A single bout of exercise with high mechanical loading induces the expression of Cyr61/CCN1 and CTGF/CCN2 in human skeletal muscle

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MECHANICAL STIMULI REGULATE the expression of diverse extracellular matrix (ECM) components (21). Aerobic exercise training has recently been shown to modulate the expression of several ECM genes, including vascular endothelial growth factor (VEGF)-A-related genes (38). VEGF-A and its receptors play an important role in promoting angiogenesis during cardiovascular development and in certain pathological states, e.g., cancer (3). In addition to being a strong angiogenic factor, VEGF-A seems to have a role in coordinating the growth and phenotype of myofibers and their interaction with capillaries (39, 40). Several studies have reported increased expression of VEGF-A in skeletal muscle after exercise (1, 12, 31) or electrical stimulation (16, 37). Hypoxia or reduced oxygen tension has been proposed to be an important trigger for VEGF-A expression and subsequent angiogenesis. However, mechanical stretch without hypoxia can stimulate VEGF-A expression and angiogenesis in vitro. In cardiac myocytes and coronary microvascular endothelial cells (42), as well as in bladder smooth muscle cells (43), cyclic stretch induces VEGF-A production. Results from skeletal muscle overload studies suggest that this is also the case in vivo (9, 33). Hypoxia-inducible factor-1α (HIF-1α) is induced by hypoxia e.g., in ischemic tissues, but HIF-1α mRNA expression has also been shown to increase in response to mechanical stress without ischemia in skeletal muscle capillaries (25). In the myocardium, however, no change was observed in HIF-1α mRNA levels after mechanical stress under nonhypoxic conditions (19), but it increased at the protein level.

In addition to VEGF-A, the VEGF protein family contains VEGF-B, VEGF-C, VEGF-D, and placental growth factor, which all have angiogenic and/or lymphangiogenic properties (35). VEGF-B plays a role in the vascularization of adult and embryonic tissues, particularly in cardiac and skeletal muscle (28). VEGF-C and VEGF-D are considered the main growth factors for lymphatic vessels (18), and the mature form of VEGF-D is also a potent inducer of angiogenesis (32). Lymphatic vessels are part of the circulatory system and function in close association with blood vessels. However, data regarding the lymphatic part of the circulatory system in skeletal muscle, and especially its relation to exercise, are very limited. Furthermore, the effects of exercise on the expression of other VEGFs than VEGF-A have not been reported.

ECM-associated CCN family proteins cysteine-rich protein 61 (Cyr61/CCN1) and connective tissue growth factor (CTGF/CCN2) are important regulators of angiogenesis, endothelial cell function, and ECM modulation (2, 29). They also regulate the activity and production of other angiogenic proteins like VEGF-A (29, 43). In myocardium, Cyr61 has been shown to increase in response to pressure overload and ischemia (15). Also, in mechanically challenged vascular (11) and bladder (43) smooth muscle cells, a marked increase in Cyr61 expression and release has recently been reported, and this increase subsequently mediates VEGF-A expression (43). In human fibroblasts, mechanical stress increased the expression of both Cyr61 and CTGF mRNA (34). In skeletal muscle, increased expression of Cyr61 and CTGF was observed as a part of microarray data from three subjects after eccentric exercise (6). The results from other types of muscle tissues and cells and the...
preliminary findings from the skeletal muscle microarray data raise the question whether these proteins are also affected in mechanically challenged skeletal muscle.

The purpose of the present study was to examine the effects of jumping exercise, which causes high mechanical loading on the thigh muscles, on the expression of ECM-associated angiogenic factors Cyr61, CTGF, VEGF family members, and HIF-1α in human skeletal muscle. We hypothesized that, as in cardiac and smooth muscle cells, mechanical stress would induce the production of CCN proteins in skeletal muscle. These proteins are suggested to induce genetic reprogramming of angiogenic (e.g., VEGF-A), structural, and ECM proteins in other tissues. Furthermore, exercise with eccentric actions was expected to cause edema in the muscles, thus challenging the lymphatic system a few days postexercise and possibly affecting the expression of VEGF-C and VEGF-D.

METHODS

Subjects. Eight healthy men volunteered for the study. The mean age of the subjects was 26 ± 4 yr, height 179 ± 7 cm, and body mass 78 ± 9 kg (means ± SD). They had different training backgrounds, but none of them were involved in any systematic training during the study period or the year before it. All subjects were informed of the purpose, nature, and potential risks of the study, and they gave their written consent prior to participation in the study. The study was performed according to the Declaration of Helsinki, and the ethical committee of the University of Jyväskylä approved the study protocol.

