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Short communication

Low-pH preparation of skeletal muscle satellite cells can be used to study activation in vitro

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Abstract

When skeletal muscle is stretched or injured, satellite cells are activated to enter the cell cycle, and this process could be mediated by hepatocyte growth factor (HGF) and nitric oxide (NO) as revealed by primary culture technique. In this system, which was originally developed by Allen et al. [Allen, R. E., Temm-Grove, C. J., Sheehan, S. M., & Rice, G. (1997). Skeletal muscle satellite cell cultures. *Methods Cell Biol*., *52*, 155–176], however, some populations of satellite cells would receive activation signals during the cell isolation procedure; the high baseline level of activation diminishes the magnitude of the observed effect of HGF and NO. In this study, we modified the cell isolation procedure by lowering pH of muscle and isolation media from 7.2 (original) to 6.5. This modification was designed to block the activation signal generation, based on our previous observations that the satellite cell activation in response to mechanical stimulation only occurred between pH 7.1 and 7.5. Satellite cells prepared at low-pH showed a low baseline level of activation in bromodeoxyuridine incorporation and MyoD expression assays on control cultures, and demonstrated a large activation response to mechanical stretch, exogenous HGF and NO donor. Cell yield and myogenic purity were not affected by the modifications. The low-pH procedure could provide an improved satellite cell model for in vitro activation experiments.

Keywords: Hepatocyte growth factor; Muscle regeneration; Nitric oxide; Satellite cells; Stretch-activation

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; HGF, hepatocyte growth factor; HS, horse serum; NO, nitric oxide; PBS, phosphate-buffered saline; SNP, sodium nitroprusside

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1. Introduction

Satellite cells, resident myogenic stem cells, found between the basement membrane and the sarcolemma in post-natal skeletal muscle (Mauro, 1961), are normally quiescent most of the time in adult skeletal muscles. But when muscle is injured, overused or mechanically stretched, these cells are activated to enter the cell cycle, and are responsible for regeneration and work-induced hypertrophy of muscle fibers (Kennedy, Eisenberg, Reid, Sweeney, & Zak, 1988; McCormick & Schultz, 1992; Winchester, Davis, Alway, & Gonyea, 1991). Therefore, a mechanism must exist to translate mechanical changes in muscle tissue into chemical signals that can activate satellite cells.A variety of growth factors influence satellite cell proliferation and differentiation, as demonstrated in vitro and to a lesser extent in vivo (Bischoff, 2004). Of all the growth factors studied, thus far, hepatocyte growth factor (HGF) has been shown to stimulate quiescent satellite cells to enter the cell cycle in primary culture and living muscle (Allen, Sheehan, Tayler, Kendall, & Rice, 1995; Tatsumi, Anderson, Nevoret, Halevy, & Allen, 1998) and to stimulate proliferation in injured muscle (Miller, Thaloor, Matteson, & Pavlath, 2000). The second component involved in satellite cell activation is nitric oxide (NO). Anderson (2000) pointed to production of NO burst as a key signal responsible for the satellite cell hypertrophy and detachment from the adjacent fiber following crush injury. At the cellular level, shear force generated by contraction or retraction of damaged fibers within the basal lamina is thought to stimulate constitutive nitric oxide synthase (cNOS) as revealed by muscle fiber culture technique (Anderson & Pilipowicz, 2002; Anderson & Vargas, 2003; Anderson & Wozniak, 2004; Wozniak, Kong, Bock, Pilipowicz, & Anderson, 2005). We have reported that mechanically stretching cultured satellite cells (Tatsumi, Hattori, Ikeuchi, Anderson, & Allen, 2002; Tatsumi, Sheehan, Iwasaki, Hattori, & Allen, 2001) or living skeletal muscles (Tatsumi et al., 2006) stimulates satellite cell activation by rapid release of HGF from its tethering in the extracellular matrix and its subsequent presentation to the c-met receptor. These experiments also show that HGF release is dependent on NO production by NOS in satellite cells and/or muscle fibers in response to the mechanical perturbation, and that HGF is liberated from the matrix when un-stretched cells are incubated with a NO donor, sodium nitroprusside (SNP).

