Journal of Cellular Biochemistry 100:750–761 (2007)

Static Strain Stimulates Expression of Matrix Metalloproteinase-2 and VEGF in Microvascular Endothelium Via JNK- and ERK-Dependent Pathways

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Abstract VEGF and MMP protein production are both required for exercise-induced capillary growth in skeletal muscle. The underlying process by which muscle activity initiates an angiogenic response is not established, but it is known that mechanical forces such as muscle stretch are involved. We hypothesized that stretch of skeletal muscle microvascular endothelial cells induces production of MMP-2 and VEGF through a common signal pathway. Endothelial cells were grown on Bioflex plates and exposed to 10% static stretch for up to 24 h. MMP-2 protein level was measured by gelatin zymography and VEGF, MMP-2, and MT1-MMP mRNA levels were quantified by real-time quantitative PCR. ERK1/2 and JNK phosphorylation and VEGF protein levels were assessed by Western blotting. Effects of mitogen-activated protein kinases (MAPKs) (ERK1/2, JNK) and reactive oxygen species (ROS) on stretch-induced expression of MMP-2 and VEGF were tested using pharmacological inhibitors. Stretching of endothelial cells for 24 h caused significant increases in MMP-2 protein and mRNA level, but no change in MT1-MMP mRNA. While MMP-2 protein production was enhanced by H_2O_2 in unstretched cells, ROS inhibition during stretch did not diminish MMP-2 mRNA or protein production. Inhibition of JNK suppressed stretch-induced MMP-2 protein and mRNA, but inhibition of ERK had no effect. In contrast, inhibition of ERK but not JNK attenuated the stretch-induced increase in VEGF mRNA. Our results demonstrate that differential regulation of MMP-2 and VEGF by MAPK signal pathways contribute to stretch-induced activation of microvascular endothelial cells. J. Cell. Biochem. 100: 750–761, 2007.

Key words: proteolysis; angiogenesis; mechanical strain; MAP kinases; ROS

Angiogenesis occurs in adult skeletal and cardiac muscle in response to repeated bouts of exercise, and serves to maintain tissue oxygenation in the face of increased cellular metabolic activity [Hudlicka et al., 1992; Prior et al., 2004]. Events that are prerequisite for capillary sprouting include upregulation of VEGF, and production of matrix metalloproteinases MMP-2 and MT1-MMP [Haas et al., 2000; Amaral et al., 2001; Milkiewicz et al., 2001]. VEGF, via interaction primarily with VEGFR2, drives endothelial cell proliferation, migration, and invasion. MMPs are zinc-dependent proteases with varied specificity for multiple extracellular matrix proteins (collagen, laminin, fibronectin) as well as non-matrix substrates (cytokines, growth factors, and adhesion molecules) [Birkedal-Hansen, 1995; Chang and Werb, 2001], and they provide the proteolytic machinery to allow sprouting of endothelial cells through the basement membrane and the interstitial matrix. Because the interstitial extracellular matrix serves as adhesion sites for both capillaries and skeletal myocytes, myocyte contraction and relaxation creates tensional forces within the muscle that significantly impact on the microvascular network. For instance, stretching of skeletal muscle induces longitudinal stretching of capillaries, and results in capillary growth [Ellis et al., 1990;

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Grant sponsor: Canadian Institutes of Health Research.

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Received 30 March 2006; Accepted 7 June 2006
DOI 10.1002/jcb.21055

Egginton et al., 1998, 2001]. It is feasible that stretch may act directly on the capillary endothelium, thus initiating the process of abluminal capillary sprouting. However, previous analyses were performed on muscle homogenates, so that the cell type(s) and signal pathways responsible for stretch-induced VEGF and MMP production remain to be defined.

Endothelial cells are mechanosensitive, detecting altered tension through interactions between extracellular matrix components, transmembrane integrin receptors, and cytoplasmic scaffolding and signaling proteins [Hynes, 1992; Stromblad and Cheresh, 1996]. The mitogen-activated protein kinase (MAPK) family plays important roles in the transduction of mitogenic and differentiation signals from the cytoplasm to the nucleus in response to mechanical strain [Chess et al., 2000; Li et al., 2003]. An immediate but transient phosphorylation of ERK1/2, JNK, and p38 kinases occurs in endothelial cells exposed to cyclic strain [Azuma et al., 2000]. Previous studies in a number of cell types showed a positive relationship between ERK1/2 and/or JNK activation and MMP-2 production [Montesano et al., 1999; Kurata et al., 2000; Boyd et al., 2004; Ispanovic and Haas, 2004]. Reactive oxygen species (ROS) also are able to regulate intracellular pathways in response to mechanical stress, and are known to promote MMP-2 production in some cell types [Yoon et al., 2002; Grote et al., 2003].

While it has been established that angiogenesis in skeletal muscle is stimulated by mechanical stimuli [Hudlicka et al., 1992], the molecular events controlling production of VEGF and MMPs in physiological angiogenesis remain poorly understood. We hypothesized that mechanical stretch would induce the coordinate expression of MMPs and VEGF in skeletal muscle microvascular endothelial cells. We found evidence that divergent branches of the MAPK family control the production of these key contributors to skeletal muscle angiogenesis.