Exercise protocol. The subjects started with a 5-min warm-up on a bicycle ergometer, after which maximum voluntary contraction (MVC) of both legs was measured unilaterally. Thereafter, the subjects were seated on a special sled ergometer (23) inclined at 20.3° from the horizontal. This apparatus has been used routinely for loading the leg extensor muscles in stretch-shortening-type action (27). After determination of optimal dropping height (highest rebound jump), subjects performed 100 maximal unilateral drop jumps at a rate of one every 5 s. Maximal jumps were immediately followed by continuous jumping to submaximal height (50% of individual maximum) until exhaustion. All the jumps were performed by the right leg while the left leg served as a control. Unilateral MVC of both legs was measured again immediately and 48 h after the exercise.

Muscle biopsies. Muscle needle biopsies were obtained under local anesthesia (lidocaine-epinephrine, 1%) 30 min and 48 h after the exercise from the middle region of the vastus lateralis muscle of the exercised leg. A control biopsy was taken from the nonexercised leg under local anesthesia (lidocaine-epinephrine, 1%) 30 min and 48 h after the exercise from the middle region of the vastus lateralis muscle of the nonexercised leg. Before the exercise, a control biopsy was taken from the same site of the nonexercised leg. A control biopsy was also taken before, immediately after, and 2 days after the exercise protocol.

Western blotting. Muscle samples were weighted and homogenized in RIPA buffer (PBS, 1% Igepal, 0.5% Na-deoxycholate, 0.1% SDS with protease inhibitors PMSF, aprotinin, and leupeptin) to produce a 6% homogenate. Total protein concentration was measured with a DC protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s protocol. The samples (15 μg) were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (GE Healthcare Biosciences, Uppsala, Sweden). Filters were blocked with rabbit goat-neonatal serum in 1% BSA in PBS and incubated with polyclonal goat-anti-human (Cyr61 or CTGF, primary antibodies; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-VEGF-A (Hs00189521_m1), HIF-1α (Hs00173634_m1), VEGF-C (Hs00153458_m1), VEGF-D (Hs00119521_m1), HIF-1α (Hs00153153_m1), Cyr61 (Hs00155479_m1), and CTGF (Hs00170014_m1) and designed and synthesized by Applied Biosystems (ABI Gene expression assays). Primer pairs were designed so that they overlapped an exon-exon boundary to avoid interference from possible genomic DNA contamination. They were also designed to detect all splicing variants of the gene. Expression data were normalized to the levels of GAPDH (Hs99999905_m1) mRNA. GAPDH was chosen because it is considered the most stable internal control in exercise studies (17). We also calculated GAPDH expression in relation to the amount of total RNA, and no differences in GAPDH expression were found between the control and experimental samples. All samples were analyzed in triplicate.

Immunohistochemistry. Transverse sections of the muscle samples (10 μm) were cut in a cryostat and used for the immunohistochemical staining of Cyr61 and CTGF. Also, routine hematoxylin-eosin staining was performed. For immunohistochemistry, the sections were air-dried, fixed with 4% formaldehyde, and incubated in 3% BSA-PBS to block nonspecific binding. Subsequently, sections were incubated overnight at +4°C with primary antibodies: polyclonal goat anti-human Cyr61 or goat anti-human CTGF (Santa Cruz Biotechnology) with monoclonal mouse anti-human CD31 (BD Biosciences Pharmingen, San Diego, CA) or mouse anti-human myosin heavy chain 1 (MHC1; A4.951). The monoclonal antibody against MHC1 was developed by H. Blau et al. and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development. Primary antibodies were detected using donkey anti-goat secondary antibodies with fluorescent Alexa Fluor 488 and 555 labels (Molecular Probes, Leiden, The Netherlands). Localization of Cyr61 and CTGF was analyzed with a confocal microscope (FV-1000, Olympus, Tokyo, Japan). Negative controls were done by omitting the primary antibodies.

Cyr61 and CTGF mRNA in muscle fibers. To verify the expression of Cyr61 and CTGF in the muscle fibers, which was seen with immunohistochemistry, single muscle fibers were separated from the mouse flexor digitorum brevis muscle. Muscles were incubated with collagenase, fibers were purified from other cell types with column purification and cultured on Matrigel-coated dishes (30). During purification and before RNA isolation, the dishes were inspected under a microscope to be sure that no cells other than muscle fibers were present. Total RNA was extracted with a Pico Pure RNA isolation kit (Arcturus, Mountain View, CA) and transcribed to cDNA with Sensiscript RT kit (Qiagen, Hilden, Germany). Real-time PCR reactions were performed as described above with TaqMan primers and probes (Applied Biosystems) for mouse Cyr61 (Mm00487498_m1) and CTGF (Mm00515790_g1).

