Satellite cell activation in stretched skeletal muscle and the role of nitric oxide and hepatocyte growth factor


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Tatsumi, Ryuichi, Xiaosong Liu, Antonio Pulido, Mark Morales, Tomowa Sakata, Sharon Dial, Akihito Hattori, Yoshioide Ikeychi, and Ronald E. Allen. Satellite cell activation in stretched skeletal muscle and the role of nitric oxide and hepatocyte growth factor. Am J Physiol Cell Physiol 290: C1487–C1494, 2006; doi:10.1152/ajpcell.00513.2005.—In the present study, we examined the roles of hepatocyte growth factor (HGF) and nitric oxide (NO) in the activation of satellite cells in passively stretched rat skeletal muscle. A hindlimb suspension model was developed in which the vastus, adductor, and gracilis muscles were subjected to stretch for 1 h. Satellite cells were activated by stretch determined on the basis of 5-bromo-2’-deoxyuridine (BrdU) incorporation in vivo. Extracts from stretched muscles stimulated BrdU incorporation in freshly isolated control rat satellite cells in a concentration-dependent manner. Extracts from stretched muscles contained the active form of HGF, and the satellite cell-activating activity could be neutralized by incubation with anti-HGF antibody. The involvement of NO was investigated by administering nitro-L-arginine methyl ester (L-NAME) or the inactive enantiomer N’-nitro-o-arginine methyl ester HCl (o-NAME) before stretch treatment. In vivo activation of satellite cells in stretched muscle was not inhibited by o-NAME but was inhibited by L-NAME. The activity of stretched muscle extract was abolished by L-NAME treatment but could be restored by the addition of HGF, indicating that the extract was not inhibitory. Finally, NO synthase activity in stretched and unstretched muscles was assayed in muscle extracts immediately after 2-h stretch treatment and was found to be elevated in stretched muscle but not in stretched muscle from L-NAME-treated rats. The results of these experiments demonstrate that stretching muscle liberates HGF in a NO-dependent manner, which can activate satellite cells.

SKELETAL MUSCLE HAS THE ABILITY TO respond to contractile demands through fiber hypertrophy, and it is an amazingly resilient tissue that can be repaired rapidly after damage resulting from a variety of mechanical and chemical insults. Central to both processes is the activity of resident myogenic stem cells or satellite cells. The role of satellite cells in muscle growth and regeneration has been appreciated for many years (1, 7, 8, 11, 22). The mechanisms responsible for activating satellite cells in muscle tissue in response to mechanical changes, however, have not been delineated clearly to date.

Subj ecting muscle to mechanical stretch has been demonstrated to cause muscle hypertrophy (15, 27). Effects of stretch on muscle protein hypertrophy have been reported to entail the stimulation of transcription factors MyoD and myogenic regulatory factor family isoform 4 as well as myogenin expression (17) and the expression of serum response factor (9). Perrone (20) demonstrated that in vitro stretch increased myotube protein synthesis and that autocrine IGF-I was implicated in the mechanism. Baar et al. (5) studied 70-kDa S6 kinase phosphorylation in stretched myotubes in vitro and reported that the response was mediated through autocrine action of factors released into the medium of stretched muscle tissue cultures, but more recent work from the same laboratory suggests that mechanoreceptors directly activate intracellular signaling cascades (12). In vivo experiments have also been shown to stimulate expression of several FGFs and FGF receptors (19).

In addition to changes in gene expression and protein synthesis in stretched muscle, the proliferation of satellite cells has also been observed (15, 27). The relationship between stretched muscle and satellite cell activation was studied in vitro by Tatsumi et al. (25) in experiments in which quiescent satellite cells from uninjured adult rats were shown to become activated to enter the cell cycle early by subjecting the cultured cells to cyclic stretch. It was further demonstrated that stretch (2 h) caused the release of hepatocyte growth factor (HGF) into the medium and that released HGF stimulated satellite cell entry into the cell cycle. These experiments also demonstrated that HGF was associated with satellite cells at 12 h poststaining, the time when stretch treatment was applied. These experiments provided evidence that HGF was released from its binding sites in the extracellular domain and that HGF release does not involve new growth factor synthesis.