In previous experiments from our laboratory, activation was monitored by 5-bromo-2′-deoxyuridine (BrdU)-incorporation into satellite cells, which were prepared from adult (9-month-old) male rats according to the method of Allen, Temm-Grove, Sheehan, and Rice (1997). In rats of this age group, satellite cells are predominantly quiescent in the tissue at the time of harvest. However, the baseline of activation index in control cultures was always significantly higher than the theoretical low percentage that would be predicted for quiescent satellite cells from normal adult muscle. A typical example of this problem is shown in Fig. 1 (open circle). The increasing baseline level possibly represents cells that received activation signals during the cell isolation procedure, which involves mechanical perturbation steps such as excising, pinching, trimming, mincing and vortexing of muscle tissues. The effect of culture preparation diminishes the ability to evaluate the true activities of HGF and NO, and leaves open the possibility that HGF/NO could just stimulate proliferation of activated cells in vitro.

In order to clarify this issue, the need for a stable baseline of low activation is indeed essential for the most

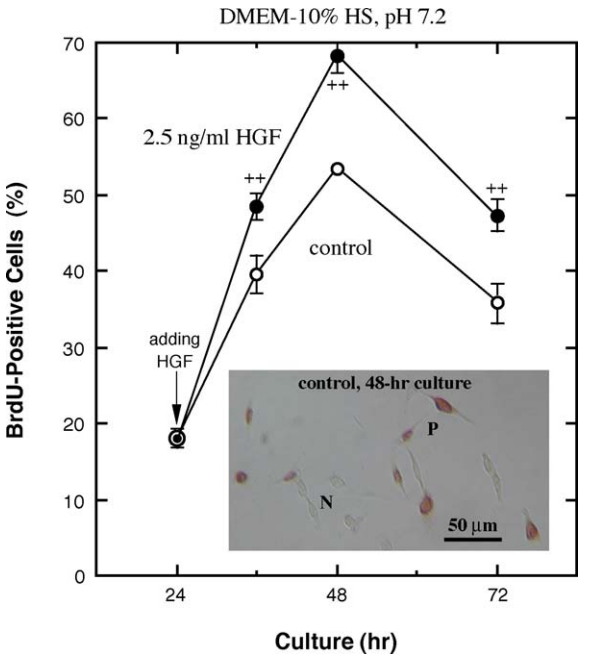

Fig. 1. HGF-induced activation profile of satellite cells prepared by the regular pH-7.2 method (Allen et al., 1997). Satellite cells were maintained in the presence (closed circle) or absence (open circle) of 2.5 ng/ml HGF during 48 h from 24 to 72 h post-plating, followed by BrdU-activation assay to count positive (P) and negative (N) cells under the microscope (see figure insert). Note that the control culture showed an increase in the BrdU-positive cell percentage with culture time from 24 to 48 h, and produced a difference as little as about 15% from the HGF culture mean at 48 h post-plating. Data points represent means and standard errors for four cultures per treatment. $^{++}$Statistically significant difference from each control culture mean at $p < 0.01$.

effective study of satellite cell activation in vitro, and for the interpretation of those experiments, as reviewed by Anderson and Wozniak (2004) and Wozniak et al. (2005). A good approach was reported in a single fiber culture model by Wozniak et al. (2003) and Wozniak and Anderson (2005), however, currently there is no good technical approach to prevent activation of satellite cells in cell culture studies. We hypothesized that acidic conditions could prevent spontaneous activation during the cell isolation procedure, according to our previous result that the satellite cell activation in response to mechanical stretch only occurred between pH 7.1 and 7.5 (Tatsumi et al., 2002). In this paper, we modified the isolation procedure of satellite cells by lowering pH of muscle and cell isolation media from 7.2 (original) to 6.5, and then re-examined their activation potentials in response to stretch, HGF and NO in vitro.

2. Materials and methods

2.1. Animal care and use

Experiments were conducted according to The Guideline for the Care and Use of Laboratory Animals, Graduate School of Agriculture, Kyushu University and with the approval of The Kyushu University Laboratory Animal Care and Use Committee (No. 05-002-01).

2.2. Satellite cell isolation and culture

Satellite cells were isolated from 9-month-old male Sprague–Dawley rats according to Allen et al. (1997) with two minor modifications: (i) pH of the cell isolation medium, phosphate-buffered saline (PBS), was switched from 7.2 (original) to 6.5 and (ii) muscles were excised after their pH was decreased down to 6.5. The muscle pH declined to the value in a curvilinear fashion within 30 min post-mortem due to accumulation of a glycogen metabolite lactic acid, as monitored by inserting a needle type electrode into longissimus thoracis and gluteus medius muscles of five individual rats examined (Fig. 2).

