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The enzymatic activity of sialidase Neu2 is inversely regulated during in vitro myoblast hypertrophy and atrophy

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ABSTRACT

Sialidase Neu2 is an exoglycosidase that removes terminal sialic acids from glycolipids and glycoproteins. In this study, we investigated Neu2 expression during muscle hypertrophy and atrophy. Neu2 mRNA and enzymatic activity were significantly increased in hypertrophic myofibers. A rise in Neu2 activity was observed after constitutive activation of AKT or Igf-1 treatment as well as in myoblasts treated with vasopressin or trichostatin, an inhibitor of histone deacetylases. In contrast, myofiber atrophy obtained by dexamethasone treatment or starvation triggered a significant loss of Neu2 activity and was paralleled by downregulation of Neu2 transcript levels. Overall, we may conclude that Neu2 enzymatic activity is causally linked to proper muscle differentiation and growth.

The enzymatic activity of mammalian cytosolic sialidase Neu2 usually increases during formation and growth of myotubes [1–3], suggesting that the modulation of sialylated glycoconjugates in the cytosol of myofibers contributes to proper differentiation. In line with this theory, Neu2 expression is stimulated predominantly via the PI3K/AKT/mTOR pathway [4], a key regulator of the myogenic program [5]. Insulin-like growth factor-1 (Igf-1) plays a predominant role in muscle growth [5,6], as its muscle-specific overexpression produces a remarkable muscle hypertrophy in transgenic mice [7]. Accordingly, conditional activation of AKT in muscle produces rapid hypertrophy [8]. Over the last few years, additional factors have been suggested to stimulate myofiber differentiation and hypertrophy through additional pathways. Among these, Arg^8-vasopressin has been shown to stimulate myogenic differentiation by activation of both calcineurin and Ca^{2+}/calmodulin-dependent kinase [9,10], whereas follistatin, a member of the TGF-β family, increases muscle cell size by increased recruitment of satellite cells into pre-existing myofibers [11]. In contrast, maintenance of muscle mass is often compromised under different physiological and pathological conditions that trigger sarcomere remodelling, leading in turn to myofiber atrophy. In this study, we employed different in vitro myoblast cell models to investigate the expression of sialidase Neu2 in relation to myofiber hypertrophy and atrophy.

Materials and methods

Materials. All reagents were from Sigma–Aldrich, if not indicated otherwise.

Cell culture and pharmacological treatments. Myoblasts were maintained at 37 °C and 5% CO_2 in DMEM supplemented with 20% FBS and 100 μg/mL penicillin–streptomycin. To induce differentiation, preconfluent C2C12 cells were cultured in DMEM supplemented with 2% HS. This medium was changed daily. To induce atrophy, C2C12 myotubes were starved in glucose- and amino acid-deprived medium (10 mM Hank's/Hepes buffer (pH 7.4)) or, alternatively, treated with 100 μM dexamethasone.

Differentiation of rat L6E9 myoblasts was induced when preconfluent cells were shifted to DMEM supplemented with 1% FBS, whereas hypertrophy was obtained after administration of 10 ng/mL Igf-1 after one day of differentiation.

Preconfluent rat L6C5 myoblasts were differentiated in DMEM supplemented with 1% fatty acid-free BSA, whereas hypertrophy was obtained after treatment with 0.1 μM Arg^8-vasopressin (AVP). Alternatively, L6C5 myoblasts were treated with 50 nM trichostatin (TSA) for 16–18 h in high-serum medium, and then allowed to undergo hypertrophy in DMEM supplemented with 1% FBS. The myoblasts were also treated with the following pharmacological agents: PI3K inhibitor LY294002 (10 μM), mTOR inhibitor rapamycin (5 ng/ml), calcineurin inhibitor cyclosporin A (2.5 μM) and the Ca^{2+}/calmodulin-dependent kinase inhibitor KN562 (8 μM). To quantify the myofiber size, 10 fields were randomly chosen and 10 myotubes were measured per field. The average diameter per myotube was the mean of 10 measurements taken along the length of the myotube. The fusion index was determined by counting the number of nuclei in a single myofiber. Ten myotubes in ten different microscopic fields were chosen to calculate the average number of nuclei.

Sialidase assay. The enzymatic activity of cytosolic sialidase was assayed as previously described [3,4]. Briefly, ultracentrifuged cytosolic fractions of myotubes were assayed using a mixture containing 60 nmol of the synthetic substrate 4-

Abbreviations: FBS, fetal bovine serum; HS, horse serum; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered solution; PI3K, phosphoinositide-3 kinase; MyHC, myosin heavy chain.

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methylumbelliferyl *N*-acetylneuraminic acid and 100 μg of BSA in 0.2 mL of 50 mM sodium acetate buffer (pH 5.8). After 3 h of incubation at 37 °C, the reaction was terminated by addition of 0.8 mL of 0.25 M glycine buffer (pH 10.4), and the amount of 4-methylumbelliferone released was determined fluorometrically with an excitation wavelengh of 365 nm and an emission of 450 nm. A pH curve has been set up for each experiment to determine whether the enzymatic activity assayed in the cytosolic fraction had the optimum in the typical range of cytosolic sialidases, usually between 5.6 and 6.

