## Muscle and bone, a common world?

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# CHAPTER 5

## Mechanical loading by fluid shear stress of myotube glycocalyx stimulates growth factor expression and nitric oxide production

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## ABSTRACT

Skeletal muscle fibers have the ability to increase their size in response to a mechanical overload. Finite-element modeling data suggest that mechanically loaded muscles *in vivo* may not only experience tensile strain but also shear stress. However, whether shear stress affects biological pathways involved in muscle fiber size adaptation in response to mechanical loading is unknown. Therefore our aim was two-fold: 1) to determine whether shear stress affects growth factor expression and nitric oxide (NO) production by myotubes, and 2) to explore the mechanism by which shear stress may affect myotubes *in vitro*.

C2C12 myotubes were subjected to a laminar pulsating fluid flow (PFF; mean shear stress 0.4, 0.7 or 1.4 Pa, 1 Hz) or subjected to uni-axial cyclic strain (CS; 15% strain, 1Hz) for 1h. NO production during 1h PFF or CS treatment was quantified using Griess reagent. The glycocalyx was degraded using hyaluronidase, and stretch-activated ion channels (SACs) were blocked using GdCl3. Gene expression was analyzed immediately after 1h PFF (1.4 Pa, 1Hz) and at 6h post-PFF treatment.

PFF increased IGF-I Ea, MGF, VEGF, IL-6, and COX-2 mRNA, but decreased myostatin mRNA expression. Shear stress enhanced NO production in a dose-dependent manner, while CS induced no quantifiable increase in NO production. Glycocalyx degradation, and blocking of SACs ablated the shear stress-stimulated NO production.

In conclusion, shear stress activates signaling pathways involved in muscle fiber size adaptation in myotubes, likely via membrane-bound mechanoreceptors. These results suggest that shear stress exerted on myofiber extracellular matrix plays an important role in mechanotransduction in muscle.

## INTRODUCTION

Skeletal muscle fibers, like many other cell types, are highly sensitive to mechanical stimuli, and respond to these stimuli by adapting their size and contractile characteristics (14, 36). Mechanical signals can be transduced by cells via trans-membrane proteins within the glycocalyx and cell membrane into biochemical signaling pathways (43, 50). The most important transmembrane proteins or "mechanosensors" involved in this process of mechanotransduction are integrins, dystroglycan sarcoglycan complexes, stretch-activated ion channels (SACs), caveoli, and/or syndecans (for review see: (3, 14, 16). Stimulation of mechanosensors activates signaling cascades resulting in modulation of protein turnover, either directly via tensegrity or indirectly via production of soluble factors like growth factors and/or cytokines (16, 48). How, and to what extent, mechanosensors and growth factors exactly contribute to mechanotransduction-associated muscle adaptation is not well known (14).

Subjecting skeletal muscle *in vivo* to high static strain is generally established as a mechanical condition that stimulates the rate of protein turnover, thereby inducing muscle hypertrophy and/or increasing muscle fiber length (8, 53, 54). This mechanical loading-stimulated muscle hypertrophy, however, does not apply to every muscle (39). Moreover, high strain does not induce hypertrophy in isolated muscle fibers surrounded by an intact basal lamina and a collagen layer of endomysium (20, 21). The discrepancy between the hypertrophic effects of high muscle fiber strain applied onto a muscle *in vivo* and those applied on isolated single muscle fibers *ex vivo* indicates that strain per se is not enough to induce adaptation of muscle fiber size This raises the question whether muscle fiber strain per se determines mechanical loading-induced adaptation or whether other mechanical cues are involved.

Shear loading of the extracellular matrix in muscle *in vivo* may occur as muscle fibers are mechanically coupled laterally, and the force generated by the sarcomeres is likely exerted to the extracellular matrix, i.e. the endomysium. Finite element modeling has shown that serial sarcomere strain within a muscle is not uniform, and that shear stresses contribute to the overall loading pattern of the muscle fiber (19, 55). Shear loading of the extracellular matrix *in vitro* (i.e. glycocalyx) has been applied to other cell types than muscle fibers, e.g. osteocytes and endothelial cells, to study mechanotransduction (35, 51). So far, studies on mechanotransduction in muscle cells *in vitro* have been restricted to the use of tensile strain as mechanical stimulus (9, 33, 41, 49) It is unknown whether muscle cells *in vitro* respond to fluid shear stress by increasing the expression of regulatory factors involved in muscle adaptation *in vivo*.

Nitric oxide (NO) and calcium are crucial second messengers converting mechanical stimuli into intracellular signaling pathways in a variety of cell types (17, 38, 41). Activation of intracellular signaling pathways by mechanical loading induces the expression of anabolic and metabolic growth factors that are involved in the adaptation of muscle fiber size to mechanical loading. Prime anabolic and metabolic growth factors involved in muscle hypertrophy include insulin-like growth factor-I (IGF-I) isoforms IGF-I Ea and mechano growth factor (MGF) (10), vascular endothelial growth factor (VEGF) (6), and myostatin (28).

Cyclo-oxygenase-2 (COX-2) and interleukin-6 (IL-6) are also well known regulators of muscle fiber size (37, 40). During exercise, the expression of these key-regulators is altered in muscle (5, 13, 24). We hypothesized that myotubes subjected to fluid shear stress activate signaling pathways involved in the adaptation of muscle fiber size to mechanical loading.

To test whether myotubes respond to fluid shear stress, the expression levels of signaling molecules involved in muscle fiber size regulation (i.e. IGF-I Ea, MGF, VEGF, myostatin, IL-6, and COX-2) were determined in myotubes subjected to a laminar fluid flow. Since the mechanosensors within the sarcolemma are surrounded by and/or connected to the glycocalyx, we further investigated whether an intact glycocalyx is required for mechanotransduction. Finally we tested whether the NO response to fluid shear stress is dependent on calcium influx via SACs within the sarcolemma.