METHODS

Animal Model

Male Sprague–Dawley rats (Charles River), 300 g body weight, were used in these studies. All surgical procedures were performed under aseptic conditions and sodium pentobarbital anesthesia, in accordance with Animal Care Procedures at York University and the APS Guiding Principles in the Care and Use of Animals. Extensor digitorum longus (EDL) muscle was subjected to sustained stretch by overload following unilateral extirpation of the agonist muscle tibialis anterior (TA). An incision was made in the leg skin from the knee joint to the foot superficial to the TA muscle. The TA tendon was released from connective tissue, gently lifted up and sectioned at its distal end leaving EDL muscle untouched. Each animal was implanted with an Alzet osmotic pump (1003D; Duret Corporation) containing vehicle or vehicle and the JNK inhibitor SP600125 (Sigma Aldrich). The vehicle was composed of (v/v): 30% PEG400; 20% polypropylene glycol; 15% cremophor EL; 5% ethanol; 30% saline (90.9% NaCl w/v in ddH_2O). This solution was mixed then filter sterilized (0.22 μm syringe filter). SP600125 (SP; 50 mg) was dissolved in 4 ml vehicle solution. Each pump and attached vinyl catheter (PE-60) was back-filled with 150 μL of the vehicle or vehicle +SP. The Alzet model 1003D, with a delivery rate of 1 μL/h, provided a cumulative delivery of 300 μg of SP per rat per day. Pumps were implanted subcutaneously at the top of the hindleg undergoing extirpation. The vinyl catheter attached to the pump was positioned under the skin of the leg to lie over the EDL muscle. Needle perforations in the final 0.5 cm of the vinyl catheter provided delivery of drug along the length of the EDL muscle.

Sutures closed the connective tissue overlying the muscle, and the skin was closed with staples. The EDL muscles from the operated legs were removed under sodium pentobarbital anesthesia (50 mg/kg; via intraperitoneal injection) 4 days following surgery.

Cell Culture

Skeletal muscle endothelial cells were isolated from EDL muscles of male Sprague–Dawley rats (300 g), as described previously [Han et al., 2003]. Cells were grown in gelatin-coated flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 0.11 mg/mL sodium pyruvate, 0.3 mg/mL Glutamax, 50 μg/mL penicillin, 50 μg/mL streptomycin. All media components were purchased from Invitrogen, Canada. Cells were maintained at 37°C

and 7% CO_2, and used in this study between the 4th and 10th passages. MAPK and ROS inhibitors were purchased from Calbiochem.

Application of Mechanical Strain

Endothelial cells were plated at a density of 1 million cells/well in six-well plates coated with type I collagen (Bioflex type I collagen culture plate, Flexcell International, Hills Borough, NC). After 18 h, cells were exposed to static stretch using a computer driven vacuum-operated, stress-providing instrument (Flexcell strain unit FX-4000 Tension Plus, Flexcell International). The vacuum-induced constant stretch with 10 or 20% elongation of the diameter of the flexible surface. The Bioflex loading station is designed to provide uniform radial and circumferential strain across a membrane surface [Sumpio et al., 1987]. The cells were exposed to stretch for varied times (0, 10, 30 min; 1, 6, or 24 h) dependent on the experiment. Control cells were plated on Bioflex plates for an equivalent time, but not subjected to stretch. For MAPK inhibition, cells were pretreated with inhibitors (0.3 μM U0126, 50 μM SP600125; Calbiochem) for 3 h prior to initiation of stretch.

Role of Reactive Oxygen Species in MMP-2 Production

Microvascular endothelial cells (1 million cells/35 mm dish) were exposed to phenazine methosulfate (PMS; 2 μM) for 48 h in order to elicit a sustained increase in H_2O_2. Some cells were incubated with 50 μM ebselen for the final 24 h of culture. Ebselen is a synthetic selenium-containing compound with glutathione peroxidase mimetic properties [Maiorino et al., 1988]. Cells were then analyzed by fluorescence or lysed for gelatin zymography. PMS-dependent increase in intracellular ROS was confirmed by measuring the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Oregon). Cells were loaded with 5 μM DCFH-DA for 15 min, then washed with PBS and visualized at 10× or 20× magnification using a Zeiss 200 M inverted microscope, and fluorescein filter set. Images were digitally acquired with a cooled CCD camera (Quantix 57) and MetaMorph software (Universal Imaging). In experiments involving ROS inhibition, SMEC were pretreated for 2 h with one of several ROS inhibitors prior to 24 h stretch (80 μM ebselen, 10 μM diphenyleneiodonium (DPI), 10 mM N-Acetyl-Cysteine (NAC), 50 μM Apocynin). DPI is a flavoprotein inhibitor that blocks electron transport in a broad range of systems including NAD(P)H oxidases, NO synthase, mitochondria Complex I, and other systems [Li and Trush, 1998]. We also tested apocynin, which was reported to be a more specific inhibitor of the neutrophil NADPH oxidase [Grimminger et al., 1995], and NAC, which is a thiol reductive agent that scavenges H_2O_2. The cells were treated with DMSO as control. Total cellular protein was isolated and analyzed by gelatin zymography.

Gelatin Zymography

Total cellular protein was extracted in a 100 mM Tris buffer containing 1% Triton-X-100 and 5% glycerol, in the presence of 0.1% protease inhibitor cocktail and 1 μM sodium orthovanadate. Total cellular protein was quantified using the bichoninic acid assay (BCA; Pierce) according to the manufacturer's instructions. Cell lysates (10 μg) prepared with non-reducing sample buffer were separated on 8% SDS–PAGE gel embedded with 0.02% gelatin. After electrophoresis, gels were washed for 1 h with Triton X-100 and with ddH_2O and incubated for 20 h at 37°C in 50 mM Tris-HCl, 5 mM $CaCl_2$. Enzyme activity was stopped using 100 mM EDTA and gels were fixed then stained with Coomassie brilliant blue. Following destaining, gelatinolytic bands were imaged using the FluorChem Imager (Alpha Innotech). Quantification of protein expression was done using Alpha Ease software. Total MMP-2 was quantified as the sum of latent and active bands.