Plasmid construction and stable trasfections. The pBABE vectors harboring a myristoylated or a kinase-dead AKT (mutated at the ATP binding site K179M) [6] were used to transfect C2C12 myoblasts. Rat Neu2 cDNA was amplified using pCDNA-Neu2 plasmid as template [3] and primers 5′-CGGGATCCCGATGGAGACCTGCCCCG TCCTCCAGAAA-3′ and 5′-CGGGATCCCGTCAAGCGTAGTCTGGGACGTCGTATGGGTAC CCCTGAGCACCATGTACTGTGGG-3′ containing BamHI restriction sites and the hemagglutinin (HA) epitope in the reverse primer. The resultant PCR product was BamHI digested and cloned in the similarly opened pBabe vector, yielding plasmid pBabe/HA-Neu2 used to transfect C2C12 myoblasts. Stable transfectants were obtained after 10–15 days of selection in medium containing the antibiotic puromycin (2 μg/mL).

RT-PCR analysis. Total RNA was obtained by Tri-reagent extraction, digested with 1 U of DNase (DNA-free, Ambion), and retrotranscribed (2 μg) with 400 U of MMLV-RT (Promega).

The primers (250 nM) were as follows: mouse Neu2 sialidase forward primer (5′-CGAGCCAGCAAGACGGATGAG-3′) and reverse (5′-GGCTCTACAAGCTTACTCAC TACCCGG-3′) were used for 29 cycles of PCR; rat Neu2 sialidase forward primer (5′-CCGTCCAGGACCTCACAGAG-3′) and reverse (5′-TCACTGAGCACCATGTACTG-3′) were used for 30 cycles of PCR; rat follistatin forward primer (5′-CTCTTCAAGTGG ATGATTTTC-3′) and reverse (5′-ACAGTAGGCATTATTGGTCTG-3′) were used for 30 rounds of PCR; mouse Murf-1 forward primer (5′-GGTGCCTACTTGCTCCTTGT-3′) and reverse (5′-CTGGTGGCTATTCTCCTTGG-3′) were used for 27 rounds of PCR; mouse Atrogin forward primer (5′-CGACCTGCCTGTGTGCTTAC-3′) and reverse (5′-CTTGCGAATCTGCCTCTCTG-3′) were used for 28 cycles of PCR. Gene expression levels were normalized to gapdh mRNA expression by 23 rounds of PCR.

Western blot analysis. Samples were separated by SDS–PAGE under reducing conditions and transferred to nitrocellulose membranes according to standard procedures. The following primary antibodies were used: anti-myogenin (clone F5D) and anti-GATA-2 (clone CG2-96) from Santa Cruz Biotechnology, anti-MyHC from the Hybridoma Bank (University of Iowa), anti-phospho-AKT (Ser^{473}) and anti-phospho-p70S6K (Thr^{389}) from Cell Signalling Technology, anti-HA, and anti-tubulin from Sigma–Aldrich. After incubation with horseradish peroxidase-conjugated secondary antibodies (Chemicon), immunocomplexes were visualized using enhanced chemiluminescence reagent (Chemicon).

Degradation of long-lived proteins. Bulk degradation of long-lived proteins was determined according to a method previously reported [12]. Briefly, cells were plated into 35 mm dishes and cultured in cysteine/methionine free media containing 5 μCi L-[^{35}S] cysteine/methionine (GE Healthcare) for 6 h at 37 °C. Unincorporated radioisotopes and degraded amino acids released from short-lived proteins were removed by rinsing three times with PBS. Cells were then chased with the culture medium containing 10% FBS and 2 mM cold cysteine/methionine. After 15 h incubation, at which time short-lived proteins were being degraded, the chase medium was replaced with serum-containing DMEM (control) or Hank's/Hepes buffer. After incubation at 37 °C for 6 h, the medium was harvested and 100% trichloroacetic acid (TCA) was added to 10% final concentration. The samples were centrifuged at 12,000*g* for 10 min and the acid-soluble radioactivity was measured by liquid scintillation counting. Meanwhile, the cells were fixed by adding 1 ml of 10% TCA directly to the culture dishes, washed with 10% TCA and dissolved in 1 ml of 0.2 N NaOH. Radioactivity in the samples was measured similarly. The percentage protein degradation was calculated by dividing the amount of acid-soluble radioactivity in the culture medium by the sum of acid-soluble and acid-precipitable radioactivities.