A second chemical signal shown to be involved in satellite cell activation is nitric oxide (NO). Anderson (3) used a crush injury model to show that mouse satellite cells are rapidly activated after crush and that blocking NO synthesis blocks activation. Recent in vitro experiments conducted at the Anderson laboratory (4, 28, 29) demonstrated that in a culture system with single fibers and their associated satellite cells, HGF and NO stimulated satellite cell activation and DNA synthesis. Stretch activation of isolated satellite cells was also shown to be dependent on NO for HGF release and subsequent activation (26). Therefore, satellite cell activation by HGF or by NO has been demonstrated at three levels of organization: whole muscle, isolated fibers with associated satellite cells, and isolated satellite cells.

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The hypothesis of the present study was that muscle stretch in vivo results in HGF release in a NO-dependent process that subsequently activates satellite cells. The purpose of the present experiments was to link the in vitro observations of stretch-induced satellite cell activation to the in vivo response of satellite cells to muscle stretch. Our results indicate that when muscle is stretched in vivo, NO synthase (NOS) is stimulated, HGF is released, and satellite cells are activated as they were in previously published stretch experiments with cultured satellite cells (25, 26) and isolated fibers (4).

MATERIALS AND METHODS

Satellite cell isolation and culture. Satellite cells were isolated from 9-mo-old male Sprague-Dawley rats according to the method described previously by Allen et al. (2). Briefly, vastus, adductor, and gracilis muscles were excised, trimmed of fat and connective tissue, hand minced with scissors, and digested for 1 h at 37°C with 1.25 mg/ml Protease Type XIV. Cells were separated from muscle fiber fragments and tissue debris by performing differential centrifugation and plated on polysyline- and fibronectin (both from Sigma, St. Louis, MO)-coated dishes in DMEM containing 10% horse serum (DMEM-10% HS; Life Technologies, Grand Island, NY), 1% antibiotic (Life Technologies), and 0.5% gentamicin (Invitrogen, Carlsbad, CA). Costar 24- or 48-well culture plates were used. Cultures were maintained in a humidified 5% CO2 atmosphere at 37°C.

For immunoneutralization experiments, goat anti-human recombinant HGF antibody (R&D Systems, Minneapolis, MN) or neutral control antibody (Cappel Research, Durham, NC) was preincubated with stretched muscle extract (SME) for 2 h at 4°C before being added to culture medium. SME, human recombinant HGF (R&D Systems), and/or goat anti-human recombinant HGF were added to culture medium at the time of plating, and the medium was replaced at 24 h. Cultures were pulse labeled for 2 h with 10 μM 5-bromo-2’-deoxyuridine (BrDU) in DMEM-10% HS from 40 to 42 h postplating and then examined using immunocytochemistry for detection of BrDU by Allen et al. (2). At 30 h postplating, cells were assayed for BrDU incorporation as described previously. The percentage of BrDU-labeled cells was used as an index of satellite cell activation and entry into the cell cycle in vivo before the rats were killed. The myogenic cell percentage was measured by assaying for the presence of desmin at 30 h postplating. The result was >97% and did not differ significantly (P < 0.01) from that of unstretched or normal control muscles from untreated rats.

Immunohistochemistry. Skeletal muscle tissue was fixed in Prefer (Anatech, Battle Creek, MI) and then dehydrated and embedded in paraffin blocks according to standard procedures. Five-micrometers-thick tissue sections were cut and applied to slides. The slides were deparaffinized and rehydrated. After rehydration, the slides were incubated in preheated 1× DakoCytomation target retrieval solution (Dako, Carpinteria, CA) and steamed for 20 min. The slides were then removed to heated, double-distilled water (dH2O) for 5 min, followed by 5 min in PBS at room temperature. After incubation, the slides were subsequently transferred to −20°C acetone solution for 10 min, rinsed with 50 mM NH4Cl for 5 min, and washed three times in PBS for 5 min each.

