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NEU3 Sialidase Is Activated under Hypoxia and Protects Skeletal Muscle Cells from Apoptosis through the Activation of the Epidermal Growth Factor Receptor Signaling Pathway and the Hypoxia-inducible Factor (HIF)-1α[S]

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Background: NEU3 sialidase removes sialic acid from gangliosides on adjacent cells.
Results: NEU3 is up-regulated upon exposure of skeletal myoblasts to hypoxic stress, and it stimulates the EGFR signaling cascade ultimately activating HIF-1α.
Conclusion: NEU3 plays a physiological role in protecting myoblasts from hypoxic stress.
Significance: NEU3 role in cell response to hypoxia may suggest new therapeutic approaches to ischemic diseases.

NEU3 sialidase, a key enzyme in ganglioside metabolism, is activated under hypoxic conditions in cultured skeletal muscle cells (C2C12). NEU3 up-regulation stimulates the EGF receptor signaling pathway, which in turn activates the hypoxia-inducible factor (HIF-1α), resulting in a final increase of cell survival and proliferation. In the same cells, stable overexpression of sialidase NEU3 significantly enhances cell resistance to hypoxia, whereas stable silencing of the enzyme renders cells more susceptible to apoptosis. These data support the working hypothesis of a physiological role played by NEU3 sialidase in protecting cells from hypoxic stress and may suggest new directions in the development of therapeutic strategies against ischemic diseases, particularly of the cerebro-cardiovascular system.

NEU3 sialidase (1, 2), hereafter simply NEU3, is the member of the mammalian sialidase family often referred to as the "ganglioside sialidase" (3) because it is known to remove sialic acid preferentially from gangliosides (4). The enzyme is linked to the plasma membrane and has been shown to possess trans-activity because it can also work on the gangliosides present on the plasma membrane of adjacent cells (5). These key features are likely instrumental for a crucial role played by the enzyme in many cellular processes, including cell proliferation and differentiation (4). In particular, the increase of sialidase expression and activity during skeletal muscle differentiation has been shown to drive the process, protecting myoblasts from apoptosis (6). Moreover, an induced overexpression of NEU3 renders murine myoblasts more resistant to cell cycle withdrawing and accelerates the differentiation process, ultimately increasing cell fusion (7). Along this line, NEU3 has been also shown to positively participate to the differentiation process leading to neurite formation in mice (8) and to the regulation and regeneration of rat hippocampus neurons (9, 10). It should be noted that activation of pro-survival pathways induced by NEU3, with concomitant suppression of apoptosis, has been mostly reported in pathological conditions (4, 11). For instance, NEU3 aberrant overexpression has been observed to occur in several neoplasms (including colon, ovarian, renal, and prostate cancer) and is considered to be one of the key triggers of tumor growth and invasiveness. Of course, NEU3, a highly conserved and ubiquitously expressed enzyme (4), is expected to play a role under physiological conditions. We moved on this direction, and the present investigation was undertaken with the aim of ascertaining the possible involvement of NEU3 in cell response to hypoxic stress and in the cell machinery opposing cell death. To this purpose, the enzyme was stably overexpressed or silenced in murine skeletal myoblasts C2C12, which were then cultured under hypoxic conditions and results compared with those of wild-type C2C12. We found that endogenous NEU3 is activated in wild-type C2C12 under hypoxic culturing conditions and that overexpression of the enzyme greatly increases cell resistance, opposing programmed cell death, whereas silencing of the enzyme facilitates apoptosis. Finding new ways of triggering and possibly enhancing the physiological cell response to hypoxia may represent a new therapeutic approach for several ischemic diseases, including heart and brain strokes.

EXPERIMENTAL PROCEDURES

Cell Culture and Stable Overexpression and Silencing of NEU3 in C2C12 Cells—C2C12 mouse myoblasts were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium with high glucose (DMEM) supplemented with 10% (v/v) fetal bovine serum

[S] This article contains supplemental Fig. 1.

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(FBS), 4 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C in 5% CO_2, 95% air-humidified atmosphere. For the 1% O_2 hypoxia experiments, cells were cultured at 37 °C in 5% CO_2, 1% O_2 incubator with nitrogen inlet. For chemical hypoxia, 100 μM deferoxamine (DFO[2]; Sigma) was dissolved in the culture medium. All cell culture reagents were purchased from Sigma-Aldrich. C2C12 stably overexpressing (L-NEU3) and stably silencing (i-NEU3) NEU3 sialidase were prepared according to our previously developed protocols (6, 7).