Reverse Transcription and Quantitative Real-Time PCR

Following the stretch protocol, cDNA from SMEC were produced without RNA isolation using Cells-to cDNA™ kit (Ambion, TX). Briefly, SMEC cells were washed three times in sterilized PBS at 4°C and then heated (75°C for 15 min) in Cell Lysis buffer II. Next, the cell lysate was treated with DNase 1 (0.004 U/μL) to degrade genomic DNA (37°C for 15 min) followed by inactivation of DNase (75°C for 5 min). The cell lysate was stored at −20°C until it was used for the reverse transcription reaction. Ten microliters of cell lysate was reverse transcribed in a 20 μL reaction using reagents from the Cells-to cDNA™ kit according

to manufacturer's protocols. The cDNA was diluted fourfold with RNAse-free water. Quantitative real-time PCR (Q-PCR) was performed by use of an ABI PRISM 7700 Sequence Detection System. VIC-labeled control ribosomal RNA and FAM-labeled MT1-MMP probe and primers sets were purchased from Applied Biosystems (catalogue numbers P/N4308329 and Mm00485054-m1, respectively). Primers and Taq Man® FAM-labeled probe for MMP-2 were designed using PrimerExpress 1.0 software (PerkinElmer Life Sciences). MMP-2 probe—6FAM—caa tgc tga tgg aca gcc ctg ca—MGBNFQ; Forward primer—cca tga agc ctt gtt tac ca; Reverse primer—ctg gaa gcg gaa cgg aaa. A 25-μL reaction mixture contained 12.5 μL of TaqMan® universal PCR master mix PCR Mix (Applied Biosystems), 4 μL of cDNA template and the appropriate concentrations of gene-specific primers and probe sets. PCR was performed with thermal conditions as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold (C_t) values were used to determine the amount of MMP-2 and MT-1 MMP mRNAs and 18S rRNA for all groups. The mean C_t values of triplicate samples from each group were determined and then $\Delta C_{T\ (Sample)}$ was calculated according to equation $\Delta C_{T\ (Sample)} = \text{average } C_{T\ (Sample)} - \text{average } C_{T\ (rRNA)}$. The fold change in MMP-2 and MT1-MMP mRNA expression was calculated using $\Delta\Delta C_T$ method as described in the Applied Biosystems manual. mRNA expression levels of target genes were expressed relative to the appropriate static control, which was set to 1.0.

Immunoblotting

Equal amounts (10 or 20 μg) of total cellular protein extracts (prepared as for the gelatin zymography) were separated through 10% SDS–PAGE gel. Then the gel was transferred to a polyvinylidine difluoride membrane (Millipore) using a semi-dry transfer apparatus. The membrane was blocked with 1× TTBS containing 3 or 5% non-fat milk and then the probed overnight at 4°C with primary antibodies; anti-phospho-ERK (NEB), phospho-JNK (Upstate or Cell Signaling) diluted 1:500 in blocking buffer. Membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (Pierce) at a dilution of 1:10,000. Protein expression was detected using enhanced chemiluminescence (SuperSignal West, Pierce) and visualized using the Fluorchem Imager (AlphaInnotech). Quantification of protein expression was done using Alpha Ease software. Subsequently membranes were stripped in 62.5 mM Tris containing 2% SDS and 0.7% β-mercaptoethanol and probed with anti-total ERK (NEB) or, anti-total JNK (Upstate or Cell Signaling) antibodies, diluted 1:500 in TTBS containing 5% non-fat milk. Band intensities for phospho ERK1/2 and JNK1/2 were normalized with total ERK and JNK, respectively.

Immunoprecipitation

Two micrograms of JNK Ab (Upstate) was added to 100–150 μg protein lysate and incubated for 1 h at 4°C. Protein A-Agarose beads (Pierce) were then added to the lysates and allowed to bind to the JNK Ab for 1 h at 4°C and then collected by centrifugation at 12,000*g*. Collected pellets were washed three times with 1 mL NET-Gel Buffer (contains; 50 mM Tris-HCl, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide). Finally the protein A-antigen/antibody complex was dissolved in 30 μL of 1× reducing sample buffer (0.312% Tris-HCl, 50% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue). Samples were centrifuged and supernatants were electrophoresed using reducing SDS–PAGE conditions. Western blotting was used to detect phospho-JNK.

Statistical Analysis

The data from a minimum of three independent experiments were expressed as mean ± SEM. Statistical significance was performed using one-way ANOVA followed by post-hoc Tukey testing for experiments consisting of more than two groups, or using two-tailed Student's *t*-test to compare experiments consisting of only two groups. A value of $P < 0.05$ was considered to define statistical significance.