Electron microscopy. Samples were fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution for 2 h and postfixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 1% OsO_4 solution for 1 h. After dehydration in an ethanol gradient (70% ethanol [20 min], 96% ethanol [20 min], 100% ethanol [2 × 20 min]), samples were incubated with propylenoxid (2 × 10 min), impregnated with a mixture of propylenoid/LX-112 (Ladd Research Industries, 1:1) and embedded in LX-112. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Jeol-100 CX II TEM at 80 kV.

Immunofluorescence microscopy. The myotubes grown on 12 mm glass coverslips were coated with 20 μg/mL laminin (Roche), fixed with ice cold methanol and then incubated with rat monoclonal anti-HA antibody (clone 3F10, Roche) followed by an anti-rat Alexa Fluor 594-conjugated secondary antibody (Molecular Probes). Alternatively, cells were incubated with anti-MyHC antibody (Hybridoma Bank, University of Iowa) followed by a biotinylated anti-mouse antibody and Alexa Fluor 488 streptavidin-conjugate (dilution 1:800). Fluorescent staining of myotubes was observed under an Axiovert S100 microscope (Zeiss).

Statistics. All data are expressed as means ± SEM. Statistical significance was determined using *t*-Student analysis. A p value of <0.05 was considered significant.

Results and discussion

In vitro myoblast differentiation and hypertrophy enhance Neu2 enzymatic activity

In this study, we analyzed the Neu2 enzymatic activity in myoblasts undergoing differentiation and hypertrophy upon different stimuli. Different pathways have been characterized to promote myoblast fusion and growth; in particular, the PI3K/AKT/mTOR pathway plays a pivotal role during myofiber growth [5,6], as its conditional activation in skeletal muscle induces rapid hypertrophy in transgenic mice [8]. Accordingly, the constitutive activation of AKT (caAKT) by expression of a myristoylated AKT form [6] led to sustained hypertrophy of C2C12 myoblasts, as revealed by morphological analysis (Fig. 1A) and myofiber size measurements (Fig. 1B, top); in caAKT myofibers, an increased phosphorylation of AKT and its direct downstream target p70S6K were detected via Western blotting as compared to control (Fig. 1B, bottom), whereas Neu2 transcript levels were upregulated (Fig. 1B, bottom). Under these conditions, a strong increase of sialidase enzymatic activity was detectable in the cytosol of hypertrophic myotubes already two days after differentiation (Fig. 1C). Inhibition of mTOR activity in caAKT myoblasts after treatment with rapamycin abolished the myofiber hypertrophy (not shown) and caused a significant loss of Neu2 activity (Fig. 1C). Also expression of a kinase inactive form of AKT (kiAKT) in C2C12 cells reduced myofiber size (not shown) [6] and downregulated Neu2 activity (Fig. 1C), indicating that the activation of the AKT/mTOR pathway stimulates sustained Neu2 enzymatic activity that may accompany muscle hypertrophy.

We further analyzed Neu2 activity in L6E9 myoblasts, a cell model which has been well characterized to develop hypertrophy upon Igf-1 treatment [13]. Igf-1 induced already at day 3 a pronounced myofiber hypertrophy as compared to untreated L6E9 myoblasts (Fig. 1D). This effect was accompanied by elevated expression of myogenin as detected via Western blotting and by increased transcription of Neu2 mRNA (Fig. 1E), followed by increased Neu2 enzymatic activity (Fig. 1F). Because PI3K and calcineurin activity are both required to switch myoblasts from a proliferative to a myogenic condition [14,11], co-administration of Igf-1 with the PI3K inhibitor LY294002 (LY) or the calcineurin inhibitor cyclosporin A (CsA) reverted the effects induced by Igf-1 and resulted in loss of Neu2 activity (Fig. 1F). To obtain additional evidence that increased Neu2 expression may depend on calcineurin activity in myotubes, we stimulated L6C5 myoblasts with Arg^8-vasopressin (AVP), a neurohypophyseal peptide that by interacting with V1 type receptors triggers hypertrophy mainly through the activation of calcineurin and Ca^{2+}/calmodulin-dependent kinase [9,10]. Myoblasts AVP-treated for 6 days formed larger myotubes as compared to control cells and showed two typical hallmarks of muscle hypertrophy [13], the reorganization of nuclei into nuclear rings (Fig. 1G) and increased expression of the transcription factor GATA-2 (Fig. 1H). Under these conditions, AVP treatment upregulated Neu2 transcription significantly (Fig. 1H) and triggered a progressive, long-lasting (6 days) increase in Neu2 activity (Fig. 1I) that was suppressed after single or simultaneous administration of two specific inhibitors of calcineurin and Ca^{2+}/calmodulin-dependent kinase from day 3 to 6 (d3–d6) (Fig. 1J), a time point during which the myogenic program was already started. Finally, we tested whether Neu2 sialidase activity was modulated during myofiber hypertrophy caused by an augmented recruitment of myoblasts into pre-existing myofibers. In particular, follistatin is considered to play a pivotal role in this process through a mechanism that involves the inhibition of classes I–II histone deacetylases (HDAC) [11,15,16]. After administration of trichostatin