RNA Extraction and Gene Expression by Quantitative PCR (qPCR)—Total RNA was isolated with RNeasy Mini Kit (Qiagen), following the protocol suggested by the manufacturer. Then, 1 μg of RNA was reverse-transcribed employing the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). For qPCR, 10 ng of total RNA was used as template with iCycler iQ5 (Bio-Rad). The PCR mixture included 0.2 μM primers (see sequences below) and iQ SYBR Green Supermix (Bio-Rad Laboratories), in a final volume of 20 μl. Amplification and qPCR data acquisition were performed using the following cycle-conditions: initial denaturation at 95 °C for 3 min, followed by 45 cycles of 5 s each at 95 °C and 30 s at 57 °C. The -fold change in expression of the different genes in L-NEU3 and i-NEU3 cells compared with control C2C12 was normalized to the expression of β-actin mRNA and was calculated by $2^{-\Delta\Delta Ct}$ with iQ5 software version 2.0 (Bio-Rad). All reactions were performed in triplicate, and the accuracy was monitored by analysis of the PCR product melting curve. Primers used were (6, 7): β-actin forward, 5′-AGAGGGAAATCGTGCGTGAC-3′ and reverse, 5′-CTCGTTGCCAATAGTGATGACC-3′; HIF-1α forward, 5′-CGCTATCCACATCAAAGCAA-3′ and reverse, 5′-GCACTAGACAAAGTTCACCTGAGA-3′; NEU3 forward, 5′-TGCGTGTTCAGTCAAGCC-3′ and reverse, 5′-GCAGTAGAGCACAGGGTTAC-3′; SP1 forward, 5′-ATGCCCCTATTGCAAAGACA-3′ and reverse 5′-TGGATGTGACAAATGTGCTG-3′; SP3 forward, 5′-TGGTAAAAGATTTACACGAAGTGATG-3′ and reverse, 5′- GGACAAACAAACTTCTTCTCACC-3′.

Sialidase Activity Assay—Wild-type C2C12 cells, and L-NEU3 and i-NEU3 cells were harvested by centrifugation and resuspended in PBS containing 1 mM EDTA, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Total cells suspensions were lysed by sonication and then centrifuged at 800 $\times$ *g* for 10 min to eliminate unbroken cells and nuclear components. The obtained supernatants were subsequently centrifuged at 200,000 $\times$ *g* at 4 °C for 20 min on a TL100 Ultracentrifuge (Beckman) to obtain cytosolic and particulate (or membrane) fractions. The sialidase activity present in the particulate fractions was assayed using 4-MU-NeuAc at pH 3.8 according to well established protocols (5). One milliunit of sialidase activity is defined as the amount of enzyme liberating 1 nmol of product (4-MU) per min.

Western Blot Analysis—Cells were lysed in ice-cold lysis buffer, containing 1% Nonidet P-40 (Sigma) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium deoxycholate, 1 tablet (1/10 ml) of protease inhibitors mixture tablets (Roche Applied Science). Lysates were incubated for 30 min on ice prior to centrifugation at 14,000 rpm for 10 min at 4 °C. Proteins in the supernatant were denatured by boiling for 5 min in sodium dodecyl sulfate (SDS) sample buffer. Protein were separated on 10% SDS-PAGE and subsequently transferred onto nitrocellulose membranes by electroblotting. Then, the membranes were incubated for 1 h in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl), 0.1% (v/v) Tween 20 containing 5% (w/v) dried milk or 5% (w/v) bovine serum albumin (BSA; Sigma) or 1% BSA for the blocking buffer. Blots were incubated with a primary antibody in the appropriate blocking solution for 1 h at room temperature or at 4 °C overnight. The following primary antibodies were used: anti-phospho-EGFR Tyr-1148, 1:1000 dilution (Cell Signaling); anti-EGFR, 1:1000 dilution (Cell Signaling); anti-AKT1/2/3, 1:100 dilution (Santa Cruz Biotechnology); anti-phospho-AKT1/2/3 Thr-308, 1:100 dilution (Santa Cruz Biotechnology); anti-HIF-1α, 1:3000 dilution (Cayman Chemical); anti-β-actin, 1:5000 dilution (Abcam); anti-*p*-70S6K, 1:1000 dilution (Cell Signaling); anti-*p*-70S6K, 1:1000 dilution (Cell Signaling). The membrane was washed four times for 10 min and then incubated with the appropriate secondary antibody conjugated (anti-mouse and anti-rabbit HRP-conjugated (Dako)) with horseradish peroxidase for 1 h. After four washes in TBS-Tween 20 solution, the protein bands were detected using an ECL detection kit (Pierce), as described by the manufacturer.