RESULTS

Static Stretch of Skeletal Muscle Endothelial Cells Upregulates MMP-2

Extirpation of TA muscle causes physiological stretching of the synergistic EDL, extending average sarcomere length from 2.4 to 2.9 μm, and this is accompanied by elongation of adjacent capillaries to the same degree [Ellis

et al., 1990; Egginton et al., 1998]. Previous work determined that skeletal muscle capillaries produce MMP-2 in response to muscle stretch [Rivilis et al., 2002]. To determine whether endothelial cells specifically respond to stretch by production of MMP-2, we subjected confluent monolayers of cultured microvascular endothelial cells to sustained static stretch, mimicking the condition of muscle stretch in vivo. MMP-2 protein and mRNA levels increased significantly in response to static stretch (Fig. 1A,B). The active form of MMP-2 (62 kDa) was observed in only one set of samples. Because MT1-MMP is the primary physiological activator of MMP-2 and previous studies showed that muscle stretch induces MT1-MMP mRNA [Rivilis et al., 2002], we used qRT-PCR to assess whether MT1-MMP mRNA was upregulated during endothelial cell stretch. This analysis failed to show significant elevation of MT1-MMP mRNA in stretched (24 h, 10% static stretch) compared to non-stretched cells (RT-PCR values: 0.98 ± 0.09 vs. control 1.00 ± 0.0; NS).

Stretch-Induced MMP-2 Production Is not Dependent on ROS

ROS production is reported to increase transiently in endothelial cells subjected to cyclic stretch [Ali et al., 2004; Chapman et al., 2005], and ROS production is linked to increased production of MMP-2 in some cells types [Yoon et al., 2002; Zhang et al., 2002; Galli et al., 2005]. Thus, we hypothesized that stretch-induced MMP-2 production occurred via activation of a ROS-dependent signal pathway. We first confirmed that ROS would increase MMP-2 production in unstretched endothelial cells, utilizing PMS to cause a sustained increase in ROS via continuous release of H_2O_2. ROS production using this protocol was validated by fluorescence detection using the cell permeable ROS-sensitive dye, DCFH-DA. Intracellular DCFH fluorescence was minimal in control cells, strongly elevated in PMS treated cells, could be reduced substantially by preincubation of the cells with the ROS scavenger ebselen (Fig. 2A). Treatment of unstretched cells with PMS resulted in significant increases in MMP-2 production, which was reduced to control levels in ebselen-treated cells (Fig. 2B), providing evidence that ROS levels can modify MMP-2 expression. However, the stretch-dependent increase in MMP-2 was not attenuated by pretreatment of endothelial cells with ebselen, or other inhibitors of ROS (Fig. 2C). In fact, using the ROS-sensitive dye DCFH as an indicator of intracellular ROS levels, the signal originating from stretched cells did not differ obviously from that detected in control unstretched cells (Fig. 2D). In both treatments, the fluorescence signal could be quenched by pretreatment with ebselen.

Effects of Strain on the Activation of ERK1/2 and JNK1/2

Because both ERK1/2 and JNK pathways have been linked to MMP-2 and VEGF

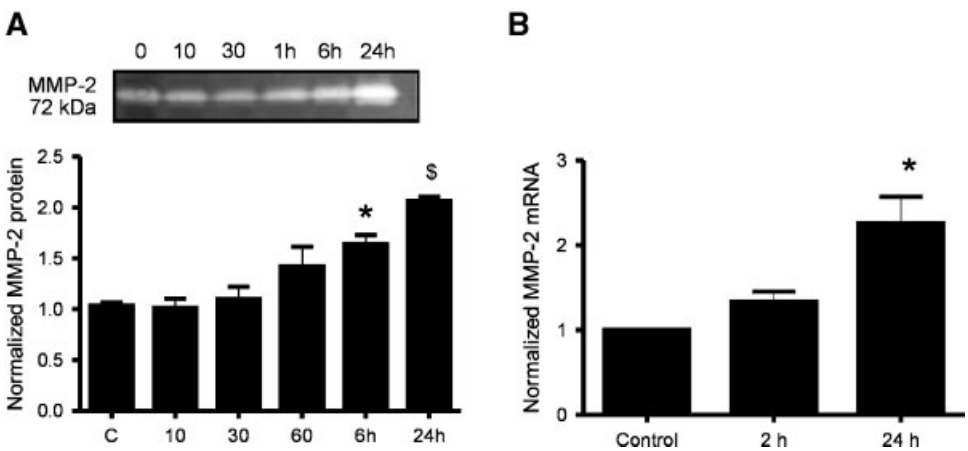

Fig. 1. Static stretch of skeletal muscle endothelial cells upregulates MMP-2. **A**: Endothelial cells were stretched for 0, 10, 30 min; 1, 6, and 24 h. MMP-2 production was measured by gelatin zymography of cell lysates, and band intensities were normalized to time 0. Values are mean ± SE, n = 3; $^{*}P < 0.05$ versus unstretched; $^{\$}P < 0.01$ versus unstretched. **B**: Cellular RNA was isolated from control, 2 and 24 h stretched samples to analyze MMP-2 mRNA by qPCR. Values are mean ± SE, n = 3 ($^{*}P < 0.05$ vs. unstretched).

