentiate into myotubes. The conditioned media from myoblasts (MB-CM) was collected after 48 h of seeding these cells when they are actively proliferating, therefore, only factors secreted by myoblasts were present in the MB-CM. For the generation of myotube conditioned media (MT-CM), C2C12 cells were plated at 6,500 cells/cm^2 in T75 flasks supplemented with 12 mL culture media/flask. Cells were induced to form MTs by switching culture medium to DMEM + 2.5 % horse serum (100 U/mL P/S) at a cell density of 80-90 % (2-3 d post-seeding). Cells were then cultured for 5-6 d until multinucleated, spontaneously contracting MTs were formed. The CM was generated during a 24 h culture period by changing the culture medium to phenol red-free DMEM/high glucose + 0.1 % BSA (100 U/mL P/S). Prior to use the produced CM was centrifuged at 500 g for 5 min at 4 °C to remove cells and cellular debris. C2C12 MB-CM was generated by seeding 20,000 cells/cm^2 per T75 using 12 mL of DMEM + 10 % FBS as culture medium. On the following day, medium was changed to 12 mL phenol red-free DMEM/high glucose + 0.1 % BSA (100 U/mL P/S), in which C2C12 myoblasts were further cultured for 24 h to collect MB-CM. A similar DNA to culture media volume ratio was achieved using the described methods to generate MT-CM and MB-CM.

Further cell cultures included NIH3T3 fibroblasts that were maintained in DMEM + 10 % CS, HeLa epithelial cells that were cultured using DMEM + 10 % FBS, and CRL-1927 mesangial cells, which were cultured in a 3:1 mixture of DMEM : Ham's F12 medium with 14 mM HEPES and 5 % FBS. All three cell types were used for the generation of CM during a 24 h culture period using phenol red-free DMEM/high glucose + 0.1 % BSA (100 U/mL P/S). A similar DNA per culture media volume compared to MT-CM was achieved by seeding 20,000 HeLa cells/cm^2 per T75 1 d prior to CM collection, 6,500 NIH3T3 cells/cm^2 per T75 4 h prior to CM collection, and 40,000 CRL-1927 cells/cm^2 per T75 1 d prior to CM collection.

Primary osteocyte isolation from mouse long bones

Osteocyte-enriched cell fractions were isolated as previously described (Kato *et al.*, 1997) and modified by Stern *et al.* (2012). Briefly, long bones from 7-day old mice (5-6) were removed with attached muscle and rinsed in HBSS. Bones were placed in αMEM (1,000 U/mL P/S) and soft tissue and periosteum were scraped off, epiphyses were cut off and bones were flushed with αMEM using a 27G½ needle-syringe to remove bone marrow. Bones were pre-digested with collagenase type IA (300 U/mL in αMEM + 1,000 U/mL P/S) for 25 min at 37 °C on an orbital shaker to remove remaining bone marrow, periosteum and fibroblasts. Bones were then cut lengthwise and into roughly 2×2×2 mm pieces. Bone pieces were incubated 3 times with collagenase type IA, each time the solution containing cells was aspirated and the enzyme activity was inactivated with 500 µL FBS and bone pieces were washed in HBSS. The first fraction (fibroblast-enriched) and fraction 2+3 (osteoblast-enriched) were cultured in 60 cm^2 collagen-coated plates with αMEM + 10 % FBS. Bone

pieces were incubated alternatively with EDTA solution (5 mM in HBSS + 0.1 % BSA) and collagenase type IA for another 3 times to generate the osteocyte-enriched fractions. The remaining bone pieces were placed in αMEM and minced in a tissue homogenizer (BD™ Medimachine, Becton Dickinson Biosciences, San Jose, CA, USA) in 3 aliquots of 1 mL, for 5min homogenization each. The bone particle suspension and the osteocyte-enriched fractions (4, 5+6, 7, 8, 9) were cultured in 60 cm^2 collagen-coated plates with αMEM + 5 % FBS + 5 % CS (1,000 U/mL P/S) culture media.

***Ex vivo* skeletal muscle contractility**

These experiments were performed as previously established (Shen *et al.*, 2009; Thornton *et al.*, 2011; Zhao *et al*., 2008). Intact extensor digitorum longus (EDL) and soleus muscles were dissected from 4-5-month old C57Bl/6 male mice. Muscles were placed inside Radnotti Chambers containing Ringer's solution for the collection of isometric force data using an eight chamber system. Experiments were driven by an ADI-PowerLab Software (Colorado Springs, CO, USA) that is customized for these experiments. Muscles were equilibrated to mimic conditions of normal activity (low duty cycle). Following 20 min equilibration, the muscles were subjected to the length-force relationship to determine optimal length at which maximal force is achieved. Next, muscles were stimulated with frequencies ranging from 1-130 Hz to generate the force *versus* frequency relationship. Muscles were then equilibrated with stimulatory trains of 500 ms, 80 Hz, repeated at every minute for 20 min. This protocol creates a duty cycle of less than 1 %, which does not induce fatigue. After this initial equilibration period, the solution bathing the muscles was discarded to eliminate any possible confounding factors released during the mounting and stretching procedure. Then, fresh Ringer's was added to the muscles and they were allowed to equilibrate for 30 min at the aforementioned conditions. This solution was collected and considered as the CM 80 Hz. Fresh solution was added again and muscles were equilibrated at 1 Hz stimulation for 30 min and this solution was the CM 1 Hz. In different experiments, we alternated the sequence of CM generation with either 1 Hz/80 Hz or 80 Hz/1 Hz, and found that altering the sequence of collection of CM had no influence on the results.

Using these experimental conditions, the muscles are unlikely to possess anoxic cores. This is based on the fact that 1) contractile force is completely stable during the course of our experiments lasting from 1-4 h, 2) a pO_2 of 250 ±10 mmHg, normally considered a normoxic condition for isolated muscles is used, 3) we have previously shown that muscle degradation, and in particular degradation of troponins does not occur and when pO_2 in the chamber was dropped to 60 mmHg or below, troponin degradation was observed (Brotto *et al.*, 2000; de Paula Brotto *et al.*, 2001), 4) LDH activity in our isolated muscle preparations is extremely low at less than 10 U/g of wet weight tissue, and most importantly it remains constant during the experiment, unless hypoxia is introduced and or hypoxia plus fatigue is introduced and 5) our studies are well in agreement with the studies of Barclay (2005) that reported that although anoxic cores were a significant problem for rat skeletal muscles, they are not for mouse muscles.

Cell viability

The percentage of cell death was quantified using the trypan blue exclusion method following previously published procedures (Ahuja *et al*., 2003; Kitase *et al*., 2010). Briefly, cells were cultured overnight, washed with phosphate-buffered saline (PBS) and treated with or without CM for 1 h at various concentrations using phenol red-free αMEM + 2.5 % FBS + 2.5 % CS (100 U/mL P/S) as culture medium. After pre-treatment, cells were exposed to phenol red-free culture media with or without dexamethasone (1 μM) for 6 h. Adherent cells were trypsinized and combined with non-adherent cells. Cells were incubated for 10 min with 0.1 % trypan blue. A Neubauer hemocytometer was used to count viable, non-stained cells and dead or dying cells that demonstrate blue staining throughout the whole cell body. Experiments contained 4-6 biological replicates and on average 100 cells per replicate were counted.