Treatment of Cell Cultures with [3-^{3}H]Sphingosine—The determination of the ganglioside pattern of wild-type C2C12, L-NEU3 and i-NEU3 cells was accomplished by radioactive metabolic labeling, according to our previous reports (5–7). [3-^{3}H]sphingosine (PerkinElmer Life Sciences) was dissolved in methanol, transferred into a sterile glass tube, and then dried under a nitrogen stream. The residue was dissolved in an appropriate volume of prewarmed (37 °C) 10% fetal bovine serum DMEM to obtain a final sphingosine concentration of 3×10^{-8} M (corresponding to 0.4 μCi/100-mm dish). A total of 1×10^6 cells were incubated in this medium for a 2-h pulse followed by a 24-h chase, a condition warranting a steady-state metabolic condition (6, 7). At the end of the 24-h chase, cells were harvested and lyophilized.

Lipid Extraction and Analyses—Total lipids from lyophilized cells were extracted twice with chloroform/methanol 2:1 (v/v) and with chloroform/methanol/water 20:10:1 (v/v/v), respectively. The resulting lipid extracts were dried under a nitrogen stream and dissolved in chloroform/methanol 2:1 (v/v) at room temperature. Lipid extracts were analyzed by HPTLC carried out with the solvent system chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11 (v/v/v). The total lipid extracts were subjected to partitioning in chloroform/methanol/water, 2:1 (v/v) and 20% water. The two phases obtained (aqueous, containing gangliosides, and organic, containing neutral sphingolipids) were counted for radioactivity and submitted to HPTLC separation depositing the same amount of proteins on each lane (determined by Lowry assay, depositing the minimum amount

[2] The abbreviations used are: DFO, deferoxamine; ANOVA, analysis of variance; EGFR, EGF receptor; GM3, NeuAcα 2,3Galβ1,4Glc-ceramide; HIF, hypoxia-inducible factor; LDH, lactate dehydrogenase; qPCR, quantitative PCR; GM2, β-D-GalNAc-(1→4)-[α-Neu5Ac-(2→3)]-β-D-Gal-(1→4)-β-D-Glc-(1↔1)-ceramide; GD1a, α-Neu5Ac(2–3)β-D-Gal-(1–3)β-D-GalNAc(1–4)[α-Neu5Ac(2–3)]-β-D-Gal-(1–4)-β-D-Glc-(1–1)-Cer.

of proteins that corresponds to at least 1400 dpm) using the solvent systems chloroform/methanol/0.2% aqueous $CaCl_2$, 60:40:9 (v/v), and chloroform/methanol/water, 55:20:3 (v/v/v), for the aqueous and organic phase, respectively. [3-^{3}H]Sphingolipids were identified by referring to radiolabeled standards and quantified by radiochromatoimaging (Beta-Imager 2000; Biospace).

Cell Apoptosis Assay—Induction of caspases-3/7 activity was measured using Caspase-Glo®3/7 Assay (Promega), according to the manufacturer's instructions. This kit is based on the cleavage of the DEVD sequence of a luminogenic substrate by the caspases-3 and -7 resulting in a luminescent signal. Cells were plated on a 96-well plate in 100 μl of culture medium at a concentration of 1×10^4 cells/well. After incubation under hypoxic conditions, 100 μl of Caspase-Glo 3/7 reagent was added to each well. Then the mixture was incubated for 1 h at room temperature, and the luminescence of each sample was measured using a plate-reading luminometer (Wallac 1420 Victor3; PerkinElmer Life Sciences).

Cytotoxicity Detection Test—Cytotoxicity was measured as the total lactate dehydrogenase (LDH) released in the medium upon cell lysis, according to the protocol provided in the Cytotoxicity Detection Kit^{plus} LDH (Roche Applied Science). Target cells were plated in triplicate in a 96-well (1×10^4 cells/well) plate and incubated under hypoxic conditions. After 24 h, 50 μl of the substrate mix was added in each well and then incubated at room temperature in the dark for 15–30 min. Before measuring, 50 μl of a "stop" solution provided in the kit was added to each well. Maximal LDH release was obtained by incubating target cells with a "lysis" solution provided in the kit which completely disrupts the cell liberating LDH in the medium. Untreated wild-type C2C12 cells were used as negative control (spontaneous release). Data were collected at 490 nm using a 96-well plate reader.

Silencing of GM3 Synthase by siRNA Transfection—Specific siRNA duplexes targeting GM3 synthase, siRNA transfection reagents, and reduced-serum transfection medium were purchased from Santa Cruz Biotechnology. The day before transfection, 2×10^5 C2C12 myoblasts were seeded in each well of a 6-well cell culture plate in DMEM containing 10% FBS without antibiotics and incubated for 24 h at 37 °C and 5% CO_2. The next day, transfection complexes were prepared using GM3 synthase siRNA, siRNA transfection reagent, and transfection medium according to the manufacturer's protocol and were added to each well. The final concentration of GM3 synthase siRNA duplexes used was 3 μg. A scrambled siRNA (Santa Cruz Biotechnology) was used as negative control. GM3 synthase primers: forward, 5′-TTCTGGGGCCATGATAAGAA-3′ and reverse, 5′-TGACTGAGGTCGTAGCCAAA-3′.