## MATERIALS AND METHODS

### Myotube culture

C2C12 myoblasts were cultured in Dulbecco's Modified Eagle Medium (D-MEM; Gibco, Paisly, UK) supplemented with 10 µg/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), 10 µg/ml streptomycin (Sigma-Aldrich), 50 µg/ml fungizone (Gibco), and 10% fetal bovine serum (FBS; Gibco), at 37°C in a humidified atmosphere of 5% CO2 in air. The medium was exchanged every 3 to 4 days. Upon 70% confluence, cells were harvested using 0.25% trypsin and 0.1% EDTA in phosphate-buffered saline, and either seeded at $2x10^4$ cells per well of laminin-coated 6-well Bioflex® plates (Dunn Labortechnik GmbH, Asbach, Germany) or seeded at 2x104 cells per plastic tissue culture slide (25x65 mm, custom made). After 3 days of culture, the medium was replaced by differentiation medium consisting of D-MEM supplemented with 10 µg/mL penicillin, 10 µg/mL streptomycin, 50 µg/mL fungizone, and 2% horse serum (Gibco). This medium was refreshed daily. After another 3 days of culture, differentiated myotubes were subjected to mechanical loading by pulsating fluid flow (PFF) or to cyclic uni-axial strain (CS) as described below.

### Pulsating fluid flow (PFF)

One hour before the start of the mechanical loading by PFF, medium of C2C12 myotube cultures was replaced by D-MEM containing 2% FBS, 10 µg/mL penicillin, 10 µg/mL streptomycin, and 50 µg/mL fungizone.

PFF was applied using a modified set-up as described earlier for osteocytes (fig. 1) (25). Briefly, PFF was generated by pumping 13 ml of D-MEM containing 2% FBS through a parallel-plate flow chamber containing the C2C12 myotubes. Cells were subjected to 1 h PFF with three different peak shear stress rates: 8.8 Pa/s (mean shear stress: 1.4 Pa; pulse amplitude: 1.4 Pa; pulse frequency: 1 Hz), 4.8 Pa/s (mean shear stress: 0.7 Pa; pulse amplitude: 0.7 Pa; pulse frequency: 1 Hz), and 2.2 Pa/s (mean shear stress: 0.4 Pa; pulse amplitude: 0.4 Pa; pulse frequency: 1 Hz). Stationary control cultures were kept in a petri dish under similar conditions as the experimental cultures, i.e. at 37°C in a humidified atmosphere of 5% CO2 in air.

### Cyclic strain

One hour before subjecting C2C12 myotubes to cyclic uni-axial strain, the medium of the myotube cultures was replaced by D-MEM containing 2% FBS, 10 µg/mL penicillin, 10 µg/mL streptomycin, and 50 µg/mL fungizone. CS was applied using the FlexCell® FX4000™ Tension system according to the manufacturer's instructions (FlexCell® Int Corp, Hillsborough, NC). The Flexcell® system was previously validated in our laboratory (22). C2C12 myotubes were subjected to a CS in a sinusoidal pattern with a frequency of 1 Hz, and a maximum elongation of 15%, for 1 h. Stationary control cultures were kept under similar conditions as the experimental cultures but without CS application.

### Glycocalyx degradation

The glycocalyx of the C2C12 myotubes was degraded by pre-incubation in D-MEM containing

230 µg/mL hyaluronidase (H2126, Sigma-Aldrich) and 2% FBS for 1 hour before treatment with PFF, at 37°C (35).

### Blocking stretch-activated ion channels (SACs)

SACs in differentiated C2C12 myotubes were blocked using gadolinium chloride ($GdCl_3$, Sigma-Aldrich). Myotubes were pre-incubated in D-MEM containing 10 µM $GdCl_3$ and 2% FBS at 37°C for 1 hour before treatment with PFF (11, 30).

### Nitric oxide production

NO production was measured as nitrite (NO2) accumulation in the medium using Griess reagent containing 1% sulfanylamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H3PO4. Serial dilutions of NaNO2 in medium were used as a standard curve. Absorbance was measured at 540 nm with a microplate reader (BioRad Laboratories Inc., Veenendaal, The Netherlands).

### RNA isolation and real-time PCR

Total RNA was isolated using RiboPure™ Kit (Applied Biosystems, Foster City, CA, USA). Total RNA concentration was measured using a BioTek Synergy™ microplate reader (BioTek Instruments, Inc., Winooski, VT). mRNA was reverse-transcribed to complementary DNA (cDNA) using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time PCR was performed to determine mouse IGF-I Ea, MGF, VEGF, and myostatin mRNA levels on the StepOne™ Real-Time PCR system (Applied Biosystems). Primers were designed using the Universal Probe Library from Roche Diagnostics. Primers for IGF-I Ea were: 5'-GTGTTGCTTCCGGAGCTGTG and 5'- CAAATGTACTTCCTTCTGAGTC; MGF: 5'-GGAGAAGGAAAGGAAGTACATTTG and 5'- CCTGCTCCGTGGGAGGCT; VEGF: 5'-CTGTAACGATGAAGCCCTGGAGTG and 5'-GGTGAGGTTTGATCCGCATGATCT; and 18S: 5´ GTAACCCGTTGAACCCCATT and 5´ CCATCCAATCGGTAGTAGCG. Data were analyzed using StepOne™ v2.0 software (Applied Biosystems) and normalized for 18S ribosomal RNA expression. IL-6, COX-2, and GAPDH mRNA expression levels were measured using Taqman® qPCR using inventoried Taqman® gene expression assays (Applied Biosystems). GAPDH was used as housekeeping gene.

### Statistical analysis

Statistical analysis was performed using SPSS 20 (SPSS Inc., Chicago, IL). Groups were compared using ANOVA with Bonferroni-adjusted t-test as post hoc test. Differences were considered significant if $p<0.05$. All data are expressed as mean ± SEM.

## RESULTS AND DISCUSSION

It is well known that *in vivo* skeletal muscle hypertrophies when it is maintained at shear stress as a mechanical component during muscle contractions *in vivo* has been predicted by finite element modeling (14, 19). In our study, shear stress was applied to C2C12 myotubes by driving laminar fluid flow in a pulsatile manner through a parallel plate flow chamber (fig. 1). To the best of our knowledge, it has not been reported before that myotubes are affected by fluid shear stress. Therefore we tested whether treatment of myotubes with PFF caused myotube damage or affected their attachment to the substrate by visual inspection and DNA content quantification (fig. 2A,B). PFF did not affect myotube attachment nor the DNA content of the cultures, indicating that a pulsating fluid shear stress with a rate of 8.8 Pa/s did not damage or detach the C2C12 myotubes.