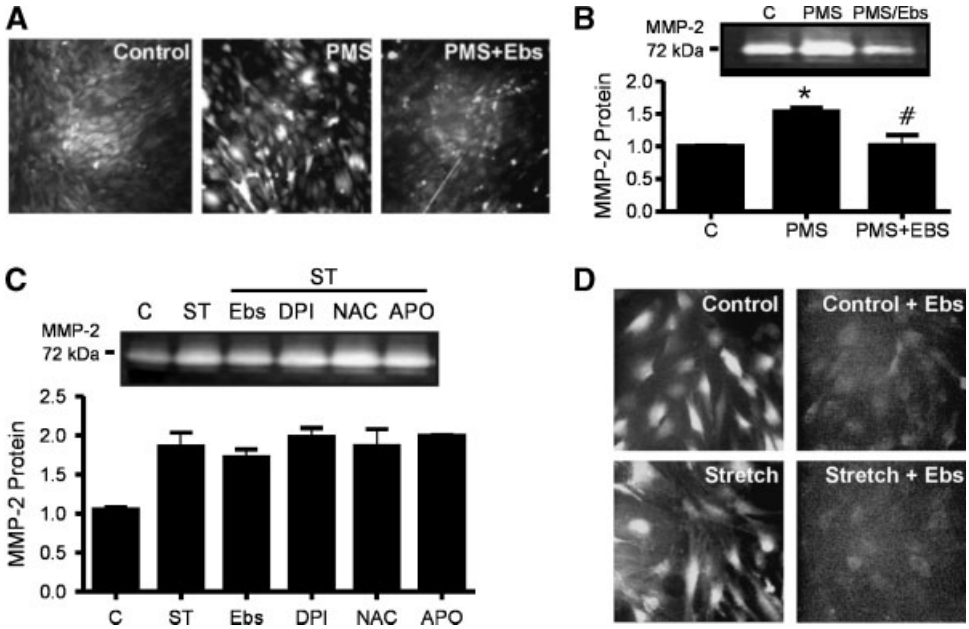

Fig. 2. H_2O_2 stimulates MMP-2 production, but does not contribute to stretch-induced MMP-2 production. **A**: Endothelial cells were plated in the absence or presence of PMS, or PMS and ebselen, for 48 h and then loaded with 5 μM DCFH-DA for 15 min. Images were acquired with fluorescence microscopy (10× objective) using identical exposure times for control, PMS and PMS + ebselen conditions. **B**: After 48 h culture time, MMP-2 protein production was measured from cellular lysates using gelatin zymography. Values are mean ± SE, n = 3–5, $^*P < 0.05$ versus control; $^{\#}P < 0.05$ versus PMS. **C**: Cells were pretreated with various ROS inhibitors/scavengers prior to stretch for 24 h. MMP-2 protein was analyzed by gelatin zymography. None of the inhibitors reduced MMP-2 production. ST, stretch; Ebs, ebselen; DPI, diphenyleneiodonium; NAC, N-acetylcysteine; APO, apocyanin. Values are mean ± SE, n = 5, $^*P < 0.05$ versus control. **D**: Endothelial cells were plated on type I collagen-coated Bioflex plates overnight, then subject to no stretch or 10% static stretch for 20 min in the presence of 5 μM DCFH-DA. Some wells were pretreated with 50 μM ebselen for 1 h prior to addition of DCFH-DA. Cells were immediately viewed using fluorescence microscopy (20× objective) and digital images were acquired using identical exposure settings for all conditions.

production, we examined whether these signaling cascades were activated in microvascular endothelial cells stimulated by static stretch. As shown in Figure 3A, static strain-induced transient phosphorylation of 44 and 42 kDa forms of ERK1/2 in a time-dependent manner. Phosphorylation of ERK1/2 was increased maximally at 10 min (3.5-fold for ERK1 and 3.9-fold for ERK2 compared with unstretched cells) but it returned to the basal level at 1 h. Static stretch also significantly increased JNK1/2 activation at 30 min, which declined to the basal level by 1 h (Fig. 3B).

JNK but not ERK Is Involved in Stretch-Induced MMP-2 Production

Next, we tested for involvement of JNK and or ERK pathways in regulating the MMP-2 response to stretch. Inhibition of ERK1/2 using 0.3 μM U0126 did not reduce the stretch-induced MMP-2 protein and mRNA expression (Fig. 4A,C, respectively). Western blot analysis confirmed an effective inhibition of ERK under these treatment conditions (data not shown). JNK inhibition using SP600125 attenuated significantly the strain-induced increases in MMP-2 protein and mRNA levels (Fig. 4B,D, $P < 0.05$ vs. ST).

ERK Regulates Stretch-Induced VEGF Production

Capillary growth depends on both proliferation and proteolysis. VEGF is known to promote these events, as well as cell migration. Skeletal muscle capillaries subjected to muscle stretch also exhibit increased VEGF production [Rivilis et al., 2002]. We tested whether endothelial cells produce VEGF in response to stretch and if so, whether MAPK signals were involved. Both VEGF protein and mRNA (Fig. 5A,B) were increased by 24 h exposure to static stretch. Stretch-induced VEGF mRNA was abrogated by ERK1/2 inhibition (using U0126), but not by JNK inhibition (Fig. 5C).