RESULTS

Effects of Hypoxia on Wild-type C2C12 Proliferation and NEU3 Expression and Activity—Wild-type C2C12 cells, hereafter simply C2C12, were cultured for 72 h in 1% oxygen or DFO, which is widely used at this concentration to chemically mimic hypoxia by chelating iron (12, 13). DFO was initially tested in the concentration range of 10–500 μM on wild-type C2C12 (supplemental Fig. 1). The final concentration of 100 μM was chosen because it was more toxic than 1% oxygen, but not completely lethal, and could allow us to test NEU3 effects under severe hypoxic stress. Exposure to 1% oxygen caused a marked reduction of proliferation compared with untreated C2C12 (Fig. 1*A* and *D*). DFO treatment caused massive cell death, as only few cells were alive after a 72 h treatment, and widespread cell debris could be observed in the culture plates (Fig. 1, *A* and *D*). NEU3 expression, measured by qPCR, underwent a progressive and remarkable increase during 1% O_2 or DFO treatment (Fig. 1*B*). NEU3 activity increased markedly over time during exposure to 1% O_2 and, to a minor extent, upon DFO treatment (Fig. 1*C*). NEU3 activity with DFO could not be determined at 72 h due to the massive extent of cell death.

Effects of NEU3 Sialidase Overexpression and Silencing on C2C12 Proliferation under Hypoxia—C2C12 cells were transduced with a lentiviral vector containing the murine NEU3 sialidase gene (7). Among five clone tested, clone 2 (hereafter named L-NEU3) showed the highest sialidase expression, which was 19-fold higher than in C2C12 (Fig. 2*A*), whereas sialidase activity was approximately 3-fold higher (Fig. 2*B*). C2C12 cells were also transduced with a lentiviral vector engineered to silence NEU3 with a shRNA targeting the coding region of the enzyme, as described previously (6). The selected clone, hereafter named i-NEU3, showed the lowest sialidase expression levels, quantified as a 2.5-fold reduction compared with wild-type C2C12 (Fig. 2*A*), whereas sialidase activity displayed a 3-fold decrease (Fig. 2*B*).

C2C12, L-NEU3, and i-NEU3 cells were then cultured for 3 days in normoxia, or 1% oxygen, or 100 μM DFO and analyzed for cell growth. As shown in Fig. 2*C*, under normoxic conditions cell growth was slightly higher (approximately 15% after 3 days) in L-NEU3 compared with C2C12 cells, whereas in i-NEU3 cells it was much lower (approximately 40% less), confirming previous observations (6, 7). In 1% O_2 conditions, L-NEU3 cells had a proliferation reduction after 3 days in culture which was much lower than control C2C12 cells (33% *versus* 58%), whereas i-NEU3 cells underwent a drop in proliferation close to 90% (Fig. 2*C*). Upon DFO treatment, proliferation appears to be blocked in all cell types, with a cell survival after 3 days of 80% for L-NEU3 cells, 30% for C2C12 cells, and 27% for i-NEU3 cells (Fig. 2*C*). *Panels D* in Fig. 2 show that L-NEU3 cells reached confluence after 3 days culture in normoxic conditions, as well as at 1% O_2, but little proliferation occurred in DFO. By contrast, i-NEU3 cells exhibited clear signs of massive cell death in both 1% O_2 and DFO because many dead cells could be found floating in the culture dish.

Caspase Activation and Cytotoxicity—C2C12, L-NEU3, and i-NEU3 cells were exposed to 1% O_2 or 100 μM DFO for 72 h, and apoptosis was determined by measuring caspase-3/7 activation at 3, 6, 24, 48, and 72 h. Results revealed that caspase-3/7 activation was markedly reduced (approximately 50%) in L-NEU3 cells compared with C2C12, particularly beyond 24 h in both 1% O_2 and 100 μM DFO (Fig. 3*A*). Instead, i-NEU3 showed a higher caspase activation compared with C2C12, which was significantly higher (approximately 20–30%) beyond 24 h (Fig. 3*A*). Cytotoxicity and cell lysis were measured by detecting LDH activity released from damaged cells. Analy-