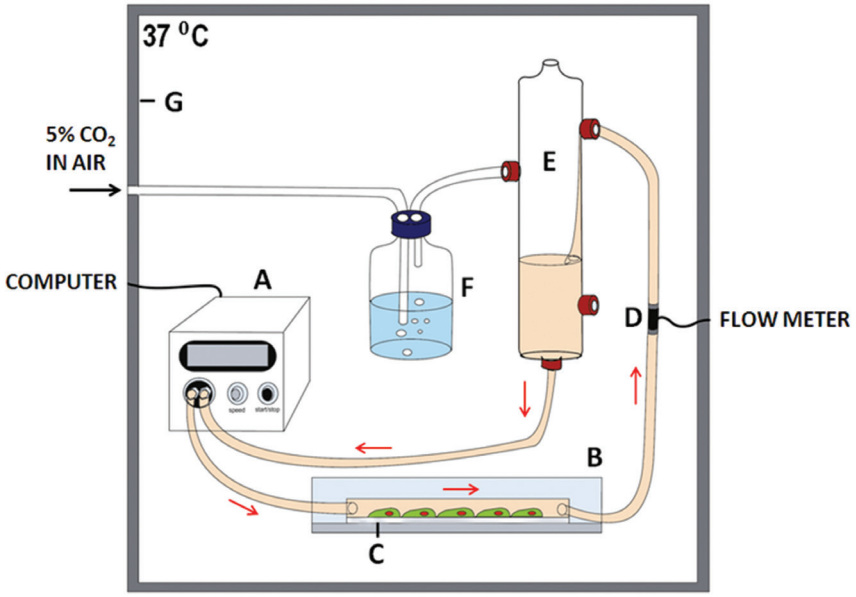


**Figure 1: Pulsating fluid flow (PFF) apparatus.** Pulsating fluid flow was generated by pumping culture medium through a parallel plate flow chamber over al glass slide with a cell monolayer. The pump was computer-controlled and the flow rate was monitored using a flow probe. A, pump; B, flow chamber; C, glass slide with cells; D, flow probe; E, medium reservoir; F, water bottle; G, incubator.

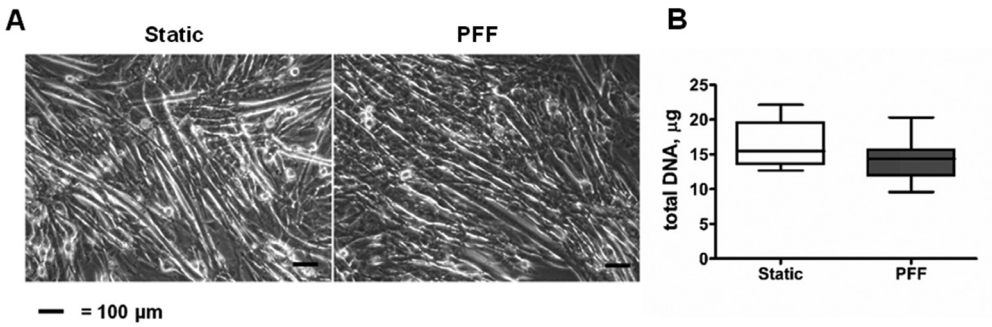


**Figure 2: PFF does not affect total DNA content or substrate attachment of C2C12 myotubes.** **(A)** Micrographs of C2C12 myotubes subjected to 1 h PFF treatment (8.8 Pa/s, 1h) or static culture. **(B)** PFF did not affect total DNA content of C2C12 myotubes. PFF, pulsating fluid flow. tValues are mean ± SEM (n=6).

### Shear stress activates biochemical pathways involved in muscle fiber size adaptation in myotubes

Shear stress stimulates the expression of growth factors in bone cells and endothelial cells (4, 23). Here we tested whether myotubes exposed to fluid shear stress express enhanced levels of growth factors and cytokines that are involved in the induction of muscle hypertrophy. Treatment of C2C12 myotubes with 1 h PFF substantially changed mRNA expression levels of several growth factors known to play a key role in muscle hypertrophy. One hour PFF increased mRNA expression levels of IGF-I Ea (1.8-fold) and the IGF-I splice variant MGF (2-fold) in myotubes immediately after 1 h PFF, but it decreased IGF-I Ea mRNA levels (2-fold) at 6 h post-PFF treatment (fig. 3A). The increases in IGF-I Ea and MGF mRNA levels in myotubes directly after 1 h PFF correspond to published data on the expression of these factors in muscles *in vivo* after mechanical loading (10). VEGF mRNA expression levels in myotubes were increased (1.8-fold) after 1 h PFF (fig. 3A). VEGF is a pro-angiogenic growth factor and also stimulates myotube hypertrophy (2, 6, 32). PFF decreased mRNA expression levels of myostatin (2-fold) at 6 h post-PFF treatment (fig. 3A). Myostatin is a negative regulator of muscle growth, and its expression in rat muscle decreases in response to mechanical loading *in vivo* (13). The relative changes in gene expression of the anabolic growth factors IGF-I Ea, MGF, and VEGF, and the catabolic growth factor myostatin by C2C12 myotubes as a result of PFF treatment were similar as those reported for muscle fibers *in vivo* in response to exercise (5, 13), suggesting that fluid shear stress elicits a physiological, anabolic response in myotubes.

The cytokine IL-6 is often considered an inflammatory cytokine, but it is also increased in skeletal muscle after exercise and induces muscle hypertrophy *in vivo* (24, 37). Fluid shear stress resulting from PFF substantially increased (9-fold) IL-6 mRNA levels in myotubes (fig. 3B). This PFF-induced increase in IL-6 gene expression is significantly