Subsequently, muscle groups from the back and upper hind limb were trimmed of fat and connective tissue in PBS-pH 6.5, hand minced with scissors, and digested for 1 h at 37 °C with 1.25 mg/ml protease type XIV (Sigma, St. Louis, MO, USA) in PBS-pH 6.5. Cells were separated from muscle fiber fragments and tissue debris by differential centrifugation and plated on poly-L-lysine and fibronectin-coated BioFlex amino culture plates (Flexcell International, McKeesport, PA, USA) in Dulbecco's modified Eagle medium (DMEM) containing 10% normal horse serum (DMEM-10% HS, pH 7.2),

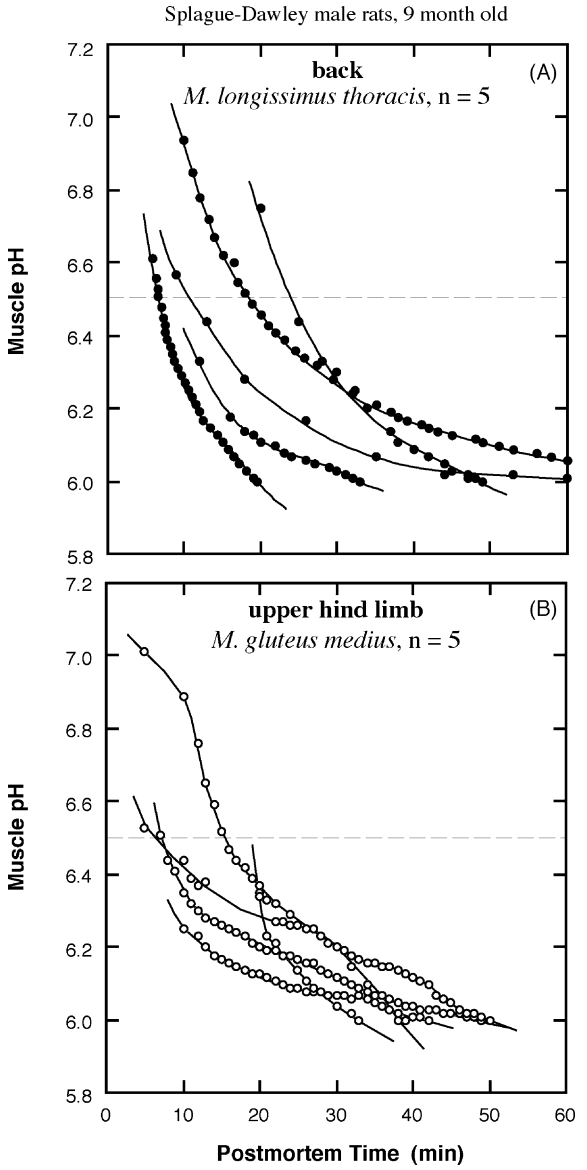

Fig. 2. Post-mortem changes in muscle pH. The change was monitored at ambient temperature of about 25 °C by directly inserting a needle type glass pH electrode into *M. longissimus thoracis* of the back (panel A) and *M. gluteus medius* of the upper hind limb (panel B) of 5 individual rats.

1% antibiotic–antimycotic mixture and 0.5% gentamicin (Life Technology, Grand Island, NY, USA).

Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h and then subjected to mechanical stretch from 24 to 72 h post-plating in culture in a vacuum-operated cyclic strain-providing instrument (FlexerCell FX-2000 System, Flexcell International). The percentage of stretch and the stretch interval length were previously optimized for the effect on satellite

cell activation (Tatsumi et al., 2001); optimum conditions of 25% stretch at 12-s intervals were used (Fig. 5, upper panel). For cell culture assays of human recombinant HGF (R&D Systems, Minneapolis, MN, USA) and sodium nitroprusside (SNP; Calbiochem-Novabiochem, La Jolla, CA, USA), a NO donor, satellite cells were cultured for 24 h on poly-L-lysine and fibronectin-coated 48-well cluster culture plates (Costar, Cambridge, MA, USA), and then maintained in the presence of 2.5 ng/ml HGF or 30 μM SNP from 24 to 72 h post-plating.