For laminin staining, the slides were incubated with goat anti-rat laminin α-1 (M-20) IgG (1:100 dilution in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) or its inactive enantiomer N03-nitro-α-arginine methyl ester HCl (d-NAME; Calbiochem-Novabiochem) at a concentration of 0.75 mg/100 g body wt 30 min before stretch. All experiments involving animals were conducted according to The Guidelines for the Care and Use of Laboratory Animals (enacted August 13, 1997, by the Graduate School of Agriculture, Hokkaido University, Hokkaido, Japan) and with the approval of the University of Arizona Institutional Animal Care and Use Committee. At prescribed times after their muscles were stretched, rats were administered ip injections with BrdU (Sigma) at a concentration of 5 mg/100 g body wt. For l-NAME treatment, rats were administered ip injections containing 3.75 mg/100 g body wt l-NAME 30 min before their muscles were stretched. The rats’ muscles were stretched for 2 h according to the method described previously. At 18, 30, 36, and 48 h poststretch, the rats were administered ip injections containing BrdU and killed 16 h after injection. Skeletal muscle tissues from stretched and unstretched side legs were collected. Skeletal muscles from rats that did not undergo any treatment were collected as control samples.

Satellite cells were prepared from the left and right vastus, adductor, and gracilis muscles according to a method described previously by Allen et al. (2). At 30 h postplating, cells were assayed for BrdU incorporation as described previously. The percentage of BrdU-labeled cells was used as an index of satellite cell activation and entry into the cell cycle in vivo before the rats were killed. The myogenic cell percentage was measured by assaying for the presence of desmin at 30 h postplating. The result was >97% and did not differ significantly (P < 0.01) from that of unstretched or normal control muscles from untreated rats.

In vivo muscle stretch and satellite cell activation assay. For each in vivo stretch experiment, three 9-mo-old Sprague-Dawley male rats were used. Rats were anesthetized with halothane, and the ankles of both forelimbs and the left hindlimb were wrapped with cloth tape and plastic straps were placed around the taped legs. The forelimbs were used. Rats were anesthetized with halothane, and the ankles of both forelimbs and the left hindlimb were wrapped with cloth tape and plastic straps were placed around the taped legs. The forelimbs were used. Rats were anesthetized with halothane, and the ankles of both forelimbs and the left hindlimb were wrapped with cloth tape and plastic straps were placed around the taped legs. The forelimbs were used. Rats were anesthetized with halothane, and the ankles of both forelimbs and the left hindlimb were wrapped with cloth tape and plastic straps were placed around the taped legs. The forelimbs were used. Rats were anesthetized with halothane, and the ankles of both forelimbs and the left hindlimb were wrapped with cloth tape and plastic straps were placed around the taped legs. The forelimbs were used. Rats were anesthetized with halothane, and the ankles of both forelimbs and the left hindlimb were wrapped with cloth tape and plastic straps were placed around the taped legs. The forelimbs were used. Rats were anesthetized with halothane, and the ankles of both forelimbs and the left hindlimb were wrapped with cloth tape and plastic straps were placed around the taped legs. The forelimbs were used. Rats were anesthetized with halothane, and the ankles of both forelimbs and the left hindlimb were wrapped with cloth tape and plastic straps were placed around the taped legs. The forelimbs were used. Rats were anesthetized with halothane, and the ankles of both forelimbs and the left hindlimb were wrapped with cloth tape and plastic straps were placed around the taped legs. The forelimbs were used.
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mouse dystrophic IgG PAb (1:100 dilution in PBS; Santa Cruz Biotechnology). After incubation for 60 min, slides were washed with three changes of PBS for 5 min each, followed by incubation in secondary antibody solution (1:100 dilution of rabbit anti-goat IgG Alexa Fluor 594 in PBS; Invitrogen) for 30 min at 30°C in the dark.