Results from the trypan blue assay were validated using the gold standard for apoptosis, nuclear fragmentation. Cells were fixed for 10 min at 4 °C in 2 % neutrally buffered paraformaldehyde and after treatment nuclei were stained with DAPI (0.25 mg/mL) for 5 min. The percentage of apoptotic cells was determined counting cells with impaired nuclear membrane that demonstrate nuclear blebbing *versus* non-impaired cells. Experiments contained 4-6 biological replicates and an average 100 cells per replicate were counted.

Immunolabeling of active β-catenin for nuclear translocation

MLO-Y4 cells were seeded onto positive-charged glass slides and cultured overnight. MT-CM or blank media was added to produce 10 % final concentration. Cells were incubated for 2 h. Cells were rinsed 2 times in cold PBS, fixed in 2 % paraformaldehyde containing 0.2 % Triton X-100 for 10 min and then washed 3 times in PBS at ambient temperature for 10 min each. Slides were blocked with 2.5 % BSA and 1 % non-immune donkey serum in PBS overnight at 4 °C. Primary antibody against the active form of β-catenin was used at 1:200 dilution for 4 h at ambient temperature. Slides were washed 3 times with PBS and incubated for 1 h with Cy-3-conjugated donkey anti-mouse antibody, Alexa 488 phalloidin, and DAPI nuclear stain.

The quantification of intensity of β–catenin immunofluorescence was performed as described previously (Noursadeghi *et al.*, 2008). Images were obtained at 16 bit and analyzed using ImageJ Software (NIH). The DAPI and phalloidin images were thresholded using the Hybrid 2D Mean Filter plugin and the Isodata threshold method. A binary mask was created and subtracted from the β-catenin images, leaving only the nuclear or the cytoplasmic compartment of the cell. The nuclear and cytoplasmic histograms from each image were used to quantify staining intensity.

β-Catenin silencing using siRNA and western blot of total β-catenin

This procedure was performed as previously described (Kitase *et al.*, 2010). Briefly, MLO-Y4 cells were seeded (5,000 cells/cm^2) and cultured overnight in normal growth media (no P/S). The next day, cells were used for transfection using 50 nM siRNA (β-catenin; Risc-free) and 1 µL Oligofectamin/100 µL OptiMEM. Therefore, siRNA and Oligofectamin were first mixed separately with an aliquot of OptiMEM according to manufacturer's procedures and then incubated together to form complexes for 15 min at ambient temperature. The transfection mixture was added to the cells using a 500 µL/24-well format. After 4 h transfection at 37 °C, serum was added to the cultures resulting in a final concentration of 2.5 % FBS and 2.5 % CS, and cells were cultured for 48 h prior to experiment. Each experiment included the controls non-transfected, vehicle (Oligofectamin alone) and Risc-free (control) siRNA + Oligofectamin.

Effective silencing of β-catenin was confirmed by Western Blot 48 h post-transfection. Therefore, cells were lysed in a minimal amount of lysis buffer containing 1 mM EDTA, 0.5 % Triton X-100, 5 mM NaF, 6 M urea, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate, 10 µg/mL pepstatin, 100 µM PMSF, and protease inhibitor cocktail from Sigma Aldrich in PBS. Membrane fraction was removed by centrifugation for 5 min at 12,000 g and protein concentrations were determined using a BCA kit. Per lane 50 µg total protein were run on an SDS-PAGE. Western blot was done using PVDF membrane and Signal Enhancer solution. Primary antibody against total β-catenin was used at 1:1,000 dilution for 4 h incubation at ambient temperature.

Results

C2C12 myotubes and mature skeletal muscle cells secrete factor(s) that prevent dexamethasone-induced MLO-Y4 cell death

To investigate the effect of secreted factor(s) from C2C12 cells, MLO-Y4 cells were pre-incubated for 1 h with 10 % MB-CM or MT-CM and compared to 10 % culture media with and without dex. This percent CM was determined by dose response experiments up to 50 % conditioned media (data not shown). As no effect was observed with the MB-CM at 10 % and no differences were observed between 50 and 10 % MT-CM, all the following experiments were performed with 10 % CM. Dex-induced cell death (13.3 % ±2.2) was significantly higher than control levels (6.4 % ±0.8) (Fig. 1). MT-CM protected against the dex-induced cell death leading to an average cell death of 6.9 % ±1.1 (MT-CM) and 7.1 % ±0.8 (MT-CM with dex). Fig. 1 also shows that only CM from mature C2C12 MTs significantly prevents the cell death induced by dex. CM from MBs alone had a baseline cell death of 6.6 % ±1.0 which increased to 8.9 % ±0.7 with dex-treatment. MB-CM pretreatment followed by dex was the only treatment group that was not significantly different from dex treatment alone.

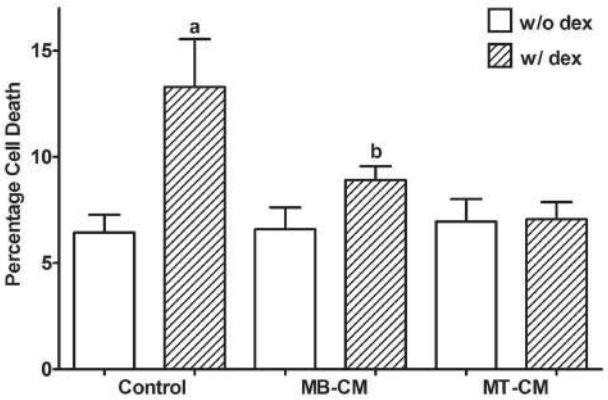

Fig. 1. The effect of skeletal muscle cell conditioned media on MLO-Y4 viability. MLO-Y4 cells were pre-incubated for 1 h with 10 % blank or conditioned media (CM), prior to 6 h incubation with or without dexamethasone (dex). Graphs represent the average percentage of cell death and the standard error of the mean determined by trypan blue assay. Effect of 10 % C2C12 myoblast (MB) and myotube (MT) conditioned media on dexamethasone-induced MLO-Y4 cell death. a: statistical difference compared to all but 'b' using an ANOVA and Tukey *post-hoc* test ($p \leq 0.05$; n = 4-6 experiments with 4 replicates each).

C2C12 MTs prevent dexamethasone-induced apoptosis

The nuclear fragmentation assay was performed to validate results using the trypan blue assay. Fig. 2A shows the DAPI staining on the different treatment groups – I) control; II) dex; III) MT-CM; IV) MT-CM + dex. The white arrows indicate cells with nuclear blebbing that were determined as apoptotic cells. Fig. 2B shows that the increase in percentage of apoptotic MLO-Y4 cells from 3.0 % ±0.2 to 6.5 % ±0.3 with dex treatment. This significant increase is not seen with pre-incubation of 10 % MT-CM, which resulted in an average of 2.6 % ±0.2 apoptotic cells.