2.3. Myogenic purity

Companion satellite cell cultures were immunostained for c-met and PAX7 at 30 h post-plating in order to determine the percentage of myogenic cells present. Briefly, cells were fixed for 10 min in cold methanol-0.1% H_2O_2 or 3.7% paraformaldehyde in PBS, treated with 0.5% TritonX-100 in PBS for 10 min, blocked with 10% HS-1% bovine serum albumin (BSA) in PBS for 30 min, and incubated overnight at 4 °C in primary antibody against c-met (R&D Systems, 1:100 dilution in 0.1% BSA-PBS) or PAX7 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA, 1:1000 dilution in 0.1% Tween 20-PBS (TPBS)). Triton treatment was omitted for c-met staining. Cells were subsequently incubated with a biotinylated anti-mouse or goat IgG affinity-purified secondary antibody (Vector Laboratory, Burlingame, CA, USA, 1:10,000 dilution in 0.1% BSA-PBS) and horseradish peroxidase–avidin (Vector Laboratory, 1:500 dilution in PBS) for 2 h at an ambient temperature, followed by measurements of 3,3′-diaminobenzidine-stained cell percentage. Cultures with less than 95% positive cells were not used for experiments.

2.4. In vitro activation assay

Cultures were pulse-labeled with 10 μM 5-bromo-2′-deoxyuridine in DMEM-10% HS during the final 2 h at each time point, followed by immunocytochemistry for detection of BrdU using a G3G4 mouse monoclonal anti-BrdU antibody (Developmental Studies Hybridoma Bank, 1:100 dilution in 0.1% BSA-PBS) and an affinity-purified horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, 1:500 dilution) according to Tatsumi et al. (1998). The percentage of BrdU labeled cells was used as an indicator of activation and entry into the cell cycle.

Cell activation was monitored also by immunocytochemistry for detection of MyoD protein using a monoclonal primary antibody (BD Biosciences, 1:1000 dilution in TPBS, overnight at 4 °C), biotinylated anti-mouse IgG secondary antibody (Vector Laboratory, 1:10,000 dilution in 0.1% BSA-PBS) and horseradish peroxidase–avidin (Vector Laboratory, 1:500 dilution in PBS, for 2 h at an ambient temperature); 3,3′-diaminobenzidine-stained cell percentage was measured under the microscope.

2.5. Western blotting

Conditioned media (DMEM) from 2-h stretched and un-stretched satellite cell cultures were subjected to sodium dodecylsulfate-10% polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970). Separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK), which were then blocked with 10% powdered milk in 0.1% Tween 20-Tris buffered saline (TTBS) prior to incubation with 1:250 dilution of a goat polyclonal anti-HGF primary antibody (R&D Systems) overnight at room temperature. Membranes were subsequently treated with a biotinylated rabbit anti-goat IgG affinity-purified secondary antibody (Vector Laboratory) at a 1:5000 dilution in 1% powdered milk in TTBS for 1 h, then with horseradish peroxidase–labeled avidin (Vector Laboratory) at a 1:500 dilution in TTBS for 30 min at room temperature, followed by enhanced chemiluminescence detection onto Kodak BIOMAX-AR X-ray films according to manufacture's recommendation (Amersham Biosciences).

2.6. Statistical analysis

General analysis of variance procedures were employed to analyze experimental results using general linear model procedures of SRISTAT2 for Windows software (Social Survey Research Information, Tokyo, Japan). Least-squares means for each treatment were separated based on least significant differences. Data were represented as means and standard errors for four cultures per treatment, and statistically significant differences at $p < 0.01$ were indicated by (++) or (**).