After being stained for laminin or dystrophin, slides were washed three times in PBS and then incubated in G34G MAb supernatant for BrdU for 60 min at 30°C. After being washed three times with PBS, the slides were incubated in a 1:100 dilution of rabbit anti-mouse IgG Alexa Fluor 488 in PBS (Invitrogen) for 30 min at 30°C in the dark. After being washed three times in PBS and once in ddH2O, the slides were incubated in 4',6'-diamidino-2-phenylindole solution (0.2 μg/ml) for 3 min and then rinsed with ddH2O. The slides were then dehydrated and coverslips were mounted. Images were captured using a Nikon Eclipse TE300 microscope equipped with CoolSNAP software (Princeton Instruments/Acton Research, Trenton, NJ). The number of BrdU-positive satellite cells and the total number of muscle fibers per view were counted.

Stretched muscle extract. SME was prepared by removing from 9-mo-old male rats adductor, gracilis, and vastus group muscles that had been stretched for 2 h. Muscles from each side were pooled and incubated in PBS at a concentration of 1 g of muscle/ml PBS for 1.5 h at 4°C with gentle shaking and then spun down at 10,000 rpm for 3 min. The extract was dialyzed against DMEM (Life Technologies) for 2 h at 4°C using a Slide-A-Lyzer cassette (Pierce Chemical, Rockford, IL) and was filtered sterilized using a 0.2-μm filter for subsequent culture experiments or for SDS-PAGE. For NOS assays, the dialysis step was omitted. Protein concentration was determined using the Bradford method with a protein assay kit (Bio-Rad Laboratories, Hercules, CA) calibrated with BSA as the standard (Gene Technology, St. Louis, MO).

Western blot analysis and ECL. SME was subjected to SDS-PAGE on 10% polyacrylamide gels under reducing conditions (25). Separated proteins were transferred onto HyBond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ), which were then blocked with 10% powdered milk in 0.1% Tween 20-Tris-buffered saline (TTBS) before being incubated with a 1:500 dilution of goat anti-human recombinant HGF PAb (R&D Systems) for 1 h at room temperature. The membranes were subsequently treated with horseradish peroxidase-conjugated rabbit anti-goat IgG (Sigma; 1:5,000 dilution in 1% powdered milk in TTBS) for 1 h at room temperature, followed by ECL detection (Amersham Pharmacia Biotech) on X-OMAT LS autoradiography film (Eastman Kodak, Rochester, NY) according to the manufacturer’s recommendations.

NOS assay. NOS activity was assessed by measuring NO2- and NO3- concentrations in SME from rats subjected to ip injection with l-NAME or d-NAME according to the method described previously by Tatsumi et al. (26). SME was collected (100 μl/vial; n = 3 cultures) in polypropylene vials (Alltech, Deerfield, IL) with Teflon liners (Alltech) and screw caps (Alltech). Samples (20 μl/vial) were applied to an automated NO detector HPLC system (Eicom, Kyoto, Japan) to determine NO2- and NO3- concentrations. Assays were standardized with 10 μM sodium nitrite and 10 μM sodium nitrate. Briefly, NO2- and NO3- in the extract were separated using a reverse-phase separation column (Eicom) packed with poly styrene polymers. NO3- was reduced to NO2- in a reduction column (Eicom) packed with Cu2+-plated Cd fillings. NO2- was mixed with a Griess reagent (Eicom), and the absorbance was read at 540 nm using a flow-through spectrophotometer. The apparent amount of NO produced was defined as the total value of NO2- and NO3- measured (NOx).

Statistical analysis. ANOVA was used to evaluate the experimental results, together with general linear model procedures of SRISTAT2 for Windows software (Social Survey Research Information, Tokyo, Japan) or SAS software (SAS Institute, Cary, NC). Least-squares means for each treatment were separated on the basis of a least significant differences test. Unless stated otherwise, the level of significance used in these experiments was P < 0.01.