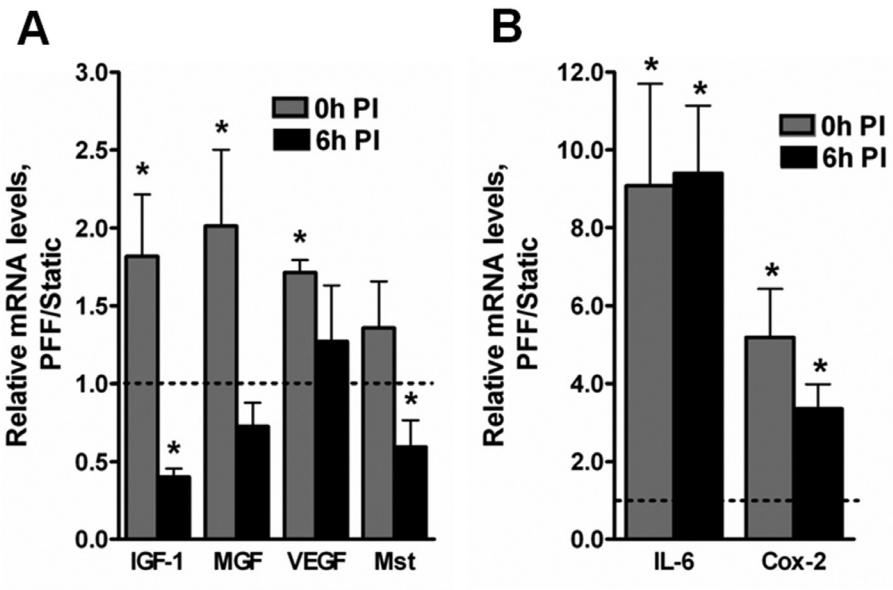


**Figure 3: PFF treatment (8.8 Pa/s, 1h) of C2C12 myotubes alters the expression of key-regulatory factors involved in muscle fiber size adaptation to mechanical loading. (A)** PFF increased IGF-IEa, MGF, and VEGF mRNA immediately after 1 h treatment. At 6 h post-PFF, myostatin and IGF-I Ea mRNA levels were decreased. **(B)** PFF increased IL-6 and COX-2 mRNA levels in C2C12 myotubes. PFF, pulsating fluid flow. Values are mean ± SEM (n=6). *Significant effect of PFF, $p<0.05$.

higher than the increase resulting from tensile strain treatment of myotubes (unpublished data), but similar to that observed in skeletal muscle in response to exercise *in vivo* (24).

Not only growth factors and cytokines, but also carboxylic acids such as prostaglandins may stimulate muscle hypertrophy (40, 49). After appropriate stimulation, prostaglandins are produced by activation of cyclooxygenases and prostaglandin synthases. The rate limiting enzyme for exercise-induced prostaglandin production is cyclooxygenase-2 (COX-2) (7). COX-2 mRNA levels were determined in C2C12 myotubes. We observed a 5-fold increase in COX-2 gene expression by myotubes immediately after 1h PFF, and COX-2 gene expression remained elevated (3-fold) until 6 h post PFF-treatment. This finding is consistent with the increased COX-2 gene expression levels observed in skeletal muscle following resistance exercise *in vivo* (52).

The PFF induced changes in gene expression of key regulators of muscle protein synthesis and degradation in myotubes suggest that fluid shear stress could act as a stimulus activating signaling pathways involved in the adaptation of exercise-induced skeletal muscle hypertrophy *in vivo*.

**Fluid shear stress, but not tensile strain, stimulates early NO production by myotubes**

It is generally known that shear stress and high tensile strain activate distinct signaling pathways in cells such as bone cells and endothelial cells (29). For instance in mechanosensitive bone cells, i.e. osteocytes, fluid shear stress but not high tensile strain rapidly, i.e. within minutes, stimulates the production of NO, a short-lived early signaling molecule involved in many biological processes (29). Such a difference in NO response to shear stress and tensile strain may also exist in muscle. Enhanced NO production in muscle mediates satellite cell activation and induces muscle hypertrophy (1). To investigate whether fluid flow-induced shear stress and tensile strain stimulate NO production in myotubes, C2C12 myotubes were subjected to shear stress by PFF (8.8 Pa/s, 1 Hz) or to tensile strain (cyclic uni-axial strain, 15%, 1 Hz). NO production was measured at subsequent time points during the first 30 minutes of mechanical stimulation. NO production was already increased by 10-fold after only 5 minutes of PFF, and the highest increase in NO production occurred during the first minutes of mechanical stimulation of myotubes (fig. 4). When C2C12 myotubes were subjected to uni-axial 15 % cyclic strain, no increase in NO production could be detected (fig. 4), while we did detect about 2-fold increases in gene expression levels of IGF-I Ea, MGF, and IL-6 (data not shown). This may indicate that in myotubes fluid shear stress resulting from PFF applied to the myotube extracellular matrix (i.e. glycocalyx) rather than cell deformation resulting from tensile strain stimulates early NO production. Unlike our results, NO release in response to stretch (>20% deformation) has been reported in mature muscle fibers *ex vivo* (47), in C2C12 myotubes *in vitro* (47), and in rat satellite cells *in vitro* (44). In these studies, the increases in NO production by mature muscle fibers (1.2-fold), C2C12 myoutbes (1.4-fold), and rat satellite cells (2-fold) were muchsmaller and/or were detected at later timepoints (ranging from 2h to 20h after the first stretch stimulus) than the increase in NO production we observed by C2C12 myotubes in response to fluid shear stress (44, 47). It is possible that 15% CS did not induce an effect of on NO production by myotubes since the changes in NO concentrations in the medium may have been below the detection limit of the Griess assay. Alternatively, NO production in response to CS may have occurred more than 30 minutes after the start of the mechanical stimulus. Thus the early increase in NO production seems much more pronounced in response to fluid shear stress than in response to cell deformation resulting from tensile strain.

Our results show that PFF-induced shear stress on myotubes modulated gene expression of key factors regulating muscle fiber size. Furthermore, myotubes responded differently to fluid shear stress than to substrate strain with regard to early NO production, suggesting that a shear load onto the extra cellular matrix of myotubes causes signaling through other and/or additional pathways than tensile strain per se.

Since it is unknown what the magnitude of shear stress is that is experienced by a muscle fibers *in vivo*, we tested whether myotubes are able to sense differences in the shear stress magnitude. C2C12 myotubes were subjected to different peak fluid shear stress rates (i.e. 2.2, 4.8, and 8.8 Pa/s). The NO production by myotubes increased with increasing shear stress rates (fig. 5). Since the myotubes were able to sense and discriminate between different magnitudes of shear stress rate and because

supraphysiological stimuli would result in cell damage and a saturated NO response, this suggests that the magnitude of shear stress rate we used is likely in the physiological range.