C2C12 MT-CM and its effect on primary osteocyte-enriched cells

If MLO-Y4 osteocytic cells can be protected from dex-induced cell death by MT-CM, we sought to investigate whether this is also true for primary osteocyte-enriched cells. Therefore, cells isolated from long bones were challenged with dex to induce cell death as performed with the MLO-Y4 cells. Pre-incubation for 1 h with 10 % MT-CM was compared to pre-incubation with 10 % blank culture media. The baseline level of apoptosis in primary isolated osteocyte-enriched cells was 21.2 % ±2.5 (Fig. 3A), this baseline is much higher than in the MLO-Y4 cell line. Dex-induced apoptosis in primary cells resulting in an average of 35.4 % ±2.5. The dex-induced increase was significantly reduced by MT-CM. Primary osteocyte-enriched cells with MT-CM pre-incubation showed an average apoptosis of 24.9 % ±2.6 and incubation with MT-CM followed by dex resulted in an average of 25.5 % ±1.5 cell apoptosis.

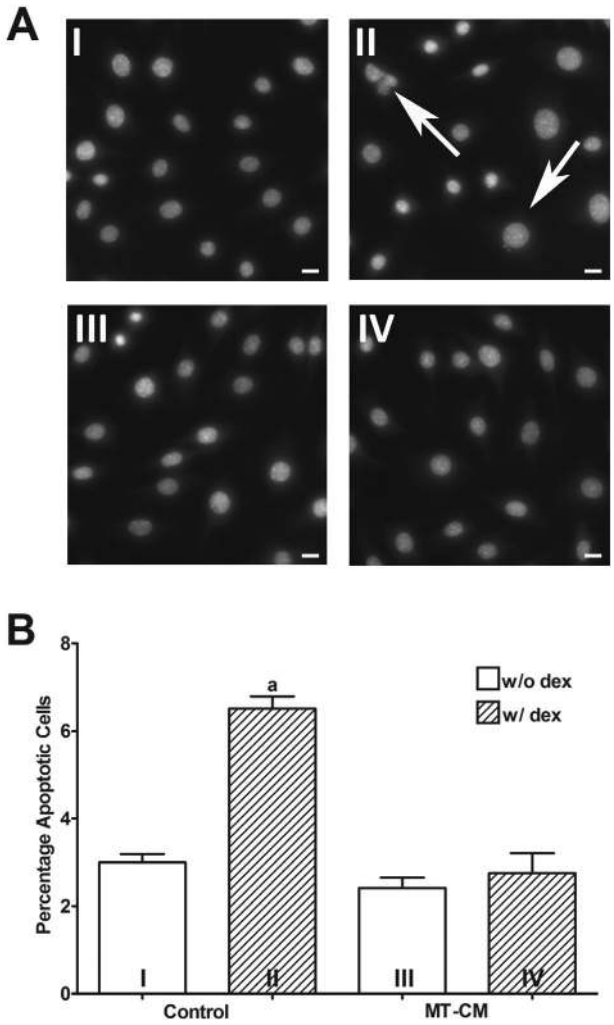

Fig. 2. The effect of C2C12 myotube conditioned media on MLO-Y4 apoptosis. MLO-Y4 cells were pre-incubated for 1 h with 10 % blank or conditioned media (CM), prior to 6 h incubation with or without dexamethasone (dex). The effect of 10 % myotube (MT) conditioned media on dexamethasone-induced MLO-Y4 apoptosis was determined by nuclear fragmentation assay. **(A)** Representative images of DAPI stained cells under treatment conditions – I) control; II) dexamethasone; III) myotube conditioned media; IV) myotube conditioned media + dexamethasone. Scale bar represents 10 µm. **(B)** Graph shows the average and standard error of the mean of apoptotic cells in each treatment condition. a: statistical difference compared to all using an ANOVA and Tukey *post-hoc* test ($p \leq 0.05$; $n = 3$ experiments with 4 replicates each).

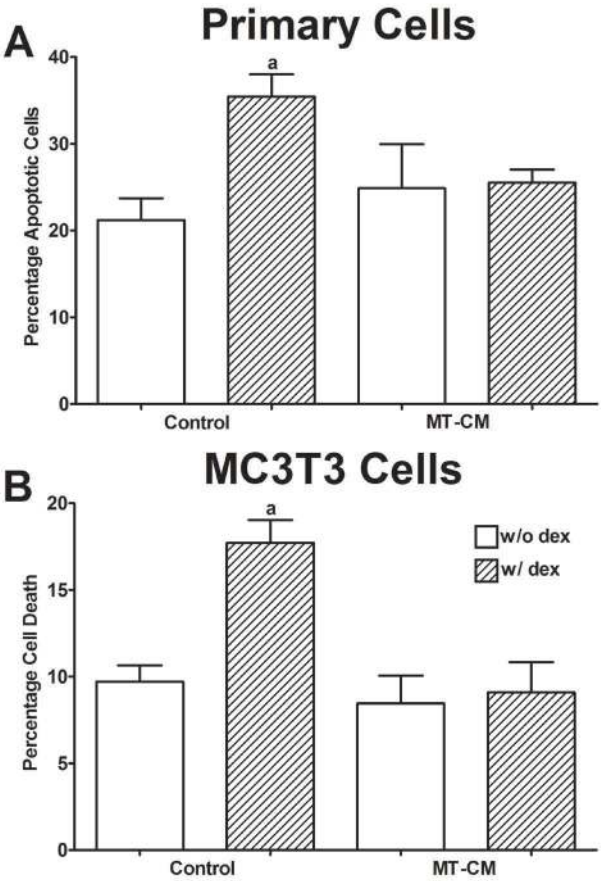

Fig. 3. The effect of C2C12 myotube conditioned media on viability of primary-derived osteocyte-enriched cells and osteoblastic MC3T3 cells**.** Primary osteocyte-enriched cells and MC3T3 osteoblastic cells were pre-incubated for 1 h with 10 % blank or conditioned media (CM), prior to 6 h incubation with or without dexamethasone (dex). **(A)** Graph shows the effect of 10 % C2C12 myotube (MT) conditioned media on dexamethasone-induced apoptosis in primary osteocyte-enriched cells (average and standard deviation). a: statistical difference compared to all using an ANOVA and Tukey *post-hoc* test ($p \leq 0.05$; $n = 1$ experiment with 6 replicates). **(B)** Graph shows the effect of 10 % C2C12 myotube conditioned media on dexamethasone-induced cell death in MC3T3 cells (average and standard error of the mean). a: statistical difference compared to all using an ANOVA and Tukey *post-hoc* test ($p \leq 0.05$; $n = 3$ experiments with 4 replicates each).

C2C12 MT-CM also protects MC3T3 osteoblastic cells against dexamethasone-induced apoptosis

Next, we determined that MT-CM is also able to prevent cell death induced by dex in MC3T3 osteoblastic cells. Baseline level of MC3T3 cell death was on average 9.7 % ±0.9 in these experiments (Fig. 3B). The baseline level was significantly increased to 17.7 % ±1.3 with dex treatment. MT-CM protected against dex-induced cell death and was not significantly different from control levels (average 8.8 % ±0.9).