Creatine kinase activity and blood lactate. Blood samples were taken before, immediately after, and 2 days after the exercise protocol. Five milliliters of blood were withdrawn, serum separated, and further analyzed for creatine kinase (CK) activity using a commercial kit.
EXERCISE-INDUCED EXPRESSION OF CCN PROTEINS


(Biochemical Boehringer, Mannheim, Germany). Whole blood lactate was measured at rest, after the preexercise warm-up, and postexercise with LactatePro analyzer (Arkay, Tokyo, Japan).

Statistical analysis. The data are presented as means ± SD. The mRNA expression results are reported as relative fold-change to the control (nonexercised leg) value, which is set to 1. All data were tested for normality. ANOVA for repeated measures was used to determine significant changes in mRNA and protein expression. Pearson’s correlation coefficient was used to study associations between the responses in mRNA expression and physiological characteristics. Data were analyzed with SPSS for Windows statistical software release 13.0.1 (SPSS, Chicago, IL), and differences were considered significant at \( P < 0.05 \).

RESULTS

Physiological characteristics. The average number of jumps (maximal + submaximal) performed during the exercise protocol was 876 ± 609 (mean ± SD, range 403–2,032). The mean duration of jumping was 19.3 ± 13.2 min. Isometric maximal force of the exercised leg decreased \( (P < 0.001) \) 30 min postexercise \( (847 ± 92 \; \text{vs.} \; 584 ± 86 \; \text{N}) \) and returned close to the preexercise levels 48 h after \( (727 ± 125 \; \text{N}; \; P = 0.051) \). No change occurred in the MVC in the control leg. Mean serum CK activity increased significantly \( (P < 0.05) \) immediately after the exercise \( (350 ± 244 \; \text{before} \; \text{and} \; 443 ± 306 \; \text{IU/l after}; \; \text{means} ± \text{SD}) \). It was further increased at 48 h \( (824 ± 843 \; \text{IU/l}; \; P = 0.069) \), but the increase was not statistically significant due to the large variation. This was mainly due to one subject who had markedly higher value \( (2,865 \; \text{IU/l}) \) than the other subjects. Lactate increased from 1.4 ± 0.3 mM before the exercise to 2.03 ± 0.35 mM after the exercise \( (P < 0.001) \). Necrotic muscle fibers were not observed in any of the muscle samples at 30 min or 48 h after the exercise, although slight swelling of the fibers was present at 48 h.

Messenger RNA expression. Exercise induced a 14-fold increase in the mRNA level of Cyr61 30 min postexercise \( (P < 0.001) \) compared with the control leg (Fig. 1A). The mRNA expression of CTGF (Fig. 1A) increased by 2.5-fold \( (P < 0.001) \). Cyr61 mRNA remained elevated at 48 h postexercise (threelfold compared with the control; \( P < 0.05 \)), whereas CTGF mRNA levels were returned almost to control values at that time point. The mRNA levels of VEGF-A, -B, -C, and -D or HIF-1α did not change significantly at either 30 min or 48 h postexercise (Fig. 1, B and C). However, there were large individual differences in the responses of VEGF-A and VEGF-B mRNA after the exercise, and the group variation increased markedly. In VEGF-C, VEGF-D, and HIF-1α, no change was observed, and the variation remained small at all time points.

The individual changes in mRNA expression did not show any significant correlation with the exhaustion time, number of jumps performed, or changes in blood lactate concentration and serum CK activity.

Cyr61 and CTGF protein expression. Cyr61 protein levels increased both 30 min and 48 h postexercise compared with the control samples \( (P < 0.05) \) (Fig. 2). A similar trend was observed in the CTGF protein, but owing to the larger variation it was not statistically significant \( (P = 0.16) \).

Localization of Cyr61 and CTGF proteins. Cyr61 and CTGF proteins localized both to muscle fibers and the ECM (Fig. 3). There were differences in staining intensity between the fibers, and double staining with slow myosin (MHC1) showed that Cyr61 and CTGF were expressed more in fast than in slow muscle fibers. Isolated muscle fibers from mouse expressed both Cyr61 and CTGF mRNA. This suggests that CTGF and
Cyr61 are produced in muscle fibers, thereby supporting the findings from immunohistochemistry. Negative controls did not show any background staining.