RESULTS

To determine the mechanism of stretch activation of satellite cells in vivo, a model was developed to apply stretch for a relatively short time to the vastus, gracilis, and adductor hindlimb muscles of adult male rats. The time course of BrdU incorporation into satellite cells was investigated by suspending the rats for 1 or 2 h, followed by ip injection of BrdU at 18, 30, 36, or 48 h poststretch. Muscles were harvested 16 h after BrdU injection, and muscle sections were stained for BrdU and for laminin (Fig. 2, A–C). Activated satellite cells were identified as BrdU-positive cells within the laminin-positive basal lamina surrounding muscle fibers. Figure 2, A–C, shows double- labeled sections from control contralateral muscle (Fig. 2A), stretched muscle (Fig. 2B), and stretched muscle from l-NAME-treated rats (Fig. 2C). Numerous labeled satellite cells were observed in stretched muscle, but few were found in muscle from the contralateral side or in stretched muscle from l-NAME-treated rats. In Fig. 2D, we show that activated satellite cells in stretched muscles continue activation through myogenesis and differentiate to fuse into muscle fibers as indicated by the presence of BrdU-labeled nuclei inside the dystrophin-labeled sarcolemma of muscle fibers 10 days after stretch. It is important to note that extensive infiltration of cells into stretched muscles was not observed (data not shown), in contrast to the inflammatory response observed in association with crush or chemical injury.

Figure 2, E and F, displays the results for stretched and contralateral unstretched legs as a function of BrdU injection time. Figure 2E demonstrates a significant increase (P < 0.01) in stretched muscles compared with contralateral muscles from the same rats regarding the number of BrdU-positive satellite cells per muscle fiber cross-section at 30 and 36 h. Furthermore, satellite cell activation was increased in the stretched and contralateral unstretched muscles compared with control rats that were not subjected to stretch treatment. Figure 2F includes data from animals that were stretched after injection of the NOS inhibitor l-NAME. When BrdU was administered at either 30 or 36 h poststretch, there was no significant difference in satellite cell activation in stretched or contralateral unstretched muscles. There was a small but significant (P < 0.01) increase by 48 h poststretch. d-NAME treatment, which does not block NOS, did not block stretch-induced satellite cell activation (data not shown).

We examined satellite cell activation with a second assay that was originally used by Tatsumi et al. (24) to demonstrate satellite cell activation in response to direct injection of HGF into uninjured adult muscles. Muscles were stretched as before, and rats were administered ip injection with BrdU 30 h poststretch. Satellite cells from each leg were cultured 16 h after BrdU injection. The percentage of BrdU-labeled cells in triplicate cultures reflected the activation of satellite cells in vivo in response to stretch treatment. As demonstrated in Fig. 3, the results were virtually identical to those detected using immunocytochemistry of muscle sections shown in Fig. 2. More than 30% of the satellite cells harvested from stretched muscles had incorporated BrdU in vivo before harvest. Fewer than 1% had been labeled in unstretched adult rat muscles. As with the data from muscle sections, satellite cell activation in the contrastr-
eral unstretched muscles was significantly greater than that in control rat muscle, but the percentage of BrdU-labeled satellite cells from stretched muscles was greater than that from the contralateral control muscles. The results observed in L-NAME- and D-NAME-treated rat muscles were also the same as those in sectioned muscles (Fig. 3). L-NAME treatment abolished satellite cell activation in stretched and contralateral unstretched muscles as shown qualitatively in Fig. 2C. D-NAME treatment did not prevent satellite cell activation in stretched or contralateral muscles. It is important to note that the percentage of desmin-positive satellite cells from all treatments and times has been determined to be $>95\%$ (data not shown). Therefore, both assays demonstrated the activation of satellite cells in stretched muscles and a low level of satellite
cell activation in unstretched muscles from the same animals, suggesting that there could be a circulating agent released in response to stretch.