Non-skeletal muscle CM did not protect against dexamethasone-induced MLO-Y4 cell death

Conditioned media generated from non-skeletal muscle cell cultures were tested for their capacity to prevent dex-induced cell death in MLO-Y4 cells. NIH3T3 fibroblast-CM, HeLa epithelial cell CM, and CRL-1927 mesangial cell CM were used at 10 % total volume of MLO-Y4 culture medium and MLO-Y4 cells were pre-incubated with each CM for 1 h prior to 6 h dex-exposure. While the results in Fig. 4 show that dex-induced cell death (5.2 % ±0.3 with control and 11.1 % ±0.6 with dex) could be significantly blocked by pre-incubation with MT-CM (5.1 % ±0.5 with MT-CM and 5.4 % ±0.1 with MT-CM plus dex), we did

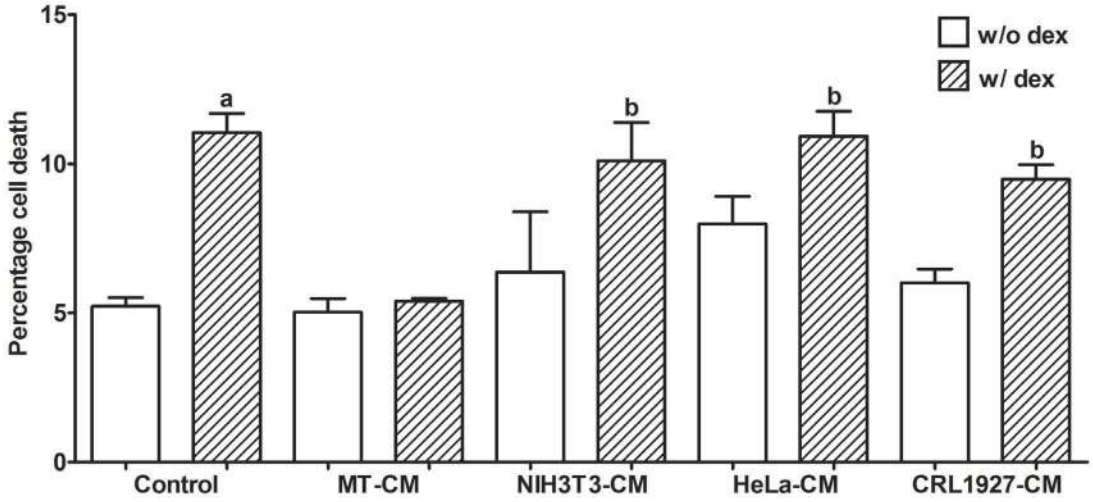

Fig. 4. Conditioned media from NIH3T3s, HeLa and CRL-1927 cells did not protect against dexamethasone-induced MLO-Y4 cell death. NIH3T3 fibroblast, HeLa epithelial cell, and CRL-1927 mesangial cell conditioned media (CM) were used at 10 % for pre-incubation of MLO-Y4 cells prior to 6 h incubation with or without dexamethasone (dex). The graph shows that only pre-incubation with myotube (MT) conditioned media significantly blocked by dexamethasone-induced cell death in MLO-Y4 cells. a: statistical difference compared to all but 'b' using ANOVA and Tukey *post-hoc* test ($p \leq 0.05$; n = 3-5 experiments with 4 replicates each).

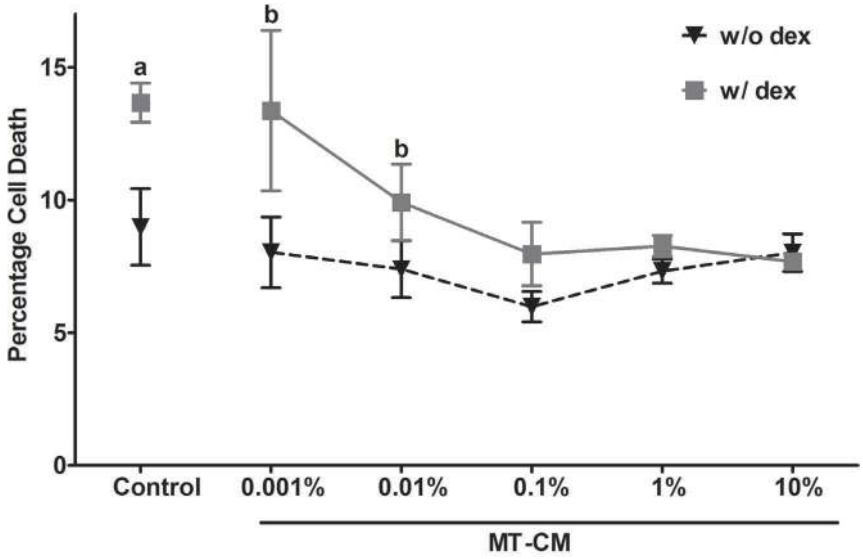

Fig. 5. Dose response curve of C2C12 myotube conditioned media to prevent dexamethasone-induced cell death in MLO-Y4 cells. MLO-Y4 cells were pre-incubated for 1 h with dilutions as indicated of myotube conditioned media (MT-CM), prior to 6 h incubation with or without dexamethasone (dex). Graph shows the average cell death and standard error of the mean of MLO-Y4 cell death under the treatment conditions. Dilution down to 0.1 % myotube conditioned media is still effective in preventing dex-induced cell death. a: statistical difference compared to all but 'b' using an ANOVA and Tukey *post-hoc* test ($p \leq 0.05$; n = 3 experiments with 4 replicates each).

not find that CM from the non-skeletal muscle cells tested resulted in a protective effect. Moreover, incubation with HeLa-CM alone did result in a slightly increased baseline cell death in MLO-Y4 cultures.

Dilutions of C2C12 MT-CM are effective down to 0.1 % in prevention of dexamethasone-induced MLO-Y4 cell death

To investigate the potency of C2C12 MT-CM we performed a dose response curve using MLO-Y4 cell death induced by dex as end point. The graph in Fig. 5 shows the effect of dex to induce cell death (9.0 % ±1.4 to 13.7 % ±0.7). Pre-incubation with 10 % MT-CM was able to prevent dex-induced increase in cell death significantly (average 7.8 % ±0.2). This protective effect of MT-CM was detectable down to 0.1 %, where MT-CM pre-incubation lead to an average of 8.0 % ±1.2 cell death with dex incubation and to an average of 6.0 % ±0.6 cell death without dex incubation.