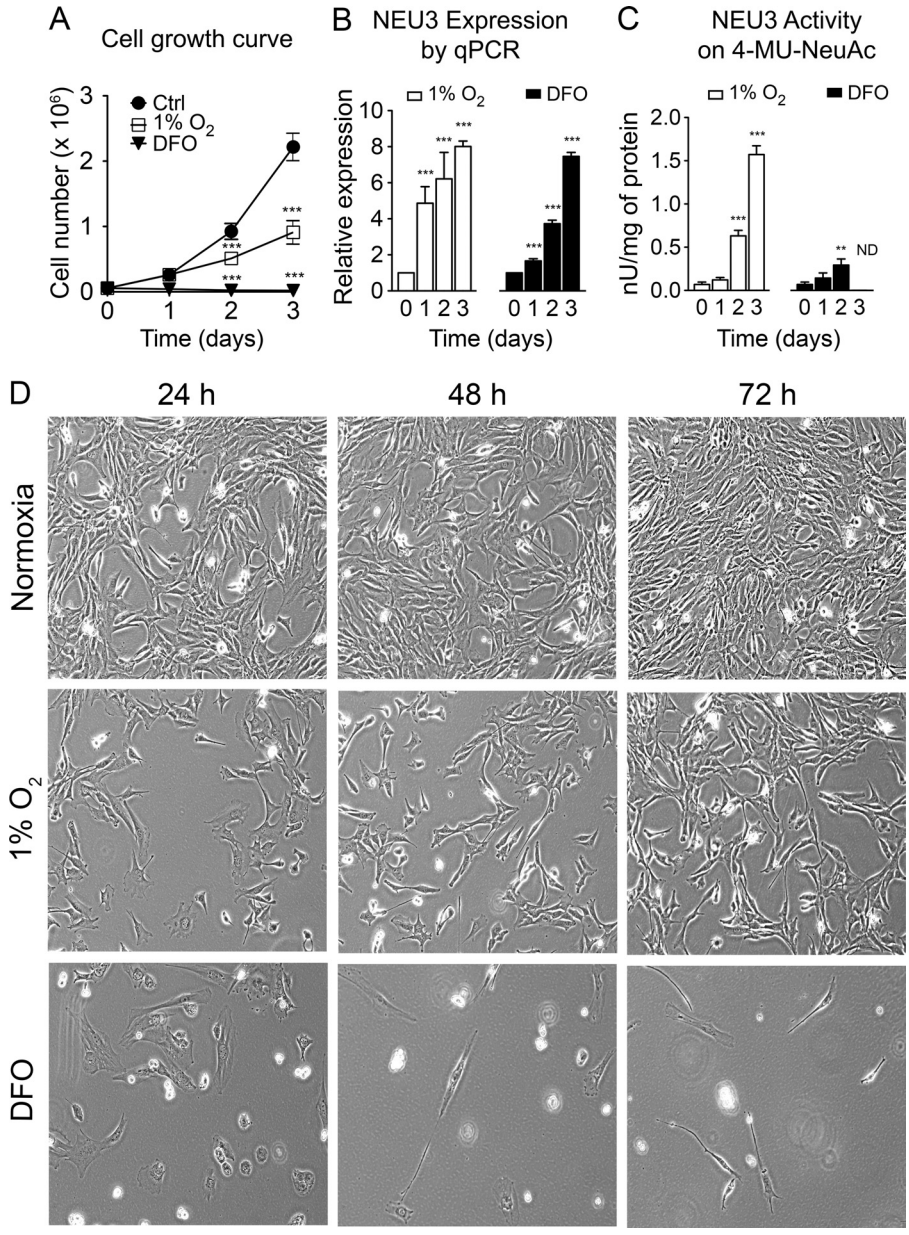

FIGURE 1. **Effects of hypoxia on C2C12 cell proliferation and NEU3 expression and activity.** Proliferating C2C12 cells were cultured for 24, 48, and 72 h in normoxia or in 1% O_2 and 100 μM DFO hypoxic conditions. *A*, cell growth curves. *B*, NEU3 expression by qPCR. *C*, NEU3 activity on 4-MU-NeuAc. All data are means ± S.D. (*error bars*) of three different experiments. Statistical differences were determined by one-way ANOVA. **, $p < 0.001$; ***, $p < 0.0001$ compared with initial values. *D*, phase-contrast microscopic images at 10× magnification.

sis of LDH activity, as a marker of cytotoxicity upon exposure to 1% O_2 and DFO up to 72 h, showed a much lower (25–30%) degree of cytotoxicity in L-NEU3 compared with C2C12, whereas the opposite, although at a lower degree, was observed for i-NEU3 cells (Fig. 3*B*).