We had hypothesized that stretch may release soluble activating activity as was observed in association with crushed muscle (6) or isolated stretched satellite cells in vitro (25). To test this hypothesis, SMEs were prepared from stretched (2 h) and contralateral control muscles and were assayed for satellite cell activation in adult rat muscle satellite cell cultures (Fig. 4A). The BrdU labeling index increased in a dose-dependent manner with increasing concentrations of SME from 0 to 0.5 mg/ml, whereas extracts from control muscles did not stimulate satellite cell activation. HGF (10 ng/ml) served as a positive control for activation.

To further explore the relationship between NOS activity and satellite cell activation, extracts were prepared from stretched and unstretched muscles from L-NAME- and D-NAME-treated rats, and satellite cell activation was compared in control rat satellite cell cultures (Fig. 4B). In untreated rats, extracts from stretched muscles (Fig. 4B,b) stimulated BrdU labeling above that of control muscle extract (Fig. 4B,a). The same results were found with the respective extracts from D-NAME-treated rats (Fig. 4B,c and d). In extracts from L-NAME-treated rats, however, extracts from unstretched muscle (Fig. 4B, e) and stretched muscle (Fig. 4B, f) were comparable. The addition of 10 ng of HGF/ml to SME from L-NAME-treated rats stimulated activation (Fig. 4B, g), demonstrating that L-NAME did not inhibit satellite cell activation directly.

To verify that NO was synthesized in response to stretch and that L-NAME inhibited NOS activity, NOS activity was assayed by measuring NOx in stretched and unstretched muscles in the presence and absence of D-NAME and L-NAME (Fig. 5). NOx in muscle extracts from unstretched contralateral control muscles was not significantly different from that from normal control muscles from rats that were not subjected to stretch treatment. In stretched muscles and in stretched muscles from D-NAME-treated rats, NOS activity was significantly increased (P < 0.01). NOS activity in stretched and contralateral unstretched muscles from L-NAME-treated rats was comparable to that in control muscle, indicating that L-NAME injection effectively inhibited NOS in these in vivo experiments.

In previous experiments with crushed muscle extract, we demonstrated that the activation was due to the release of HGF into the extract (24). Therefore, we sought to determine whether the activity of SME was attributable to the presence of HGF. The presence of HGF in SME was examined using immunoblot analysis, and the results are presented in Fig. 6. The muscle mass per volume of PBS was held constant in all samples to assess the relative amount of HGF released per mass unit. Lanes SME and USME in Fig. 6 are immunoblots of SMEs from vastus, gracilis, and adductor muscles (Fig. 6, lane SME) and from contralateral unstretched muscles (Fig. 6, lane USME). Because small amounts of HGF were detectable in the unstretched control samples, we hypothesized that stretch may release HGF that enters the circulation and can be detected in other muscles that are not stretched. To test this hypothesis, extracts were prepared from extensor digitorum longus (EDL)
Fig. 5. Effect of muscle stretch on NOS activity. NOS activity was assayed in stretched (lanes b) and unstretched (lanes a) muscle on the basis of NO$_2^-$ and/or NO$_3^-$ concentrations measured in PBS extracts. Treatments were applied in normal control rats not subjected to stretch treatment, in unstretched contralateral muscle from rats subjected to stretch (lane a), in stretched muscle (lane b), in stretched muscle from NPR-treated rats (lane c), and in stretched (lane b, L-NAME) and unstretched (lane a, L-NAME) contralateral muscles from L-NAME-treated rats. Points represent means ± SE of muscles from 3 rats/treatment.

Fig. 6. Presence of HGF in SME. Extract was prepared by incubating muscle in PBS (1 g/ml PBS) for 1.5 h at 4°C. HGF content was assessed using immunoblot analysis of vastus, gracilis, and adductor muscles: lane STD, molecular weight standard; lane SME, stretched muscle extract; and lane USME, unstretched muscle extract. Extracts of extensor digitorum longus (EDL) muscles from the control side of stretch-treated rats (lane a), from the stretched side of stretch-treated rats (lane b), and from an untreated rat (lane c) are shown. Lane CNT, SME without primary anti-HGF antibody (control).