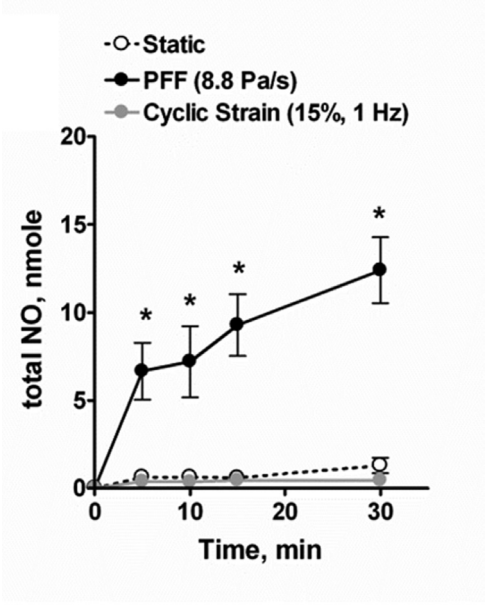


**Figure 4: PFF, but not uni-axial cyclic strain, increases NO production by C2C12 myotubes.** PFF was applied for one hour at with a peak shear stress rate of 8.8 Pa/s. NO production was induced already after 5 min of PFF. In contrast, no increase in NO production by C2C12 myotubes was detected in response to uni-axial cyclic strain (15%). PFF, pulsating fluid flow. Values are mean ± SEM (n=6). *Significant effect of PFF, p<0.05.

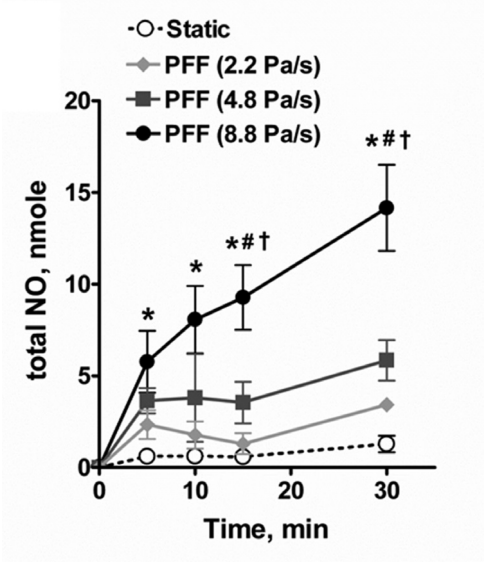


**Figure 5: PFF-induced NO production by myotubes is dependent on the shear stress rate.** PFF treatment varied rapidly stimulated the production of NO by myotubes in a shear stress rate-dependent manner. PFF, pulsating fluid flow. Values are mean ± SEM (n=6). *Significant effect of PFF, p<0.05. #Significantly higher than PFF at 2.2 Pa/s, p<0.05. †Significantly higher than PFF at 4.8 Pa/s, p<0.05.

**Shear stress-induced NO production in myotubes requires an intact glycocalyx, and functional SACs**

NO is produced by different isoforms of nitric oxide synthases (NOS), i.e. endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (34, 42). In the muscle fiber, NOS may be activated via membrane structures such as caveolae, sydecans, and/or dystroglycan-sarcoglycan-complexes that are probably connected to or lie within the hyaluronic acid-rich glycocalyx (26, 50). In addition, activation of NOS is also possible via $Ca^{2+}$/calmodulin signaling; nNOS and eNOS are both activated by the interaction with calcium and calmodulin (31, 45). We therefore investigated whether the NO response to mechanical loading by C2C12 myotubes relies on $Ca^{2+}$-influx via SACs, and whether an intact glycocalyx is required for transduction of mechanical stimulation by PFF, resulting in an NO-response. NO production by myotubes did not increase in response to PFF after disruption of the glycocalyx (fig. 6A). Also, when SACs were blocked by GdCl3, the PFF-induced NO production was ablated (fig. 6B). These results indicate that the PFF-induced NO production within myotubes was mediated by both the glycocalyx and by SACs.

The importance of the glycocalyx in transducing shear stress into a biochemical response has been reported for endothelial cells (46). Without a glycocalyx, fluid shear stress does not cause an integrated torque underneath the endothelial cell membrane. This integrated torque onto the cell and deformation of the actin cytoskeleton are probably required for the activation of mechanosensors such as caveolae, SACs, and/or dystroglycan-sarcoglycan complexes. Muscle fibers in skeletal muscle are connected laterally with other muscle fibers via collagen and proteoglycans within the extracellular matrix (12, 18). We hypothesize that shear stress applied to the extracellular matrix in muscle *in vivo*, caused by unequally

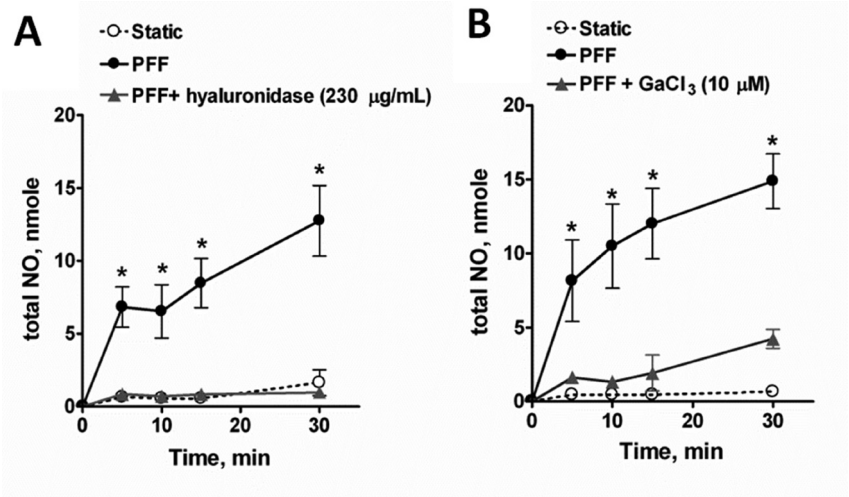


**Figure 6: Mechanical loading-induced NO production requires an intact glycocalyx, and SACs.** **(A)** Glycocalyx degradation by hyaluronidase, and **(B)** SAC blocking by GdCl3 ablated PFF-induced NO production by C2C12 myotubes. PFF, pulsating fluid flow. Values are mean ± SEM (n=6). *Significant effect of PFF, $p<0.05$.

distributed deformations within a bundle of fibers, is possibly transmitted via the glycocalyx to the mechanosensors such as SACs, and in turn results in enhanced NO production via activation of NOS.