Sphingolipid Pattern Analysis—The glycosphingolipid profile of control C2C12, L-NEU3, and i-NEU3 cells was determined by administration of [3-^{3}H]sphingosine, which led to an extensive and stable 3-^{3}H-labeling of cell sphingolipids, namely gangliosides, neutral glycosphingolipids, sphingomyelin, and ceramide, at steady-state conditions (5). Analysis of the ganglioside pattern revealed a 35% reduction of GM3 in L-NEU3 cells, whereas a 2-fold increase of the same ganglioside could be observed in i-NEU3 cells, making GM3 approximately 97% of the total ganglioside content, as reported previously (6, 7) (Fig. 4, *A* and *B*). However, no significant variation could be detected for the other major gangliosides in L-NEU3 cells, whereas i-NEU3 showed a significant reduction in GM2 (−89%) and GD1a (−96%). Conversely, but as expected (6, 7), the radioactive patterns of the sphingolipids contained in the organic phase did not exhibit significant differences upon overexpression or silencing of NEU3, with only a minor increase of GlcCer, LacCer, and SM in L-NEU3 cells (Fig. 4, *C* and *D*).

NEU3 Controls the EGFR-mediated Signaling Pathway Ultimately Leading to Activation of HIF-1α—Because ganglioside GM3 is known to inhibit EGFR autophosphorylation (14–17), we tested whether NEU3-induced reduction of GM3 would affect the activity of key proteins of the EGFR-mediated signal-