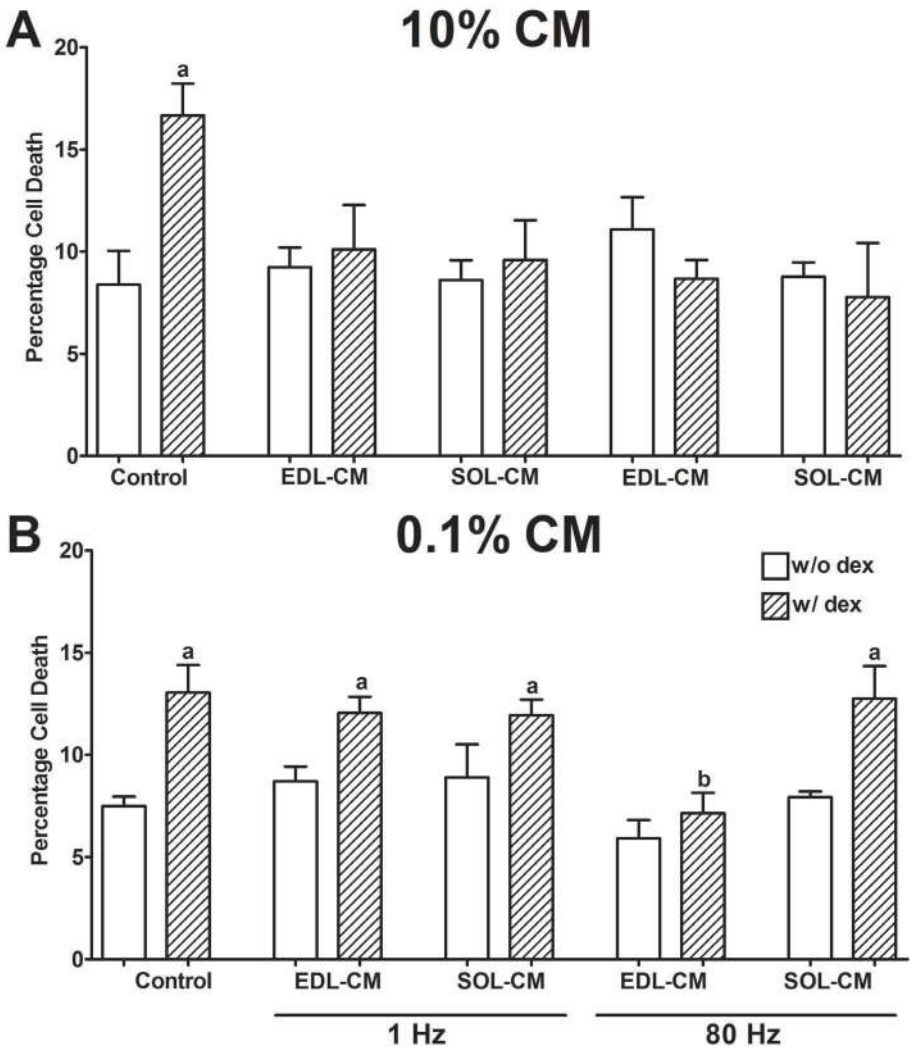

Fig. 6. Effect of skeletal muscle cell conditioned media derived during a high and low frequency loading regime on MLO-Y4 viability. Extensior digitorum longus (EDL) and soleus (SOL) from 5 months old C57BL6 were contracted at 1 Hz or 80 Hz to generate conditioned media (CM). MLO-Y4 cells were pre-incubated for 1 h with 10 % blank or conditioned media (**A**) or 0.1 % blank or conditioned media (**B**), prior to 6 h incubation with or without dexamethasone (dex). Graphs represent the average percentage of cell death and the standard error of the mean as determined by trypan blue assay. **(A)** a: statistical difference compared to all using an ANOVA and Tukey *post-hoc* test ($p \leq 0.05$; $n = 3$ experiments with 4 replicates each). **(B)** a: dex-treated groups are statistical indifferent to each other apart from 'b' using an ANOVA and Tukey *post-hoc* test ($p \leq 0.05$; $n = 3$ experiments with 4 replicates each).

CM from EDL contracted at 80 Hz is more potent than from EDL contracted at 1 Hz and more potent than soleus CM contracted at either frequency

Next, we asked whether muscle secreted factors are affected by the relative level of work executed by muscles. Therefore, intact EDL and soleus muscles were electrically stimulated *ex vivo* at 2 different frequencies – 1 Hz and 80 Hz – to simulate higher muscle working demands, but without the induction of muscle fatigue. Fig. 6 shows the graphs for MLO-Y4 cell death after pre-incubation with CM followed by exposure to dex. When osteocytes were pre-treated with 10 % CM, both EDL and soleus muscles stimulated at either 1 Hz or 80 Hz, protected against dex-induced osteocyte cell death. Only dex treatment alone significantly increased cell death from 8.4 % ±1.7 (control) to 16.7 % ±1.6 (dex) (Fig. 6A). Pre-incubation with EDL or soleus CM (contracted at either frequency) followed by dex treatment showed an average of 9.0 % ±0.6 average cell death that was not significantly different from the control. In contrast to this result, Fig. 6B shows the results of pre-treatment with 0.1 % CM. In this case, only the CM from EDL stimulated at 80 Hz was able to prevent the dex-induced cell death in MLO-Y4 cells and maintain an average cell death of 7.1 % ±1.0, which is not significantly different from control level (7.5 % ±0.5) or EDL 80 Hz alone (5.8 % ±0.8).

C2C12 MT-CM protects against dexamethasone-induced cell death through the activation of β-catenin

β-Catenin has been shown by our group to be one of the major intracellular signal transduction molecules in osteocytes that is activated and translocated to the nucleus