### Implications for a role of shear stress in muscle fiber mechanotransduction

A muscle fiber *in vivo* is subjected to different types of mechanical stimuli. The distribution of deformations of muscle fibers during exercise is likely not uniform (15), and both tensile strains and shear stresses contribute to the overall deformation (for review see (3)). Here we show that shear stress applied to myotubes stimulates the expression of anabolic regulatory factors supporting the notion that shear loading of the endomysium within a muscle *in vivo* is an important cue in mechanical loading-induced adaptation of muscle fiber size. Since high strain alone does not induce hypertrophy in mature isolated muscle fibers (20, 21), fluid shear stress activates hypertrophic pathways in myotubes, we hypothesize that shear stress applied to the extracellular matrix is a crucial cue for mechanotransduction in muscle, and subsequent activation of signaling pathways involved in muscle adaptation.

The present results raise questions regarding the mechanisms by which muscle cells sense shear stress that is applied to the extracellular matrix, and regarding the pathways by which this mechanical stimulus alters protein turnover (fig. 7). Figure 7 shows a systematic scheme of how fluid shear stress is sensed and translated into changes in protein synthesis and degradation. Since the glycocalyx seems crucial to allow a biochemical response such as NO production, further research is required to investigate the exact role of the glycocalyx in mechanotransduction. Does the structure of the glycocalyx strengthen and/or amplify the shear stress? Which mechanosensors need an intact glycocalyx in order to fulfill their function? Possible candidates are integrins, syndecans, and/or sarcoglycan complexes sticking out of the sarcolemma which might lose their structure in the absence of an intact glycocalyx. Does fluid flow induce shear stress and a shear strain of the extracellular matrix only, or does the whole muscle fiber undergo shear deformations? Does the extracellular matrix transduce shear stress onto the muscle fiber, as has already been suggested by others using a theoretical model (43)? Are SACs activated directly by shearing of the extracellular matrix, or indirectly via biochemical effects or traction forces of the cytoskeleton? Does the shear stress-induced calcium-influx stimulate growth factor expression such as IGF-I or MGF, and inhibit expression of myostatin (27)? The shear stress-induced growth factor expression in myotubes might also be dependent on NO production, like in bone cells, where NO regulates VEGF production (23). Since IL-6 expression in skeletal muscle is mediated through NO (37), we question the precise role of shear stress in the expression of IL-6. Answers to these questions will increase insight in the role of shear stress in mechanotransduction in skeletal muscle.

## Summary and Conclusion

This is the first study investigating the effect of shear stress on the biochemical response of myotubes. We have demonstrated that C2C12 myotubes respond to fluid shear stress by PFF with changes in the expression of key regulatory factors involved in muscle fiber size adaptation (i.e IGF-I, MGF, VEGF, IL-6, COX-2, myostatin). Moreover, we have shown that myotubes stimulate the production of NO in response to PFF in a shear stress rate-dependent manner. Furthermore, an intact glycocalyx and SACs are required to translate a shear stress stimulation into an NO response. Our data suggest that shear stress may be an important stimulus in the adaptation of muscle fiber size to mechanical loading.

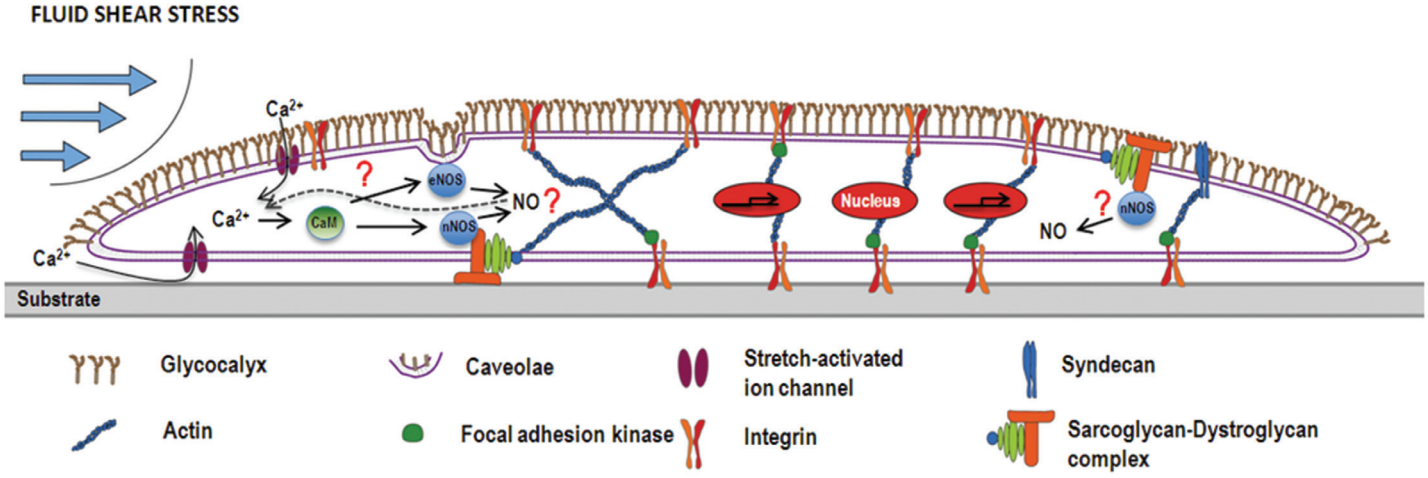


**Figure 7: Mechanotransduction in myotubes in response to fluid shear stress, a conceptual model.** Fluid flow will cause a fluid shear stress onto the extracellular matrix of mytotubes, which will induce a shear deformation of the extracellular matrix, i.e. the glycocalyx. As the glycocalyx extends quite far from the cell membrane (approximately 100-300 nm (51)) it may strengthen and/or amplify the shear stress, thereby enabling sensing of mechanical stimuli by SACs, integrins, syndecans, and/or dystroglycan-sarcoglycan complexes sticking out of the sarcolemma. SACs may be activated directly by shearing of the extracellular matrix, indirectly via biochemical effects, or via opposite traction forces exerted by the actin-cytoskeleton onto the transmembrance complexes (16). Activation of NOS is required to transduce the mechanical signal into NO production. Opening of the SACs could induce an increase in [$Ca2^+$]i, followed by $Ca2^+$/calmodulin signaling, which in turn activates eNOS and/or nNOS. eNOS and nNOS may also be activated directly via caveolea or the sarcoglycan-dystroglycan complex. NO may also affect the $Ca2^+$-influx. Both $Ca2^+$ and NO may affect gene transcription. NO, nitric oxide; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; CaM, calmodulin; SAC, stretch activated ion channel.