3. Results and discussion

The purpose of this study was to establish an isolation procedure of quiescent satellite cells and to re-examine their activation potential in response to mechanical stretch, HGF and NO in vitro. We modified the original cell isolation procedure (Allen et al., 1997) by lowering pH of muscle and the cell isolation media from 7.2 to 6.5. This modification was designed to block the

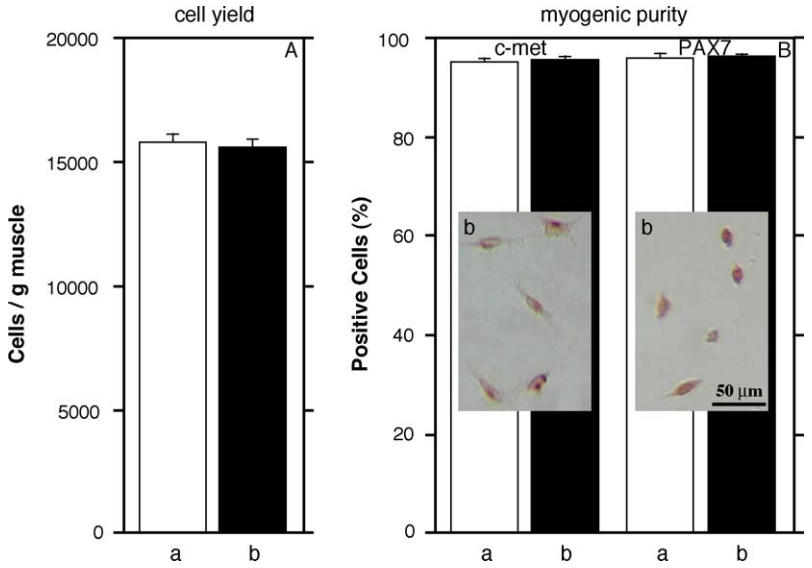

Fig. 3. Cell yield and myogenic purity. Satellite cells were prepared by the regular pH-7.2 method (Allen et al., 1997) (bar a) or the pH-6.5 method (bar b), and maintained in DMEM-10% HS (pH 7.2) for 30 h. The cell density was measured under the microscope to estimate the yield of living cells (panel A). Companion satellite cell cultures were immunostained for the presence of c-met and PAX7 using the primary antibody and biotinylated secondary antibody reagents (figure insert) in order to determine the percentage of myogenic cells present (panel B). Bars represent means and standard errors for four cultures per treatment.

activation signal generation during the cell preparation process. According to our previous investigations, satellite cell activation in response to mechanical stretch only occurred between pH 7.1 and 7.5 with the maximum stretch effect at pH 7.2, and the effect was due to changes in HGF release from the extracellular matrix (Tatsumi et al., 2002) and NOS activity (Gorren, Schrammel, Schmidt, & Mayer, 1998) as a function of pH.

Satellite cell preparations obtained by the pH-6.5 procedure were first evaluated for cell yield and myogenic

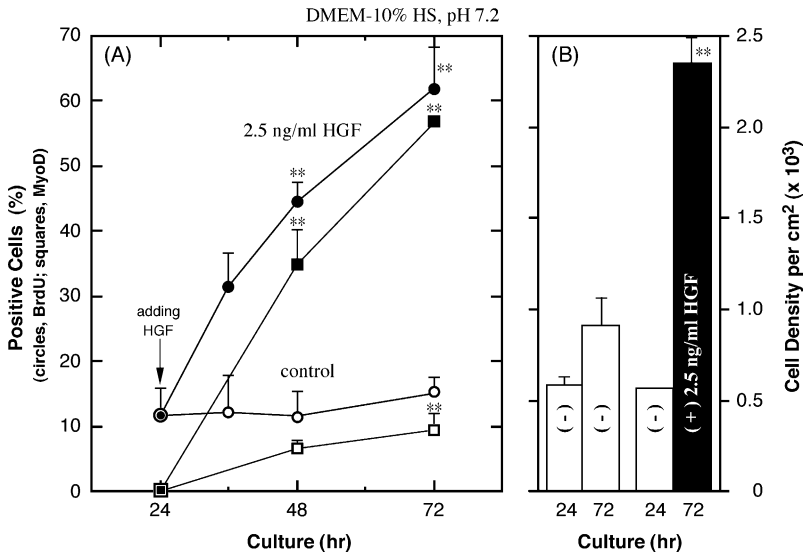

Fig. 4. Time course of HGF-induced activation of satellite cells prepared by the pH-6.5 method. Satellite cells were maintained in the presence (closed symbols and bar) or absence (open symbols and bars) of 2.5 ng/ml HGF during 48 h from 24 to 72 h post-plating. Activation activity (panel A) was evaluated by pulse labeling cells with BrdU (circles) and by immunocytochemistry for MyoD expression (squares) in culture. Panel B presents the cell density in the respective cultures at 24 h and 72 h time points. Data points and bars represent means with standard errors for four culture wells per treatment. ** Statistically significant difference from control culture mean of 24 h post-plating at $p < 0.01$.

purity (Fig. 3). The living cell yield, as measured by cell density attached to culture plates at 30 h post-plating, showed no significant difference from cells prepared separately from the same rats by the regular pH-7.2 procedure (panel A). There was also no difference in the myogenic purity, which was assayed for the presence of the c-met and PAX7 proteins at 30 h post-plating and was over 95% in both preparations (panel B).