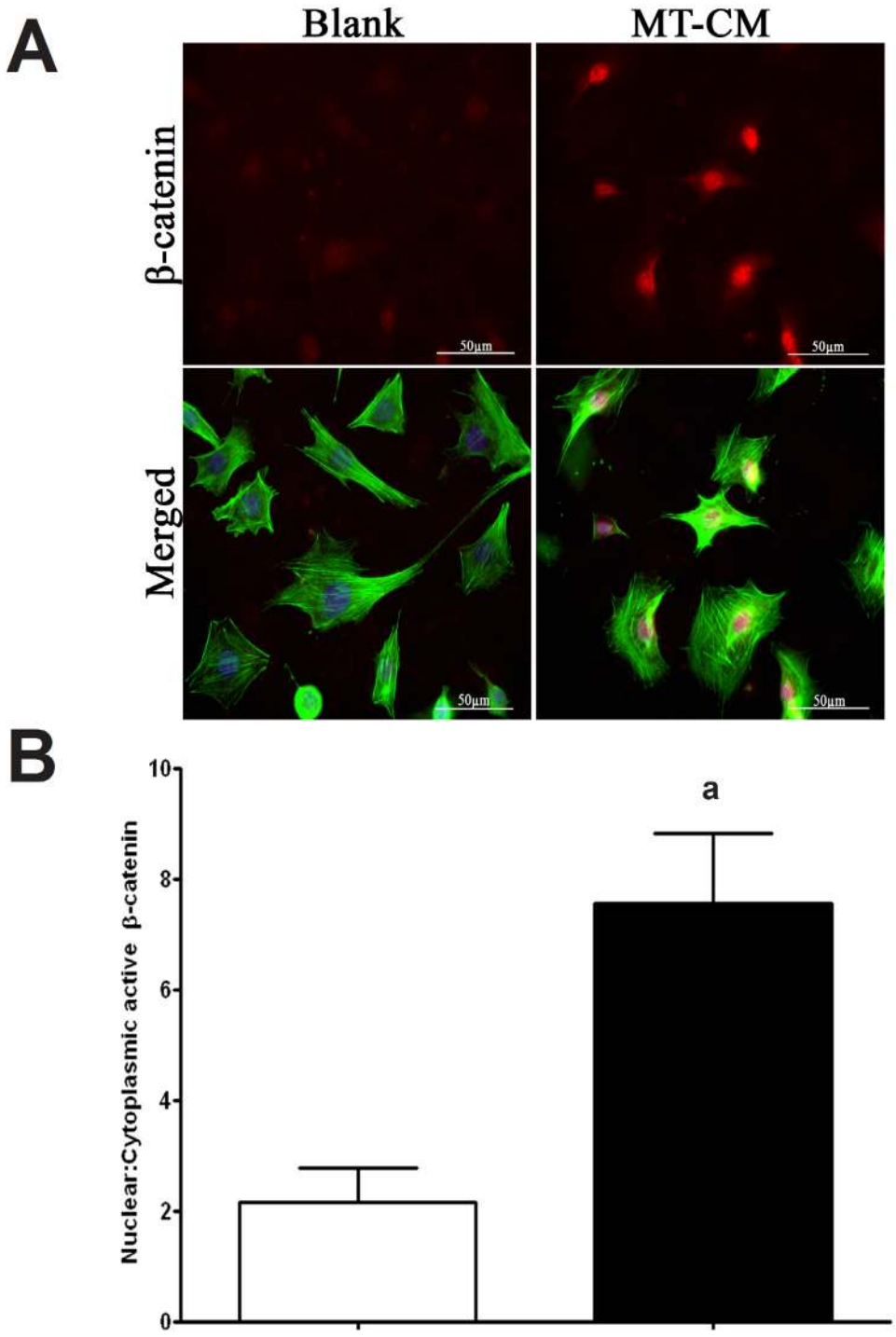

Fig. 7. C2C12 myotube conditioned media induces β-catenin translocation to the nucleus in MLO-Y4 cells. **(A)** Immunofluorescent staining of β-catenin in MLO-Y4 cells exposed to 10 % blank or myotube conditioned media (MT-CM) for 2 h. Scale bar represents 50 µm. **(B)** Graph shows the average and standard error of the mean of nuclear to cytoplasmic ratio of the intensity of the staining. a: statistical difference compared to blank media using *t*-test ($p \leq 0.05$; $n = 1$ experiment with 40 cells each).

in response to mechanical loading, i.e. in the form of fluid flow shear stress (Kamel *et al.*, 2010; Robinson *et al.*, 2006). We and others have shown that PGE_2 is secreted by osteocytes in response to fluid flow shear stress (Bonewald and Johnson, 2008; Kamel *et al.*, 2010). In previous experiments it was demonstrated that the survival effect transmitted by PGE_2 is blocked if β-catenin is silenced in MLO-Y4 osteocytes (Kitase *et al.*, 2010). In this study, we are able to demonstrate that β-catenin is translocated to the nucleus in MLO-Y4 cells with incubation with C2C12 MT-CM but not with blank media (Fig. 7A). Quantification of the nuclear β-catenin staining showed a significantly higher nuclear staining in the group treated with MT-CM (Fig. 7B).

We sought to determine whether or not β-catenin is involved in the protective effect of MT-CM on osteocyte

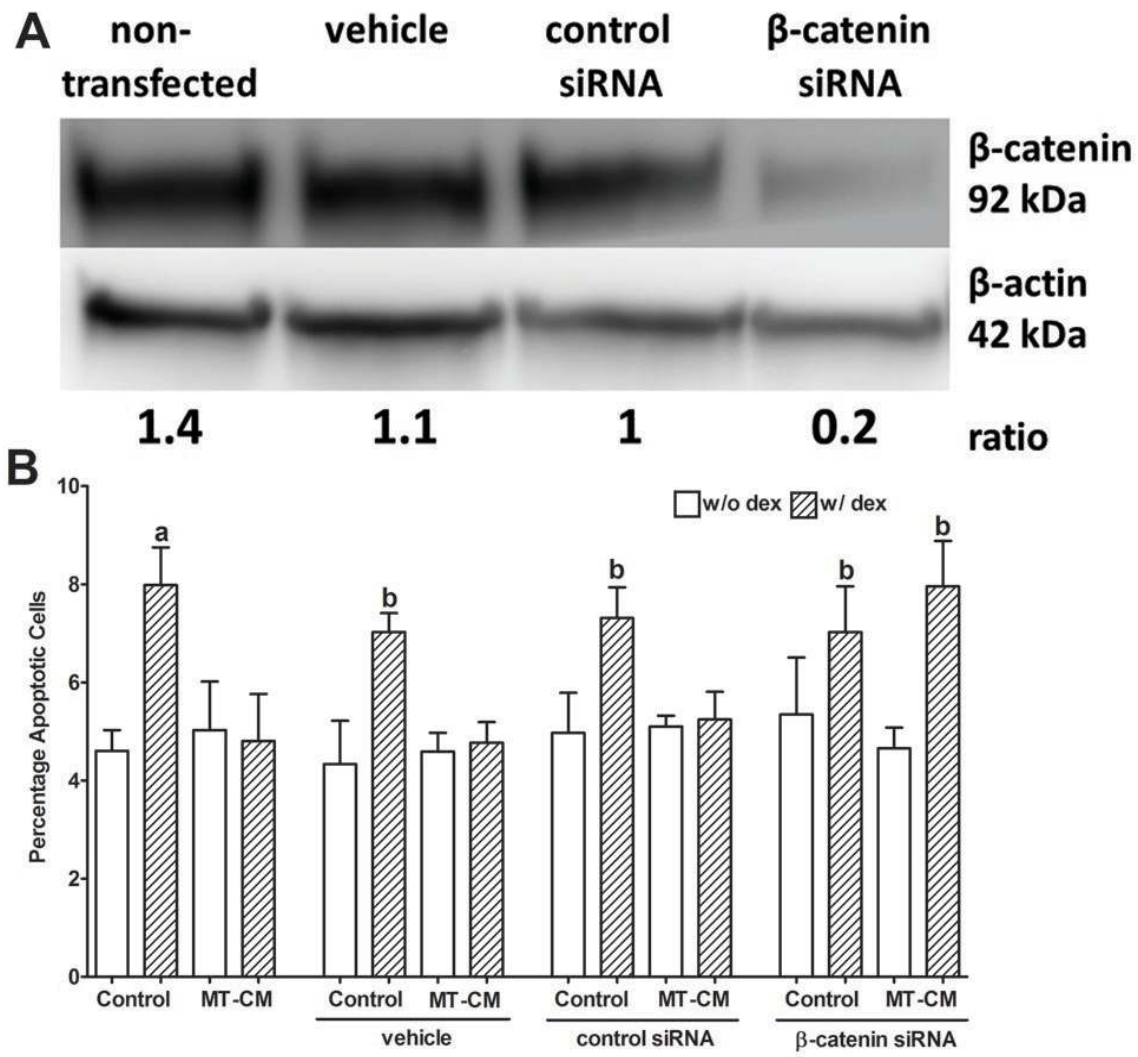

Fig. 8. β-Catenin silencing in MLO-Y4 osteocytes abrogates the effect of C2C12 myotube conditioned media. **(A)** Representative western blot of total β-catenin in non-transfected, vehicle-treated, control siRNA transfected, or β-catenin transfected MLO-Y4 cells**. (B)** MLO-Y4 osteocytes were treated with siRNA to β-catenin, control (Risc-free) siRNA, Oligofectamin (vehicle) or non-transfected 48 h prior to 1 h pre-incubation with myotube conditioned media (MT-CM) and 6 h treatment with or without dexamethasone (dex). Graph shows the average and standard deviation of the percentage of apoptotic cells as determined by nuclear fragmentation for representative experiment. a: statistical difference compared to all but 'b' using ANOVA and LSD *post-hoc* test ($p \leq 0.05$; $n = 1$ experiments with 4 replicates).

viability. Total β-catenin protein expression was silenced using siRNA to β-catenin (Fig. 8A). After 48 h, osteocytes were either directly treated with dex to induce apoptosis or pre-incubated with MT-CM. In the non-transfected group, dex treatment resulted in an increase of apoptosis from 4.6 % ±0.9 (control) to 8.0 % ±1.5 (dex) (Fig. 8B) and this increase was prevented by MT-CM pre-incubation (4.8 % ±1.9). The silencing of β-catenin diminished the protective effect of MT-CM on osteocyte survival under dex stress. If β-catenin was silenced before MT-CM and addition of dex (8.0 % ±1.8), there was no significant difference seen compared to dex alone. β-Catenin silencing before addition of MT-CM resulted in 4.7 % ±0.8 osteocyte apoptosis. The apoptosis values for the control groups vehicle and control siRNA showed comparable results to the non-transfected groups.