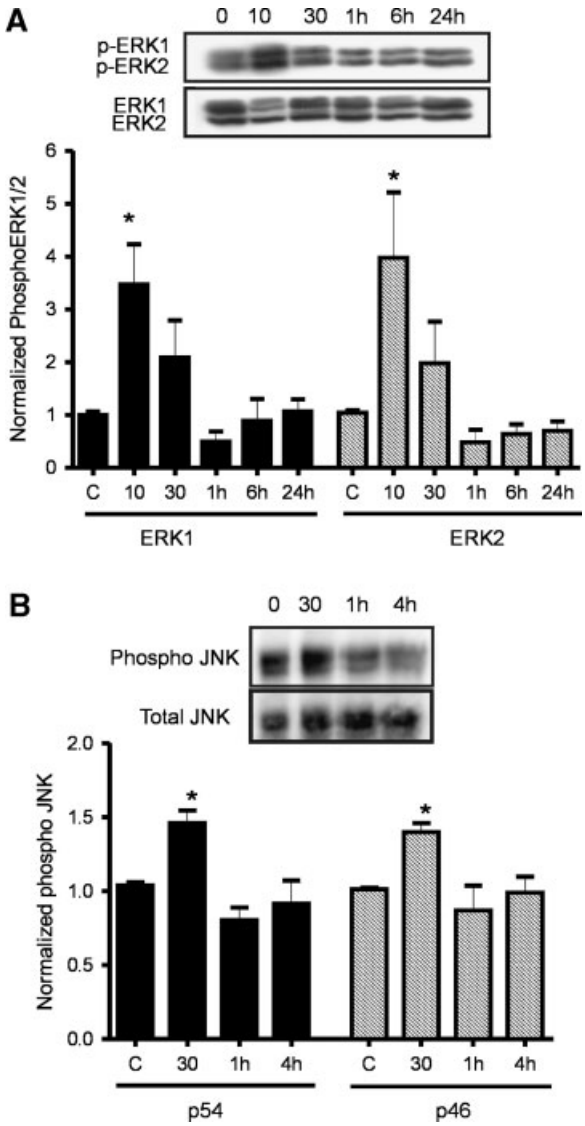

Fig. 3. Static stretch causes transient activation of ERK1/2 and JNK1/2. **A**: Endothelial cells were stretched for 0, 10, 30 min; 1, 6, and 24 h. ERK1/2 phosphorylation was quantified by immunoblotting and normalized to levels of total ERK1/2 protein. Phospho-ERK1 and phospho-ERK2 values are denoted by solid and hatched columns, respectively. **B**: JNK1/2 activation was measured by immunoprecipitation of cell extracts with JNK antibody following stretch for 0, 30 min; 1 and 4 h. Immunoblotting then was performed to detect phospho JNK. Phospho-p54 and phospho-p46 values are denoted by solid and hatched columns, respectively. Values are mean ± SE, n = 4; $*P < 0.05$.

JNK Inhibition Reduces Overload-Induced MMP-2

Based on the evidence for JNK-mediated effects on MMP-2 in cultured endothelial cells exposed to stretch, we tested whether this relationship exists in vivo. Using a rat model of muscle overload, in which stretch of the EDL is induced by removal of the larger synergistic muscle TA [Zhou et al., 1998], we examined the effects of JNK inhibition on MMP-2 production. Previous work showed significant increases in MMP-2 production 4 days after muscle overload [Rivilis et al., 2002]. The JNK inhibitor significantly abrogated the muscle overload-induced increase in MMP-2 protein, as assessed by gelatin zymography (Fig. 6).

DISCUSSION

In this study, we demonstrated that static stretch increases MMP-2 and VEGF mRNA and protein expression in microvascular endothelial cells. We provided evidence that JNK activity is involved in the upregulation of endothelial cell MMP-2 expression in vitro in response to sustained stretch. Inhibition of JNK activity in vivo also reduced stretch-induced MMP-2 production. On the other hand, stretch-induced ERK1/2 activity was not required for stretch-induced MMP-2 production but did contribute significantly to the stretch-induced increase in VEGF.

The majority of research on stretch sensitivity of endothelial cells has focused on the effects of cyclic stretch on aortic endothelial cells, with the goal of understanding endothelial cell responsiveness to cyclic distension caused by pulse pressure. However, within the context of the microcirculation, skeletal muscle stretch induces a robust angiogenesis response [Egginton et al., 1998]. Understanding the endothelial cell response to stretch thus provides a means to determine the signals that drive skeletal muscle angiogenesis, as these signal pathways are not well-elucidated. Previous studies illustrated that muscle stretch increases MMP-2 and VEGF production, and capillary growth in skeletal muscle [Egginton et al., 2001; Rivilis et al., 2002]. However, those studies were not able to determine whether stretch directly stimulated the capillary endothelial cells, or whether other cell types resident within the muscle responded to the stretch, releasing some stimulatory factors that in turn-induced endothelial cell responses. We demonstrate in the current study that microvascular endothelial cells isolated from skeletal muscle respond to static stretch through induction of MMP-2 mRNA expression and protein synthesis. Consistent with our observations, an increase in MMP-2 protein was observed in