DISCUSSION

The present exercise model was chosen to exert high mechanical loading on muscle fibers and connective tissue in the thigh muscles. In jumping exercise, muscle fibers undergo a repeated stretch-shortening cycle with both eccentric and concentric phases. This occurs also in natural human locomotion, but the loading was higher in the present model compared with, e.g., walking. The advantage of the model is that it allowed exercise of only one leg while the other served as a control. The present exercise can be considered strenuous, since immediately after the cessation of exercise blood lactate concentration was high and MVC decreased significantly. Morphologically damaged muscle fibers were not observed in any of the muscle samples, but slight swelling of the fibers occurred at 48 h postexercise in the exercised leg. This is in accordance with earlier findings from humans, which suggest that, instead of focal fiber necrosis, eccentric exercise causes remodeling of muscle fibers (41).

The present results showed that jumping exercise, most probably due to mechanical loading on muscle fibers and

Fig. 2. Protein expression of Cyr61 and CTGF in skeletal muscle. A: Cyr61 protein levels were increased significantly both 30 min and 48 h after the exercise. B: CTGF levels also showed a trend to increased expression, but this was not statistically significant (*P = 0.16). Data are presented as optical density (OD) relative to total protein content (means ± SD). *P < 0.05.

Fig. 3. Protein localization of CTGF and Cyr61 in human skeletal muscle. Confocal microscopic images show CTGF (A and B) and Cyr61 (C and D) with red color and slow muscle fibers with green. Cyr61 and CTGF are localized to skeletal muscle fibers and surrounding ECM. Fast muscle fibers expressed more CCN proteins than slow fibers (green staining for slow fibers). E: CTGF double staining with CD31 to stain capillaries (green), F: Cyr61 double staining with CD31. Similar to CTGF, Cyr61 localized also to capillaries to some extent but was mainly found in ECM and the muscle fibers. G: image with larger magnification showing CTGF staining in ECM (examples shown with arrows). H: Cyr61 staining was also found around capillaries (green) in ECM (arrows).
connective tissue, induced a rapid increase in the mRNA levels of ECM-related proteins Cyr61 and CTGF in human skeletal muscle. We also demonstrated that Cyr61 and CTGF proteins are localized in both the ECM and muscle fibers, and the expression of Cyr61 was increased after the exercise also at the protein level. The observed increase in the mRNA and protein expression of Cyr61 are in line with the observations by Hilfiker-Kleiner et al. (15), who showed that Cyr61 is induced in the myocardium by pressure overload and in cardiomyocytes after mechanical stretch. They suggested that Cyr61 might play an important role in the adaptation of the heart to cardiovascular stress. Cyr61 is classified as an immediate early gene, which is rapidly induced in response to externally applied cyclic mechanical strain in cardiac and smooth muscle cells and in fibroblasts (34, 36, 43). The present results show that this is also the case in vivo in skeletal muscle. Connective tissue growth factor belongs to the same CCN protein family as Cyr61 and has earlier been shown to be upregulated by mechanical stress in fibroblasts (34). Our results also support the microarray findings by Chen et al. (6), which showed that eccentric compared with concentric exercise induced upregulation of Cyr61 and CTGF mRNA in skeletal muscle. Interestingly, increased expression of Cyr61 and CTGF in skeletal muscle was also found in a denervation study by Magnusson et al. (24) and in our previous study on diabetic mice (20).

To verify the finding that high mechanical loading with eccentric component induces the expression of CCN proteins, we checked publicly available skeletal muscle microarray data sets from GEO (http://www.ncbi.nlm.nih.gov/sites/entrez). Acute maximal eccentric contractions (7) seemed to increase Cyr61 expression both 1 and 6 h after the exercise (no probe for CTGF in the array). Also, in a recent study by Kostek et al. (22), lengthening contractions induced Cyr61 upregulation 3 and 6 h after the exercise, and the expression of CTGF was also increased to some extent. Shortening contractions did not seem to have a marked effect. Acute voluntary running for 3 or 12 h affected neither Cyr61 nor CTGF expression in mice (8), which is in line with our recent findings from a treadmill endurance running study (19a). After long-term endurance training, increased expression was found in CTGF but not in Cyr61 in humans (38).