In experiments described in Figs. 4–6, satellite cells were subjected to treatments that have been shown to stimulate activation for 48 h from 24 to 72 h post-plating followed by pulse-labeling with BrdU during the final 2 h at each time point. Activation to enter the cell cycle was indexed by BrdU-incorporated cell percentage. In control cultures (open circles), the activation index was almost flat throughout the culture time (about 10–15%). This profile was in contrast to the increase in the activation index of cells prepared by the regular pH-7.2 procedure (Fig. 1, open circles). Cell density did not differ significantly between 24 and 72 h post-plating (Fig. 4, panel B, open bars), suggesting that most cells were maintained in quiescent state in culture. This was supported by low expression level of MyoD protein which was monitored by immunocytochemistry on control cultures (Fig. 4A, open squares).

When cultures received 2.5 ng/ml HGF, which has been shown to stimulate peak activation (Tatsumi et al., 2002), the activation index and cell density were greatly increased with culture time (Fig. 4). The activation index showed differences of about 30 and 45% from individual control culture means at 48 and 72 h post-plating, respectively. This response was greater than that of cells which were prepared by the regular pH-7.2 procedure; the activation index differed as small as about 10–15% from control means at an optimized assay time point, 36-h post-plating (Tatsumi et al., 2001, 2002). Also, MyoD expression was greatly up-regulated by 2.5 ng/ml HGF as shown in Fig. 4A (closed squares). These results indi-

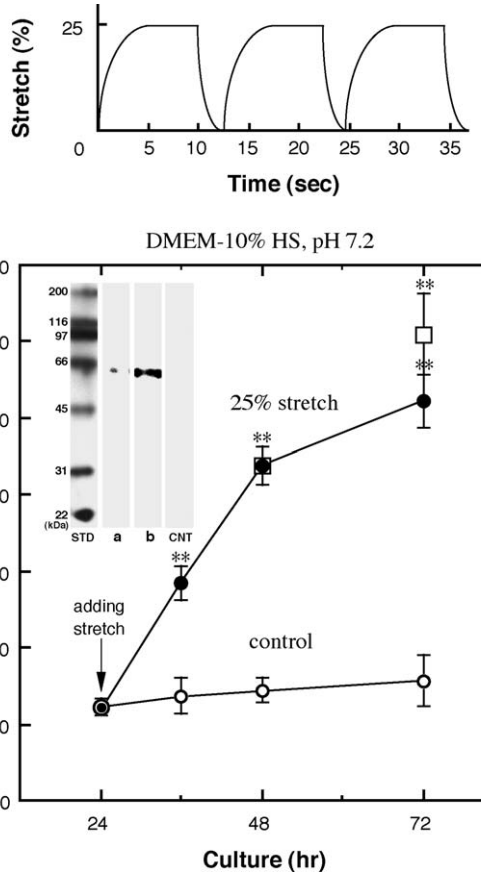

Fig. 5. Time course of mechanical stretch-induced activation of satellite cells prepared by the pH-6.5 method. Satellite cells were maintained in DMEM-10% HS (pH 7.2) in the presence (closed circle) or absence (open circle) of mechanical stretch during 48 h from 24 to 72 h post-plating in FlexerCell FX-2000 System (25% stretch at 12-s intervals as shown in upper panel). Squares, positive control cultures maintained with 2.5 ng/ml HGF from 24 to 72 h post-plating. *Insert*: Western blotting of HGF in conditioned media from control (a) and 2-h stretched (b) cells. CNT, control blot of lane b without the primary antibody treatment; STD, molecular weight standard.