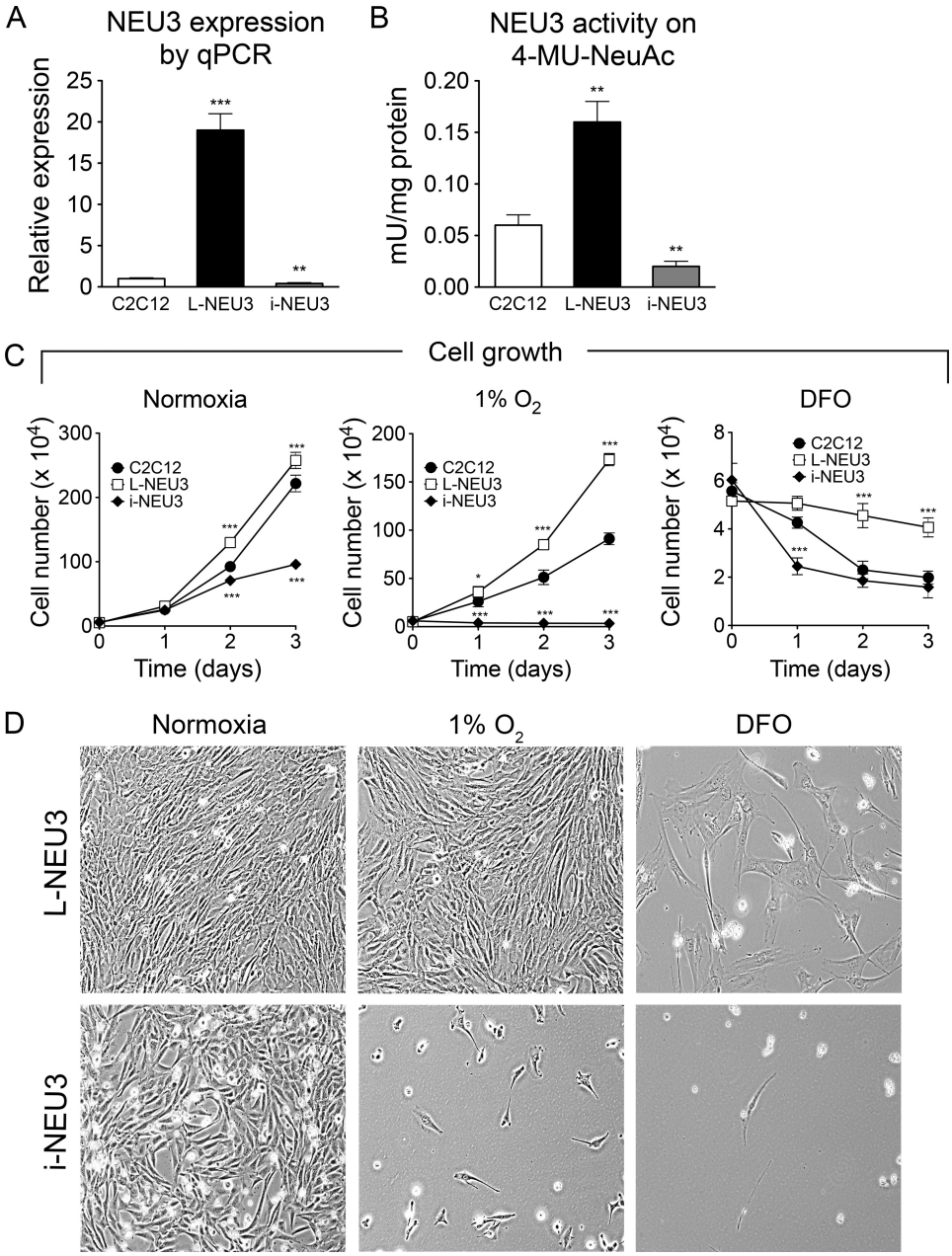

FIGURE 2. **Effects of NEU3 overexpression or silencing on C2C12 resistance to hypoxic conditions.** *A*, qPCR analysis of NEU3 mRNA expression levels. *B*, NEU3 activity on 4-MU-NeuAc. Data are means ± S.D. (*error bars*) of three different experiments, significance according to two-way ANOVA. **, $p < 0.001$; ***, $p < 0.0001$ compared with wild-type C2C12 cells. *C*, growth curves of cells cultured in normoxia, 1% oxygen. and 100 μM DFO. *D*, phase-contrast microscopy (10× magnification) of L-NEU3 and i-NEU3 cells cultured in normoxia, 1% O_2, and 100 μM DFO for 72 h.

ing cascade, in their inactive and/or active (phosphorylated) forms, thus leading to regulation of HIF-1α expression. Therefore, the levels of these proteins in all three cell lines (control C2C12, L-NEU3, and i-NEU3) under normoxic and hypoxic conditions (1% O_2 at 3, 6, and 24 h) were analyzed by Western blotting. Results revealed that HIF-1α, which is the "master operator" of cell resistance to hypoxia (18, 19), could not be detected in control C2C12 cells grown for 3, 6, and 24 h in normoxia (Fig. 5), as expected, because HIF-1α is degraded under normoxic conditions by the ubiquitin-proteasome system. Analogous results were observed in i-NEU3 cells. Conversely, L-NEU3 cells exhibited substantial levels of HIF-1α even under normoxia (Fig. 5). Upon cell culturing in 1% oxygen, HIF-1α protein level increased over time in all cells, although in L-NEU3 it reached the highest levels. In particular, in L-NEU3 cells, high levels were reached already within 3 h under hypoxia, whereas comparable levels were reached by control C2C12 only at 6 h, and by i-NEU3 at 24 h (Fig. 5). Western blot analysis of the EGFR-mediated signaling cascade, leading to HIF-1α activation, revealed similar trends, because EGFR, phospho-EGFR, AKT, phospho-AKT, p70S6K, and phospho-p70S6K were all activated earlier in L-NEU3 cells and exhibited higher levels compared with control C2C12. The increments of all measured proteins were delayed and exhibited anyhow lower levels in i-NEU3 cells (Fig. 5). The proposed mechanism of HIF-1α activation by NEU3 is schematized in Fig. 6.

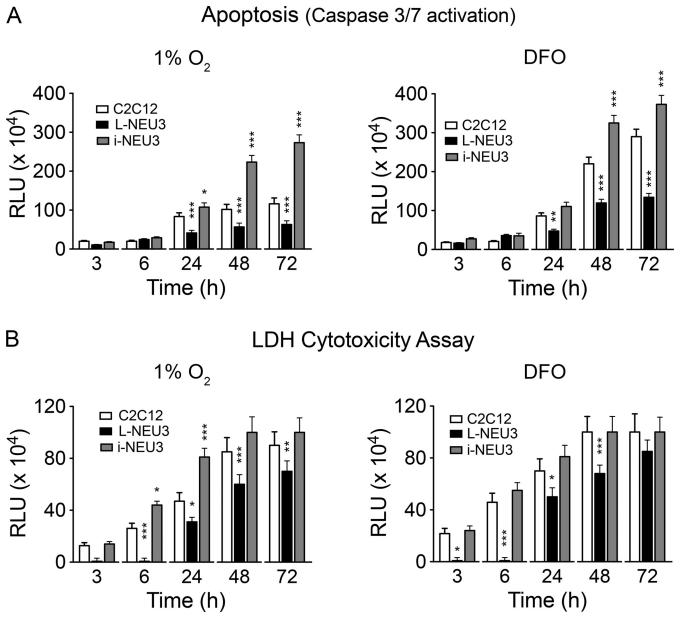

FIGURE 3. **Evaluation of apoptosis and cytotoxicity.** *A*, caspase-3 and -7 activation detected by Caspase-Glo 3/7 luminescence assay in C2C12, L-NEU3, and i-NEU3 cells exposed to normoxia, 1% O_2, and 100 μM DFO for different times. *B*, LDH activity detected by LDH Cytotoxicity Detection Kit and expressed in relative luminescence units (*RLU*). Data are means ± S.D. (*error bars*) of three different experiments. Significance is expressed with two-way ANOVA. **, $p < 0.001$ and ***, $p < 0.0001$ referred to the initial values.