Fig. 7. Immunoneutralization of HGF in SME. Satellite cell activation was evaluated in BrdU-labeled cultures at 42 h. A: SME (0.5 mg/ml) plus increasing concentrations of anti-HGF antibody from 0 to 2 µg/ml were added to satellite cell cultures. *P < 0.05. **P < 0.01. B: satellite cell activation in response to control DMEM (lane a), 10 ng/ml HGF (lane b), 0.5 mg/ml stretch muscle extract plus 2 µg/ml anti-HGF plus 40 ng/ml HGF (lane c), and 0.5 mg/ml stretch muscle extract plus 2 µg/ml control antibody (lane d). Bars and points represent means ± SE for 3 cultures/treatment. **P < 0.01.

molecules from both legs of a stretch-treated rat and from an untreated control rat (Fig. 6, lane c); EDL muscle was selected because it was not subjected to stretch on either side of the treated animal. Fig. 6, lane b, shows the presence of HGF in extracts of the EDL muscle from the stretched side of a stretch-treated rat, and Fig. 6, lane a, shows the presence of HGF in the extract of EDL muscle from the contralateral side. Similar amounts of HGF were detected in EDL muscles from the stretched and contralateral sides of stretch-treated rats, but less HGF was released into EDL extract from an untreated control rat. These results suggest that HGF is released from stretched muscle, enters the circulation, and elevates free HGF concentration in other muscles. In all extracts, the 60-kDa α-chain of mature HGF was the primary form present, although small amounts of the 90-kDa pro-HGF form could be detected.

Figure 7 displays the results from an immunoneutralization experiment in which satellite cell activation in SME was neutralized by incubation with anti-HGF antibody. In Fig. 7A, BrdU labeling of 42-h cultures of control satellite cells indicated that the addition of increasing concentrations of anti-HGF to medium containing 0.5 mg SME/ml decreased satellite cell activation. Addition of 2 µg/ml anti-HGF inactivation levels comparable to medium without SME as shown in Fig. 7B, lane a. Figure 7B also demonstrates that 10 ng of HGF/ml (lane b) produced levels of activation similar to those produced by SME with 2 µg/ml control antibody (lane d) and that the neutralization of SME with 2 µg/ml anti-HGF could be reversed by the addition of 40 ng HGF/ml (lane c).

**DISCUSSION**

These experiments were designed to examine the physiological relevance of our previously reported in vitro results. Our previous work demonstrated that isolated satellite cells from adult rats were stimulated to enter the cell cycle earlier after being subjected to cyclic stretch using a FlexerCell system (25). Furthermore, we have demonstrated that satellite cell stimulation was due to the release of HGF from its extracellular tethering to satellite cells. The last important observation from these experiments is that the release of HGF was dependent on NO synthesis (26). These results, although interesting, left open the question whether this observation was an artificial cell culture phenomenon or a reflection of the physiological response of muscle to stretch. One noteworthy difference between the satellite cell culture system and living muscle is that HGF is bound primarily around the muscle fiber in living muscle.
muscle and not necessarily to the quiescent satellite cell (24). The HGF found on cultured satellite cells as early as 12 postplating may be HGF that was released from the extracellular domain of muscle fibers during the culturing procedure. Consequently, it was important to test this model in vivo.