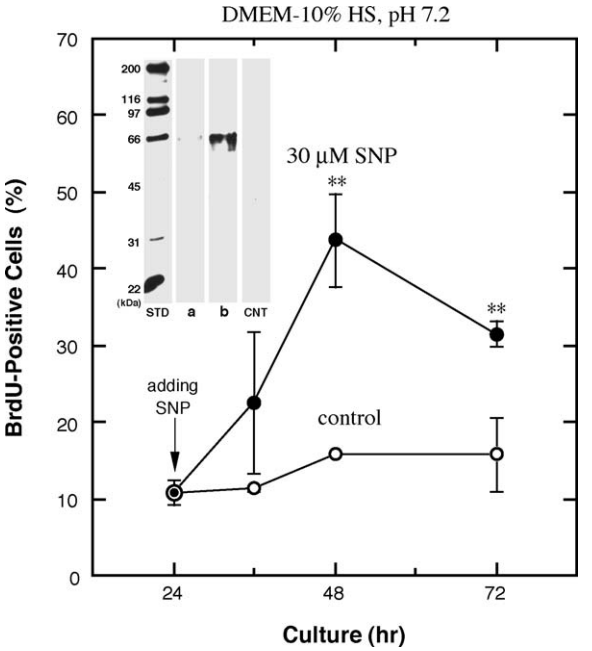

Fig. 6. Time course of NO-induced activation of satellite cells prepared by the pH-6.5 method. Satellite cells were maintained in DMEM-10% HS (pH 7.2) in the presence (closed circle) or absence (open circle) of 30 μM SNP during 48 h from 24 to 72 h post-plating. Insert, western blotting of HGF in conditioned media from control (a) and 2-h SNP-treated (b) cells. CNT, control blot of lane b without the primary antibody treatment; STD, molecular weight standard.

cate that satellite cells prepared by the pH-6.5 procedure maintain high activation potential.

This issue was further examined by assessing activation activities of the cells in response to mechanical stretch and SNP of NO donor (Figs. 5 and 6). Stretch pattern (25% stretch at 12-s intervals) and SNP concentration (30 μM) were previously optimized for the effect on satellite cell activation (Tatsumi et al., 2001, 2002). Stretch pattern applied was depicted in Fig. 5 upper panel and its cyclic performance was controlled by FlexerCell FX-2000 System equipped with BioFlex baseplate. When mechanical stretch or SNP was added to the cells, the activation index was greatly increased; activation percentage reached to a level comparable to positive control culture receiving 2.5 ng/ml HGF. These results demonstrated that the satellite cells maintain high activation potential in response to mechanical stretch and NO in vitro.

The final experiment was conducted to assess HGF releasing ability from its extracellular association, which is critical aspect of stretch-induced activation of satellite cells in addition to NO production by the cells (Tatsumi et al., 2001, 2002). Figs. 5 and 6 inserts show western blots of conditioned media that were collected from stretched or SNP-treated satellite cell cultures; cells were switched into serum-free DMEM (pH 7.2) at 24 h post-plating, the previously described stretch or SNP protocol was applied for a period of as short as 2 h, media were collected and analyzed for the presence of HGF in proteins from a constant number of satellite cells. A band corresponding to 60-kDa active HGF was clearly detected in both stretched and SNP-treated culture media; very little HGF was seen in control media. These results indicate that the HGF release takes place in response to stretch or SNP-treatment. NOS activity was also assessed in 24-h stretched and control cells as measured by the production of NO_2 and NO_3 of NO metabolites at pH 7.2. The assay was conducted by an automated NO detector-high-performance liquid chromatography system (Eicom, Kyoto, Japan) standardized with 10 μM sodium nitrite and sodium nitrate according to Tatsumi et al. (2002), and demonstrated the statistically significant increase in NO_x in conditioned media in response to mechanical stretch at $p < 0.01$ (12.2 ± 0.123 μM versus 8.96 ± 0.0790 μM of un-stretched control mean with standard error).

To summarize, in this study, we demonstrated a technique for overcoming the problem of spontaneous activation of quiescent satellite cells in culture that results from the cell isolation procedure. By lowering pH during isolation, satellite cells seem to stay in a quiescent state in control culture, as indicated by BrdU incorporation and MyoD expression assays, but they can be activated in response to stretch, HGF and NO. Cell yield, myogenic cell percentage, HGF release and NOS activities were not affected by the modifications. The pH-6.5 procedure presented here is a simple procedure that may improve the ability to study satellite cell activation in vitro.

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