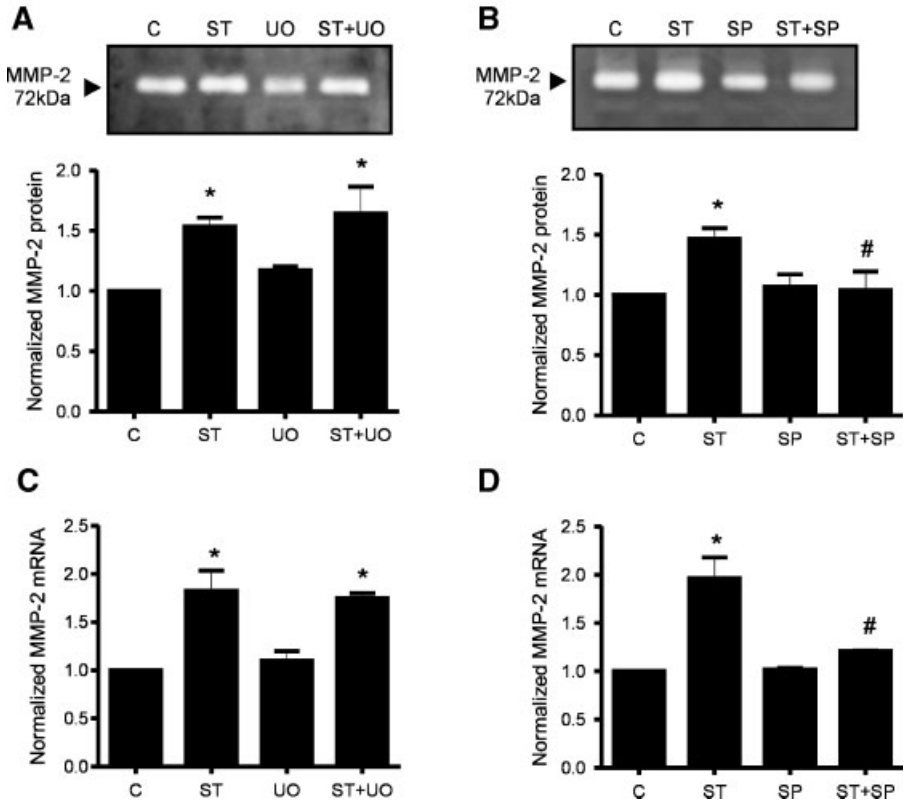

Fig. 4. JNK but not ERK is involved in stretch-induced MMP-2 protein and mRNA expression. Endothelial cells were pretreated with vehicle or 50 μM SP600125 (SP) or 0.3 μM U0126 (UO) for 3 h and then exposed to stretch (ST) for 24 h. MMP-2 production was measured by gelatin zymography (**A**, **B**). MMP-2 mRNA was quantified using qPCR (**C**, **D**). In all graphs, values are mean ± SE, n = 3 or 4. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ stretch versus stretch + SP600125.

bovine aortic endothelial cells in response to 5 or 10% cyclic stretch [von Offenberg et al., 2004]. Coronary microvascular endothelial cells also have been shown to respond directly to cyclic stretch through production of VEGF [Zheng et al., 2001].

Although a significant increase in MT1-MMP mRNA level was observed in response to muscle

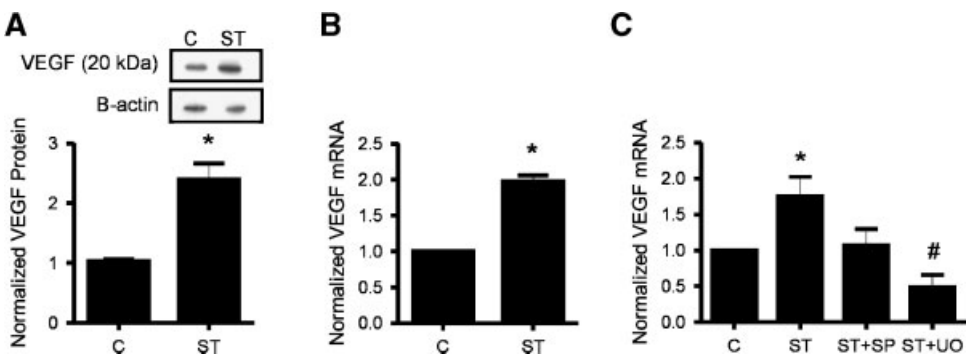

Fig. 5. VEGF protein and mRNA increase in response to stretch, predominantly via ERK1/2 activation. **A**: VEGF protein was measured by Western blotting in endothelial cell lysates following 24 h exposure to stretch, and normalized to β-actin to control for loading. $^{*}P < 0.05$ versus control, n = 3. **B**: VEGF mRNA was measured by qPCR in endothelial cell lysates following 24 h exposure to stretch. $^{*}P < 0.01$ versus control, n = 4. **C**: Cultured endothelial cells were pretreated with vehicle or 50 μM SP600125 or 0.3 μM U0126 prior to 24 h cell stretch. VEGF mRNA was measured by qPCR. $^{\#}P < 0.05$ stretch versus stretch+ UO126, n = 3.

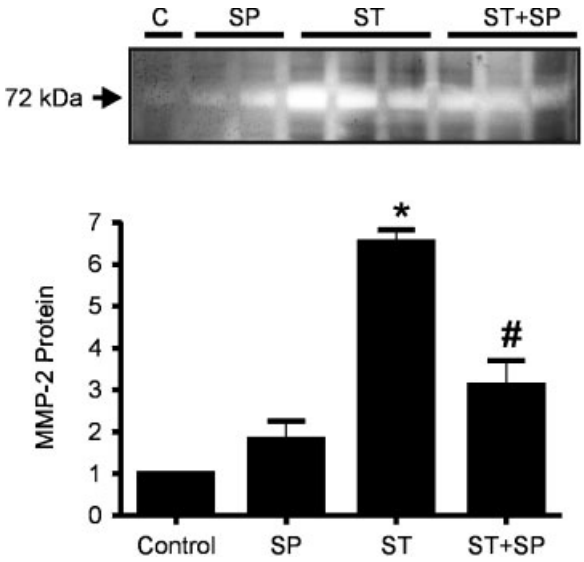

Fig. 6. Inhibition of JNK reduces overload-induced MMP-2 expression in rat skeletal muscle. Rat EDL muscles were subjected to static stretch (overload) using surgical removal of the synergistic muscle tibialis anterior. Rats were treated continuously with vehicle or SP600125 (SP) delivered locally to the muscle via osmotic pump. After 4 days, muscles were extracted and protein extracts were prepared for gelatin zymography analysis. MMP-2 protein was elevated significantly in overloaded (ST) muscles compared to control or SP treated ($^{*}P < 0.001$ vs. control, SP; n = 4). Overload and SP-treated (ST + SP) muscles had significantly lower MMP-2 protein compared to ST alone ($^{\#}P < 0.01$ ST vs. ST + SP600125, n = 4).

stretch in vivo [Rivilis et al., 2002], we detected only a trend for increased endothelial MT1-MMP mRNA in response to static ($P = 0.19$) stretch. We also did not find increases in MT1-MMP mRNA with a stronger stretch stimulus (20% static stretch; data not shown). An earlier study reported upregulation of MT1-MMP by cyclic strain in microvascular endothelium isolated from adipose tissue [Yamaguchi et al., 2002]. Defining the reason for the variable responses of MT1-MMP to stretch in different experimental models requires further investigation.