Discussion

In this study, we show that mature skeletal muscles and myotubes secrete factor(s) that protect against dexamethasone-induced cell death in osteocytes and osteoblasts, and that this protection is mediated through activation of β-catenin signaling. A very interesting result was the higher level of protection against dexamethasone-induced apoptosis in osteocytes treated with CM from the fast-glycolytic EDL muscle as compared to the slow-oxidative soleus muscle when these muscles were stimulated at 80 Hz, perhaps indicating that at this frequency of stimulation, secreted muscle factors from EDL muscle are more effective in protecting osteocytes against dexamethasone-induced cell death.

Pedersen and colleagues (Pedersen, 2011) have suggested that the profile of secreted myokines could be muscle type specific and our tantalizing results demonstrate now the need to investigate these differential effects over a wide range of stimulation protocols and experimental conditions. Equally important is our finding that the protective effects of CM are more pronounced when osteocytes are treated with CM from both EDL and soleus muscles stimulated at 80 Hz as compared to 1 Hz, suggesting that as muscles work harder, the quantity or type of secreted factors might change. Muscles appear to be able to secrete factors beyond their fascia as an increase in myokines, pyruvate, and lactate were measured in the interstitium using micro-dialysis (Kreiner *et al*., 2010). The authors postulate that these factors are derived from muscle. Experiments performed by Fritton and colleagues (Beno *et al.*, 2006) show that mouse tail vein injection of small dyes and molecules up to 70 kDa can permeate the osteocyte-lacunar-canalicular network, showing that factors in the blood stream can reach the osteocyte.

Our results support a novel concept that muscle and bone are not only communicating *via* mechanical interaction, but also that muscle can secrete factor(s) that can act in a paracrine or endocrine fashion to support bone maintenance by preserving osteocyte viability.

The differences in the number of apoptotic cells between the various cells lines and primary cells is most likely due to intrinsic differences between immortalized cells and primary cells and their culture conditions. The basal levels of apoptosis for the MLO-Y4 cells is slightly higher than as published previously (Kitase *et al.*, 2010) because a percentage of the optimal media for these cells had to be replaced with the culture media for the C2C12 cells and primary muscle culture, i.e. 10 % Ringer's solution. With regards to the basal level of apoptosis for primary osteocytes, this is most likely due to the fact that these are primary cells and not immortalized cells and the isolation procedure for primary osteocytes is most likely stressful to the cells. Osteocyte isolation from mature long bones is not trivial. The primary cells used in this manuscript underwent repetitive collagenase and EDTA digests during isolation and further replating before addition of factors. These extensive external manipulations and removal from their mineralized 3D matrix to a 2D surface is most likely responsible for the greater cell death compared to cell lines.

The close relationship between skeletal muscle and bone is well recognized based on the physical proximity of both tissues, as well as their interaction with regards to mechanical loading. Clearly, skeletal muscle applies stress on bone and bone responds to the created strain. Load plays an important role in the control of bone architecture and mass (Wolff, 1892), mechanical strain affects either bone formation or resorption (Frost, 1988), and bone adaptation is driven by dynamic loads, presumably through the induction of fluid flow within the osteocyte lacunar-canalicular system (Turner, 1998). Muscle mass is known to increase with physical activity (Lawrence *et al.*, 1962), and bone mass has been shown to respond in a similar manner, as observed in the forearms of tennis players where the bone mass of the humerus of the playing arm is significantly higher compared to the non-playing arm (Jones *et al.*, 1977).

The hypothesis that muscle produces factors that affect bone mass is supported by studies of paralyzed patients, micro-gravity and bed rest (Leblanc *et al.*, 2007), as well as concomitant development of sarcopenia and osteoporosis in the normal aging population (Karasik and Kiel, 2010). In a recent study by Daly *et al.* (2004), the relationship of muscle and bone in the forearm of tennis players was revisited. It was found that even though bone and muscle mass correlated in the playing arm, the increase in muscle area could not completely account for the increase in bone mass in the playing arm. An earlier study found a related but opposite phenomenon in women entering a 1 year weight bearing exercise regime that resulted in increased muscle strength in the forearm, yet humeral bone mineral density (BMD) stayed unchanged (Peterson *et al.*, 1991). Moreover, it was shown in the myostatin knockout mice that although increased bone mass was mainly found in muscle attachment sites, that bone shape was influenced not only in load-bearing bones but also in the non-load bearing skull (for review see Elkasrawy and Hamrick, 2010). The authors further showed that myostatin might be considered as anti-osteogenic.

Recent studies have shown that skeletal muscle acts as an endocrine organ, producing 'myokines' in response to contraction (Pedersen, 2011). Since contraction-related workload of skeletal muscle fibers increases myokine production, we tested the effects of a high (80 Hz) *versus* a low-frequency (1 Hz) stimulation protocol of intact, *ex vivo* maintained EDL and soleus muscles for their intrinsic capacity to protect osteocytes against dexamethasone-induced apoptosis. We were able to demonstrate that with increased muscle work (80 Hz compared to 1 Hz) the release of protective factor(s) increased. Conditioned media from EDL muscle stimulated at high frequency provided the highest anti-apoptotic activity, which was not the case for soleus. It is fundamental to consider the intrinsic differences between the EDL and soleus muscles from a functional and biochemical point of view. The EDL is a faster and stronger type of muscle involved in the extension of the toes that is composed by muscle fibers expressing myosin heavy chain (MHC) type IIb and IIx, the fastest, strongest, and most fatigable myosin isoforms, while the soleus is a postural muscle, composed of fibers containing MHC type I and IIa that are slower than type IIb/x, but more resistant to fatigue. The overall function and biochemistry of both muscles may be responsible for the potency of their conditioned media. With aging, there is loss of both type I and type II muscle fibers, but the loss of type II (particularly IIb and IIx) is much more accelerated. Furthermore, these are the fibers that atrophy the most during the aging process, certainly contributing to the overall loss of muscle power with aging (Romero-Suarez *et al.*, 2010; Weisleder *et al.*, 2006) (also for review see Lang *et al.*, 2010). Thus, it is possible that this accentuated loss of the faster muscle fiber types might be a direct contributing factor for development and progression of osteoporosis. Our future work will aim to investigate if aged muscle continues to secrete beneficial factor(s) for osteocyte survival.