The localization of Cyr61 and CTGF in skeletal muscle has not been reported earlier. In the present study, we showed that fast muscle fibers express more Cyr61 and CTGF compared with slow fibers. Cyr61 and CTGF staining in human skeletal muscle was also found in ECM surrounding the muscle fibers where blood vessels are also located. The mRNA results from isolated mouse muscle fibers support the finding that muscle fibers produce these proteins; however, the limitation in the mRNA findings from single fibers is that they are from mouse and not from human muscle. Because CCN proteins contain a signal peptide for secretion (29), it is possible that these proteins are produced in muscle fibers and secreted into the interstitial space to perform their functions in ECM. Cyr61 is able to execute its functions through autocrine and paracrine mechanisms by interacting with integrins and heparin sulfate proteoglycan (5). In the myocardium, Cyr61 has been shown to localize to myocytes and blood vessels (15), and vascular smooth muscle cells have been shown to produce Cyr61 (11). In the present study, Cyr61 and CTGF were expressed more in fast than slow fibers, both in the control and in the exercised muscles. Fast muscle fibers are recruited more when exercise is performed at high intensity. The exercise model used in the present study recruits fast fibers efficiently, which may explain the marked increase in Cyr61 and CTGF mRNA expression.

Mechanical stretch-induced Cyr61 expression is proposed to stimulate genetic reprogramming of angiogenic, adhesive, and structural proteins and CTGF to promote ECM accumulation, especially type 1 collagen (2, 4, 43). There is strong evidence that Cyr61 and CTGF play a role in the regulation of endothelial cell function and angiogenesis (reviewed in Ref. 2). They have both direct and indirect angiogenic actions. The indirect actions include modulation of the transcriptional rate and activity of other angiogenic molecules (e.g., VEGF-A) and modification of the structure or stability of the ECM. In mechanically challenged smooth muscle cells, increased Cyr61 resulted in a significant increase in VEGF-A expression. This increase was stretch specific and occurred without hypoxia (43). After the present jumping exercise, the changes in VEGF-A mRNA expression were not statistically significant. Although at 48 h postexercise the group mean value was 1.8-fold higher in the exercised compared with the control leg, variation between the subjects was high. The 30-min and 48-h time points in the present study were chosen to represent immediate and delayed responses, since Cyr61 and CTGF are known as immediate early genes and it was expected that the lymphatic system would be challenged a few days after the exercise when edema is normally present. This is not the optimal timing for VEGF-A, since it has been shown to peak 3–6 h after endurance-type exercise (1, 13). The variation in VEGF-B mRNA also increased after the exercise, as in VEGF-A, but in VEGF-C, VEGF-D, and HIF-1α mRNA the group means showed no change and the variation remained small at all time points. Since the control sample from the nonexercised leg was taken at the 48-h time point, it is possible that systemic responses to exercise might have masked part of the changes in VEGF-A and VEGF-B and increased the variance.

The potential functions of Cyr61 and CTGF in healthy skeletal muscle after exercise include the induction of angiogenic responses. Fataccioli et al. (10) showed with gene transfer that Cyr61 was effective in improving perfusion in ischemic hindlimb model. Its role in vascular development has also been shown with knockout mice, which suffer embryonic death due to vascular defects in placenta (26). Other possible mechanisms of CCN proteins in skeletal muscle include modulation of ECM, which is known to occur due to exercise (21). Cyr61 has also been shown to have both pro- and antiapoptotic effects in different cell types (5). The signaling pathways mediated by CCN proteins are being intensively studied at the moment, but their role in skeletal muscle is yet to be fully defined.

We also hypothesized that exercise may promote proliferation of lymphatic vessels (lymphangiogenesis) analogous to angiogenesis, because skeletal muscle lymph flow has been shown to increase during both isometric and dynamic muscle contractions (14). In addition, postexercise edema, which is often prominent a few days after exercise, indicates a need for increased removal of interstitial fluid from the muscles. VEGF-C and -D are considered to be the main lymphangiogenic growth factors, and their increased expression leads to the postnatal growth of lymphatic vessels (18). The present results, however, suggest that mechanical loading does not
affect VEGF-C or VEGF-D mRNA expression in skeletal muscles either immediately or 2 days after. This is also supported by our unpublished results from a running exercise experiment with mice, where no change was observed in VEGF-C and VEGF-D expression 3 and 6 h after the exercise (19a).

In conclusion, in the present study, we showed that the expression of Cyr61 and CTGF is induced in human skeletal muscle by exercise with high mechanical loading. These proteins were found both in the muscle fibers and surrounding ECM. On the basis of the present findings and the related literature on the myocardium and smooth muscle, it is suggested that CCN family proteins may play an important role in exercise-induced remodeling of the skeletal muscle vasculature, ECM, and myofibers since they have both direct and indirect effects on various cell types within skeletal muscle.

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