ROS is reported to increase in aortic endothelial cells exposed to cyclic stretch, which may depend on activity of NADPH oxidase [Hishikawa and Luscher, 1997] or on mitochondrial generation of ROS [Ali et al., 2004]. Those studies utilized a magnitude of strain that was much greater than that in the current study (20–25% compared to 10%), which may explain why we failed to see an effect of ROS inhibition on stretch-induced MMP-2 production despite the observation that elevated H_2O_2 in unstretched cells was capable of increasing MMP-2 production. DCFH fluorescence confirmed that production of ROS was not enhanced in stretched cells. We conclude that stretch-stimulated MMP-2 production does not rely on production of ROS.

Rapid phosphorylation of ERK1/2 increases proliferation of endothelial cells subjected to cyclic strain [Sumpio et al., 1988; Azuma et al., 2000], indicating the potential involvement of ERK1/2 in the physiological events associated with stretch-induced angiogenesis. Likewise, we observed transient activation of ERK1/2 by static stretch. In our study, inhibition of ERK1/2 using U0126 did not suppress static stretch-induced MMP-2 expression, implying that activity of ERK1/2 is not required for stretch-induced MMP-2 production in skeletal muscle microvascular endothelial cells. Recently it was shown that MMP-2 induction in response to 5% cyclic stretch is mediated via ERK1/2 in bovine aortic endothelial cells [von Offenberg et al., 2004]. This discrepancy points to the possibility of preferential use of different signaling pathways in endothelial cells dependent on species and/or tissue of origin.

On the other hand, we observed that the stretch-induced increase in VEGF mRNA was dependent on ERK1/2 activity and was not affected significantly by JNK inhibition. Our data are consistent with earlier studies that reported ERK1/2 regulation of VEGF production in non-endothelial cells [Milanini et al., 1998; Giuliani et al., 2004]. Importantly, our results provide strong evidence corroborating previous findings that endothelial cells produce VEGF [Namiki et al., 1995; Zheng et al., 2001], which may then act on the cells via an autocrine signal loop.

In contrast to the effects of ERK inhibition, we found that inhibition of JNK significantly reduced stretch-induced MMP-2 production. Several studies have reported activation of JNK1/2 in response to cyclic stretch [Ingram et al., 2000; Wang et al., 2003]. Similarly, JNK1/2 activation occurs in skeletal muscle during exercise, contributing to exercise-induced remodeling of the muscle fibers [Boppart et al., 2000, 2001]. Significantly greater activation of JNK1/2 has been observed during eccentric or static stretch compared to concentric cyclic contraction [Boppart et al., 1999; Martineau and Gardiner, 2001]. JNK1/2 activation in response to muscle overload is rapid and transient, with a

decline to basal phosphorylation by 24 h of muscle overload [Carlson et al., 2001]. Recently published studies also support a role for JNK in regulation of angiogenesis. Inhibition of c-jun, the downstream target of JNK, using DNAzymes suppressed endothelial cell production of MMP-2 and reduced formation of new capillaries [Zhang et al., 2004]. In addition, Wang et al. [2003] demonstrated that inhibition of JNK attenuated cyclic strain-induced MMP-2 expression via inhibition of AP-1 in human umbilical vein endothelial cells. Our data extend these studies by demonstrating that a physiological stimulus for angiogenesis (static stretch), can activate the JNK pathway, and promote MMP-2 production. The impact of JNK inhibition on muscle overload-induced expression of MMP-2 confirms the physiological significance of this signal pathway in regulating endothelial cell gene expression in response to mechanical stress.

Initially, we hypothesized that both stretch-dependent regulation of MMP-2 and VEGF would utilize the same signal pathway. However, our data support discrete roles of ERK1/2 in VEGF production and JNK1/2 in MMP-2 production. It is feasible that production of these two angiogenic factors is coordinated at a point upstream in the signal cascade. Potential stretch sensors include integrins, Gprotein coupled receptors, stretch-activated ion channels, and stretch-activated growth factor receptors. Further studies are required to investigate the roles of these sensors in initiating the angiogenic events associated with endothelial cell stretch.

Our data demonstrate that static stretch stimulates MMP-2 and VEGF mRNA and protein production in skeletal muscle microvascular endothelial cells. Moreover, static strain induction MMP-2 is dependent on JNK1/2, but not ERK1/2 activation or ROS production. We conclude that JNK is a key mediator of stretch-induced production of endothelial cell MMP-2, and thus a potentially important regulator of exercise-induced angiogenesis in skeletal muscle.

ACKNOWLEDGMENTS

The authors thank Gagan Dhanota for her technical support, and Tom Fudalewski for assistance with gelatin zymography analysis of muscle samples. TLH is a Canadian Institutes of Health Research New Investigator, and received funding for this project from Canadian Institutes of Health Research.

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