Conditioned media from mature skeletal muscle cells induces translocation of β-catenin to the nucleus. Moreover, silencing of the β-catenin mRNA abolishes the effect of the muscle protective factor on dexamethasone-induced apoptosis. Targeted deletion of β-catenin in osteocytes results in a bone phenotype with a 'moth-eaten' appearance, clearly showing the importance of this pathway in the maintenance of normal bone (Kramer *et al.*, 2010). Mechanical loading activates the Wnt/β-catenin signaling pathway, which protects against osteocyte apoptosis (Kitase *et al.*, 2010). Fluid flow shear stress induces the release of prostaglandin which by-passes the LRP receptors to directly activate the Wnt/β-catenin pathway (Bonewald and Johnson, 2008). β-Catenin has also been shown to bind to the connexin 43 promoter, stimulating connexin 43 expression and functional gap junctions between osteocytes (Xia *et al.*, 2010b). Therefore, β-catenin plays a key role in osteocyte regulation of bone homeostasis, in osteocyte viability in response to shear stress, and in osteocyte communication. Further studies will be required to clarify the potential interaction between skeletal muscle secreted factors and fluid flow shear stress in the preservation of osteocyte viability.

In summary, osteocytes have been shown to be multifunctional cells such as mechanosensory cells, regulators of bone resorption and formation, and regulators of mineralization and phosphate homeostasis. Osteocyte viability plays a significant role in the maintenance of bone homeostasis and integrity. Whereas blocking osteocyte apoptosis may improve diseases such as bone loss due to aging or to glucocorticoid therapy, osteocyte apoptosis appears essential for damage repair and normal skeletal replacement. The ideal anti-apoptosis factor would selectively block the former and not the latter processes. This has yet to be determined for any of the anti-apoptotic factors.

Conclusions

In conclusion, healthy skeletal muscle cells secrete factor(s) that prevent apoptosis in osteocytes stressed by glucocorticoid exposure. The identification of this factor which is underway may provide a novel means for the prevention of secondary osteoporosis caused by glucocorticoids.

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Discussion with Reviewers

Reviewer II: I would urge the authors to acknowledge that anoxic cores might be present in the *ex vivo* material (unless they have evidence to the contrary, in which case they should include it).

Authors: The concern of an anoxic core in skeletal muscles is valid because anoxic cores in muscle preparations might impair muscular function and may limit adaptation of muscle cells to increased load and/or activity. Our evidence for the absence of an anoxic core under our experimental conditions:

1. Contractile force is completely stable during the course of our experiments lasting from 1-4 h.
2. Under our experimental conditions we routinely perform experiments with a pO2 of 250 ±10 mmHg, normally considered a normoxic condition for isolated muscles (Brotto *et al*., 2000; de Paula Brotto *et al*., 2001).
3. Using the aforementioned pO2 we have previously shown that muscle degradation, and in particular degradation of troponins does not occur (Brotto *et al*., 2000; de Paula Brotto *et al*., 2001), and we have also been able to obtain essentially identical maximal contractile forces in intact muscle preparations and in skinned muscle fibers from the same preparations at the end of the intact muscle protocols, suggesting that muscle health is maintained (Brotto *et al*., 2000; Brotto *et al*., 2004; de Paula Brotto *et al*., 2001; Weisleder *et al*., 2006).
4. When pO2 in the chamber was dropped to 60 mmHg or below, troponin degradation was observed (Brotto *et al*., 2000; de Paula Brotto *et al*., 2001).
5. If an anoxic core is present one would expect that LDH activity would increase over time. We have measured LDH activity in our isolated muscle preparations and LDH is extremely low at less than 10 U/g of wet weight tissue (10 ±2 U/g), and most importantly it remains constant during the experiment, unless hypoxia is introduced and or hypoxia plus fatigue is introduced.
6. Our studies are well in agreement with the studies of Barclay *et al*. (2005) that reported that anoxic cores were a significant problem for rat skeletal muscles, but not for mouse muscles.
7. More recently, Napurus *et al*. (2012) reported that LDH accumulation under normoxic conditions of 250 mmHg using human muscle biopsies measuring 0.5 x 0.5 x 15 mm was 128 ±14 U/g, suggesting an accumulation of only 20-25 U/g per day. Only under hypoxia did these authors report significantly higher levels of LDH accumulation.
8. Furthermore, Warren *et al*. (1994) reported LDH levels of ~20 U/g when isolated mouse EDL and soleus were contracted with eccentric (lengthening) contractions to induce muscle damage.

In summary, we do not believe that anoxic cores contributed to our results. In fact, if they had contributed one would expect deleterious effects of muscle CM on bone cells and not protective effects as was the case of our studies.

Reviewer III: Why do you think that you values of apoptosis are so high in your control cultures? Do you think that the culture conditions are not optimal?

Authors: In the case of MLO-Y4 cells treated with 10 % medium or CM, the net levels of apoptosis seen with MLO-Y4 cells cultured under control conditions and after treatment with dexamethasone for 6 h are similar to previously published values (Kitase *et al*., 2010). Primary

osteocyte isolation from mature long bones is not trivial and the bones go through extensive and repetitive collagenase and EDTA treatment prior to collection of the primary cells. These extensive external manipulations likely cause stress on the primary cells in addition to the fact that cells were removed from their 3D bone matrix to be plated on a 2D collagen-coated plastic surface.

Reviewer III: How might the muscle derived factors reach the osteocytes and is it known whether osteocytes are more vulnerable to glucocorticoid treatment *in vivo* when they are further from a muscle source?
Authors: Myokines released from skeletal muscle in response to contraction can act in an endocrine fashion and target tissues such as bone, brain, and the adipose tissue, while others may act more locally *via* paracrine mechanisms (Pedersen, 2011). Myokines appear to have systemic effects that create an anti-inflammatory environment, supporting the hypothesis that muscles are endocrine organs (Scheele *et al*., 2009). Reactive oxygen species produced by muscle in response to exercise actually induce cytokines with positive instead of negative effects on muscle. A network of diseases have been recognized as the "diseasome of physical inactivity", pointing to the fact that muscles likely crosstalk to most tissues (Pedersen and Febbraio, 2008). As muscle factors can enter into the circulation, they can most likely reach osteocytes based on the fact that there is a close interaction between the osteocyte lacuno-canalicular system and the vascular system. Also, as muscle and bone are in close association, some factors may be diffusible across the intima between the two tissues.

With regards to whether osteocytes closer to the bone surface or farther away from the bone surface are more protected, this is not known. Weinstein *et al*. (1998) showed that "Osteocyte apoptosis was restricted to small groups of cells in the center of the femoral metaphyseal cortex and were absent from vertebral cortical bone. The apoptotic osteocytes were identified in close proximity to normal osteocytes, in contrast to the large homogenous areas of dead and dying cells typical of cell necrosis". No clear correlation of osteocyte apoptosis and proximity to skeletal muscle was discussed in this paper. Due to the fact that not all osteocytes exposed to glucocorticoids die *via* apoptosis, and considering that the osteocyte population *in vivo* is very heterogeneous, we would suspect that a correlation of osteocyte apoptosis and distance of the cell from skeletal muscle might be challenging.

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