## REFERENCES


1. Anderson JE. A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. Mol Biol Cell 11: 1859-1874, 2000.
2. Bryan BA, Walshe TE, Mitchell DC, Havumaki JS, Saint-Geniez M, Maharaj AS, Maldonado AE, and D'Amore PA. Coordinated vascular endothelial growth factor expression and signaling during skeletal myogenic differentiation. Mol Biol Cell 19: 994-1006, 2008.
3. Burkholder TJ. Mechanotransduction in skeletal muscle. Front Biosci 12: 174-191, 2007.
4. Chien S, Li S, and Shyy YJ. Effects of mechanical forces on signal transduction and gene expression in endothelial cells. Hypertension 31: 162-169, 1998.
5. Choi S, Liu X, Li P, Akimoto T, Lee SY, Zhang M, and Yan Z. Transcriptional profiling in mouse skeletal muscle following a single bout of voluntary running: evidence of increased cell proliferation. J Appl Physiol 99: 2406-2415, 2005.
6. Egginton S, Badr I, Williams J, Hauton D, Baan GC, and Jaspers RT. Physiological angiogenesis is a graded, not threshold, response. J Physiol 589: 195-206, 2011.
7. Einhorn TA. Cox-2: Where are we in 2003? - The role of cyclooxygenase-2 in bone repair. Arthritis Res Ther 5: 5-7, 2003.
8. Goldberg AL, Etlinger JD, Goldspink DF, and Jablecki C. Mechanism of work-induced hypertrophy of skeletal muscle. Med Sci Sports 7: 185-198, 1975.
9. Goldspink G. Changes in muscle mass and phenotype and the expression of autocrine and systemic growth factors by muscle in response to stretch and overload. J Anat 194 ( Pt 3): 323-334, 1999.
10. Goldspink G. Mechanical signals, IGF-I gene splicing, and muscle adaptation. Physiology (Bethesda) 20: 232-238, 2005.
11. Hara M, Tabata K, Suzuki T, Do MK, Mizunoya W, Nakamura M, Nishimura S, Tabata S, Ikeuchi Y, Sunagawa K, Anderson JE, Allen RE, and Tatsumi R. Calcium influx through a possible coupling of cation channels impacts skeletal muscle satellite cell activation in response to mechanical stretch. Am J Physiol Cell Physiol 302: C1741-1750, 2012.
12. Hedbom E, and Heinegard D. Binding of fibromodulin and decorin to separate sites on fibrillar collagens. J Biol Chem 268: 27307-27312, 1993.
13. Heinemeier KM, Olesen JL, Schjerling P, Haddad F, Langberg H, Baldwin KM, and Kjaer M. Short-term strength training and the expression of myostatin and IGF-I isoforms in rat muscle and tendon: differential effects of specific contraction types. J Appl Physiol 102: 573-581, 2007.
14. Huijing PA, and Jaspers RT. Adaptation of muscle size and myofascial force transmission: a review and some new experimental results. Scand J Med Sci Sports 15: 349-380, 2005.
15. Huijing PA, Yaman A, Ozturk C, and Yucesoy CA. Effects of knee joint angle on global and local strains within human triceps surae muscle: MRI analysis indicating *in vivo* myofascial force transmission between synergistic muscles. Surg Radiol Anat 33: 869-879, 2011.
16. Ingber DE. Cellular mechanotransduction: putting all the pieces together again. FASEB J 20: 811-827, 2006.
17. Ito N, Ruegg UT, Kudo A, Miyagoe-Suzuki Y, and Takeda S. Activation of calcium signaling through Trpv1 by nNOS and peroxynitrite as a key trigger of skeletal muscle hypertrophy. Nat Med 19: 101-106, 2013.
18. Jarvinen TA, Jozsa L, Kannus P, Jarvinen TL, and Jarvinen M. Organization and distribution of intramuscular connective tissue in normal and immobilized skeletal muscles. An immunohistochemical, polarization and scanning electron microscopic study. J Muscle Res Cell Motil 23: 245-254, 2002.
19. Jaspers RT, Brunner R, Baan GC, and Huijing PA. Acute effects of intramuscular aponeurotomy and tenotomy on multitendoned rat EDL: indications for local adaptation of intramuscular connective tissue. Anat Rec 266: 123-135, 2002.