Satellite cell activation in long-term stretched avian muscle in vivo was demonstrated previously (15, 27). For our present study, however, we used a shorter stretch time in rats. In agreement with the work on stretched avian muscle, we have demonstrated that satellite cells can be activated to enter the cell cycle with stretch treatment times as short as 1 h. The time course of entry into the cell cycle as monitored on the basis BrdU incorporation was essentially the same as that in injured mouse muscle as described by McGeachie and Grounds (18). These experiments also provided evidence that there may have been more activation of satellite cells in the contralateral leg than in muscles of control rats that were not subjected to stretch. Anderson (3) noted a similar effect in crush-injured mouse muscles and the respective contralateral muscles. One possible explanation is that HGF released from stretched muscle may enter the circulation and activate cells elsewhere. Elevation in circulating levels of HGF after liver (13, 16), lung (30), and muscle (23) injury were described previously.

It was also demonstrated that PBS extract of stretched muscle possessed satellite cell-activating activity when applied to cultured adult satellite cells. These observations are consistent with results derived using a crushed muscle extract as originally reported by Bischoff (6) and later verified in primary rat cultures by Johnson and Allen (14) and Tatsumi et al. (24). Furthermore, in experiments that paralleled those with crushed muscle extract (24), we have shown that HGF was present in SMEs. Western blot analysis also showed some low concentrations of HGF in extracts from the contralateral muscles of stretch-treated animals, which is in agreement with the observation that the baseline level of satellite cell activation in the contralateral muscles of stretch-treated rats is higher than that in muscles of control rats. This observation was further assessed by comparing the EDL muscles of stretch-treated and control rats, because EDL muscles were not subjected to stretch in this model. More HGF was present in EDL muscle extracts from stretch-treated rats than in that from control rats, although some HGF was found in the control rat EDL muscle extract. In control muscles extracted for only 20 min at 37°C, no HGF was detected (data not shown). The activity of HGF in SME was examined further using neutralizing antibodies. In these experiments, we demonstrated in vitro satellite cell activation was due to the presence of HGF. These results are the same as observations in crushed muscle extract, which is prepared from muscle after being removed from the animal and being crushed with forceps, although an important difference is that mechanical stretch was applied to muscle in living animals.

The second aspect of this study was the exploration of the relationship between stretch activation of satellite cells and NO synthesis. Anderson (3) proposed a model for satellite cell activation after crush injury in which NO synthesis occurred early after injury as the damaged fiber contracted and retracted rapidly within the basal lamina. The shear created was hypothesized to stimulate synthesis of NO by NOS colocalized with the dystrophin-glycoprotein complex beneath the sarcolemma (10). In Anderson’s experiments, the inhibition of NO production in crush-injured muscle interfered with satellite cell activation and HGF colocalization with its signaling receptor, c-met, on satellite cells. These results were verified in vitro using an isolated muscle fiber and/or satellite cell culture assay (4). Furthermore, Tatsumi et al. (26) previously demonstrated that inhibition of NO synthesis resulted in inhibition of satellite cell activation and HGF release in in vitro satellite cell stretch experiments. Our in vivo muscle stretch experiments bridge the gap between the in vivo crushed muscle experiments of Anderson and the isolated satellite cell culture stretch experiments by demonstrating that inhibition of NO synthesis in vivo, before muscle stretch, prevented satellite cell activation. Therefore, our current experiments suggest that there is a pathway in living muscle that responds to mechanical stretch by synthesizing NO. NO synthesis subsequently causes the release of HGF from its binding to the surface of muscle fibers, which makes it available to bind c-Met receptors to satellite cells. The result is satellite cell activation. These observations are in agreement with those made previously with stretched satellite cell cultures, isolated muscle fibers and their associated satellite cells, and crush-injured muscles (3, 4, 25, 26).

Our results are consistent with in vitro observations reported previously by Perrone et al. (20) and Baar et al. (5) that stretch treatment of cultured myotubes releases factors that can act on cells in an autocrine manner. Mechanical stretch also has been shown to release growth factors from cardiomyocytes and fibroblasts (21). Consequently, the mechanism of HGF release in response to stretch may be an example of a general cellular response to stretch or to mechanical perturbation. A variety of growth factors are known to bind to proteoglycans outside cells and in the ECM, and it is conceivable that a common mechanism mediates the release of many factors associated with cells in response to mechanical insult.

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