20. Jaspers RT, Feenstra HM, van Beek-Harmsen BJ, Huijing PA, and van der Laarse WJ. Differential effects of muscle fibre length and insulin on muscle-specific mRNA content in isolated mature muscle fibres during long-term culture. Cell Tissue Res 326: 795-808, 2006.
21. Jaspers RT, Feenstra HM, Verheyen AK, van der Laarse WJ, and Huijing PA. Effects of strain on contractile force and number of sarcomeres in series of Xenopus laevis single muscle fibres during long-term culture. J Muscle Res Cell Motil 25: 285-296, 2004.
22. Juffer P, Jaspers RT, Bakker AD, and Klein-Nulend J. Cellular studies, fluid flow and strain in bone. In: Experimental Research Studies Thieme, in press.
23. Juffer P, Jaspers RT, Lips P, Bakker AD, and Klein-Nulend J. Expression of muscle anabolic and metabolic factors in mechanically loaded MLO-Y4 osteocytes. Am J Physiol Endocrinol Metab 302: E389-395, 2012.
24. Keller P, Keller C, Carey AL, Jauffred S, Fischer CP, Steensberg A, and Pedersen BK. Interleukin-6 production by contracting human skeletal muscle: autocrine regulation by IL-6. Biochem Biophys Res Commun 310: 550-554, 2003.
25. Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, and Burger EH. Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts--correlation with prostaglandin upregulation. Biochem Biophys Res Commun 217: 640-648, 1995.
26. Loufrani L, and Henrion D. Role of the cytoskeleton in flow (shear stress)-induced dilation and remodeling in resistance arteries. Med Biol Eng Comput 46: 451-460, 2008.
27. McCall GE, Allen DL, Haddad F, and Baldwin KM. Transcriptional regulation of IGF-I expression in skeletal muscle. Am J Physiol Cell Physiol 285: C831-839, 2003.
28. McCroskery S, Thomas M, Maxwell L, Sharma M, and Kambadur R. Myostatin negatively regulates satellite cell activation and self-renewal. J Cell Biol 162: 1135-1147, 2003.
29. McGarry JG, Klein-Nulend J, Mullender MG, and Prendergast PJ. A comparison of strain and fluid shear stress in stimulating bone cell responses--a computational and experimental study. FASEB J 19: 482-484, 2005.
30. Nakamura TY, Iwata Y, Sampaolesi M, Hanada H, Saito N, Artman M, Coetzee WA, and Shigekawa M. Stretch-activated cation channels in skeletal muscle myotubes from sarcoglycan-deficient hamsters. Am J Physiol Cell Physiol 281: C690-699, 2001.
31. Nathan C, and Xie QW. Nitric oxide synthases: roles, tolls, and controls. Cell 78: 915-918, 1994.
32. Olfert IM, Howlett RA, Tang K, Dalton ND, Gu Y, Peterson KL, Wagner PD, and Breen EC. Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. J Physiol 587: 1755-1767, 2009.
33. Pennisi CP, Olesen CG, de Zee M, Rasmussen J, and Zachar V. Uniaxial cyclic strain drives assembly and differentiation of skeletal myocytes. Tissue Eng Part A 17: 2543-2550, 2011.
34. Reid MB. Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. Acta Physiol Scand 162: 401-409, 1998.
35. Reilly GC, Haut TR, Yellowley CE, Donahue HJ, and Jacobs CR. Fluid flow induced PGE2 release by bone cells is reduced by glycocalyx degradation whereas calcium signals are not. Biorheology 40: 591-603, 2003.
36. Rittweger J, and Felsenberg D. Recovery of muscle atrophy and bone loss from 90 days bed rest: results from a one-year follow-up. Bone 44: 214-224, 2009.
37. Serrano AL, Baeza-Raja B, Perdiguero E, Jardi M, and Munoz-Canoves P. Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. Cell Metab 7: 33-44, 2008.
38. Shi ZD, and Tarbell JM. Fluid flow mechanotransduction in vascular smooth muscle cells and fibroblasts. Ann Biomed Eng 39: 1608-1619, 2011.
39. Soares AG, Aoki MS, Miyabara EH, Deluca CV, Ono HY, Gomes MD, and Moriscot AS. Ubiquitin-ligase and deubiquitinating gene expression in stretched rat skeletal muscle. Muscle Nerve 36: 685-693, 2007.

40. Soltow QA, Betters JL, Sellman JE, Lira VA, Long JH, and Criswell DS. Ibuprofen inhibits skeletal muscle hypertrophy in rats. Med Sci Sports Exerc 38: 840-846, 2006.
41. Soltow QA, Lira VA, Betters JL, Long JH, Sellman JE, Zeanah EH, and Criswell DS. Nitric oxide regulates stretch-induced proliferation in C2C12 myoblasts. J Muscle Res Cell Motil 31: 215-225, 2010.
42. Stamler JS, and Meissner G. Physiology of nitric oxide in skeletal muscle. Physiol Rev 81: 209-237, 2001.
43. Tarbell JM, and Shi ZD. Effect of the glycocalyx layer on transmission of interstitial flow shear stress to embedded cells. Biomech Model Mechanobiol 12: 111-121, 2013.
44. Tatsumi R, Hattori A, Ikeuchi Y, Anderson JE, and Allen RE. Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. Mol Biol Cell 13: 2909-2918, 2002.
45. Tatsumi R, Wuollet AL, Tabata K, Nishimura S, Tabata S, Mizunoya W, Ikeuchi Y, and Allen RE. A role for calcium-calmodulin in regulating nitric oxide production during skeletal muscle satellite cell activation. Am J Physiol Cell Physiol 296: C922-929, 2009.
46. Thi MM, Tarbell JM, Weinbaum S, and Spray DC. The role of the glycocalyx in reorganization of the actin cytoskeleton under fluid shear stress: a "bumper-car" model. Proc Natl Acad Sci U S A 101: 16483-16488, 2004.
47. Tidball JG, Lavergne E, Lau KS, Spencer MJ, Stull JT, and Wehling M. Mechanical loading regulates NOS expression and activity in developing and adult skeletal muscle. Am J Physiol 275: C260-266, 1998.
48. van Wessel T, de Haan A, van der Laarse WJ, and Jaspers RT. The muscle fiber type-fiber size paradox: hypertrophy or oxidative metabolism? Eur J Appl Physiol 110: 665-694, 2010.
49. Vandenburgh HH, Hatfaludy S, Karlisch P, and Shansky J. Mechanically induced alterations in cultured skeletal muscle growth. J Biomech 24 Suppl 1: 91-99, 1991.
50. Weinbaum S, Tarbell JM, and Damiano ER. The structure and function of the endothelial glycocalyx layer. Annu Rev Biomed Eng 9: 121-167, 2007.
51. Weinbaum S, Zhang X, Han Y, Vink H, and Cowin SC. Mechanotransduction and flow across the endothelial glycocalyx. Proc Natl Acad Sci U S A 100: 7988-7995, 2003.
52. Weinheimer EM, Jemiolo B, Carroll CC, Harber MP, Haus JM, Burd NA, LeMoine JK, Trappe SW, and Trappe TA. Resistance exercise and cyclooxygenase (COX) expression in human skeletal muscle: implications for COX-inhibiting drugs and protein synthesis. Am J Physiol Regul Integr Comp Physiol 292: R2241-2248, 2007.
53. Williams PE, and Goldspink G. Changes in sarcomere length and physiological properties in immobilized muscle. J Anat 127: 459-468, 1978.
54. Yang H, Alnaqeeb M, Simpson H, and Goldspink G. Changes in muscle fibre type, muscle mass and IGF-I gene expression in rabbit skeletal muscle subjected to stretch. J Anat 190 ( Pt 4): 613-622, 1997.
55. Yucesoy CA, Koopman BH, Huijing PA, and Grootenboer HJ. Three-dimensional finite element modeling of skeletal muscle using a two-domain approach: linked fiber-matrix mesh model. J Biomech 35: 1253-1262, 2002.