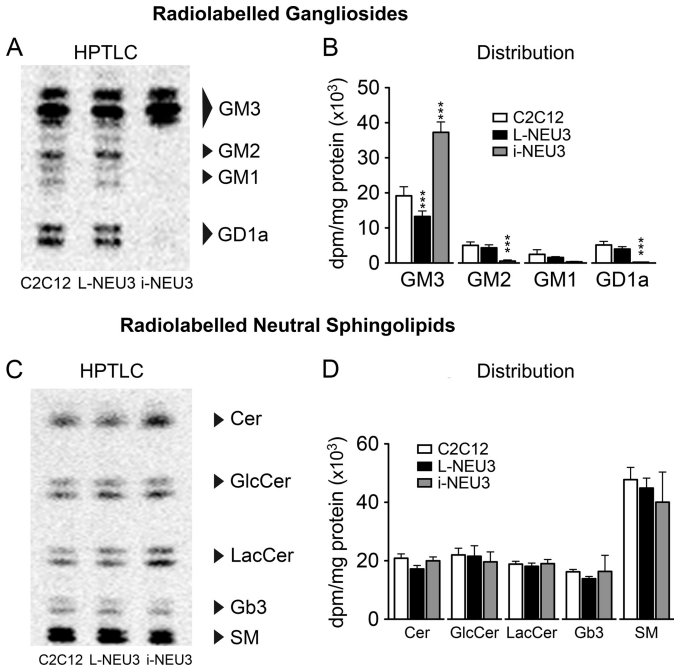

FIGURE 4. **Effects of NEU3 activity on the sphingolipid pattern.** Proliferating C2C12, L-NEU3, and i-NEU3 cells were metabolically labeled with [3-^{3}H]sphingosine. *A*, radiochromatoscanning images of HPTLC separation of gangliosides contained in the aqueous phase. *B*, ganglioside distribution expressed as dpm/mg of protein. *C*, radiochromatoscanning images of HPTLC separation of sphingolipids contained in the organic phase. *D*, sphingolipid distribution expressed as dpm/mg of protein. Data of *B* and *D* represent means ± S.D. (*error bars*) of five different experiments (significance according to two-way ANOVA: **, $p < 0.001$ and ***, $p < 0.0001$ compared with wild-type C2C12 cells).

Silencing of GM3 Synthase—To test whether the observed effects go through NEU3-induced GM3 depletion and consequent EGFR activation, we tested whether we could mimic NEU3 effects by silencing GM3 synthase, thus reducing GM3 cell content. To this purpose, wild-type C2C12 were transfected with specific siRNA duplexes targeting GM3 synthase. Analysis by qPCR revealed a 50% reduction in GM3 synthase expression compared with controls (Fig. 7*A*). Analysis of the sphingolipid pattern by administration of [3-^{3}H]sphingosine (Fig. 7, *B* and *C*), revealed a significant decrease of ganglioside GM3, which was approximately 35% less than controls, whereas both lactosylceramide and globotriaosyl ceramide were markedly increased. Analysis of cell growth under normoxia revealed a significant, although minimal, increase in cell proliferation in GM3 synthase-down-regulated C2C12 (Fig. 7*D*). Cells were then subjected to 1% O_2 and monitored for 48 h. Cell growth analysis showed a significant cell number reduction after 48 h in hypoxia in control C2C12, as expected. However, down-regulation of GM3 synthase rendered myoblasts more resistant to hypoxia, as we could detect a 25% increase of alive cells after 48 h in hypoxia, compared with control cells (Fig. 7*E*).

Activation of SP1/SP3 under Hypoxia—Wild-type C2C12 cells were subjected to 1% O_2 for 48 h to test the activation of transcription factors SP1 and SP3, which have been recently found to bind to NEU3 promoter region (20). Analysis of SP1 and SP3 expression by qPCR revealed a significant increase of both factors under hypoxia (Fig. 8, *A* and *B*), supporting a possible mechanism of endogenous activation of NEU3 under hypoxia (Fig. 8*C*).

DISCUSSION

Oxygen deprivation under ischemic conditions, which is typical of many and widespread human diseases, causes functional impairments of cells and very often structural tissue damages. A therapeutic option against ischemic lesions, based on the use of cytokines, was assayed in randomized clinical trials providing inconclusive results (21). Another approach may be based on the activation of HIF-1α, a transcription complex that responds to changes in oxygen concentration, supplying cells with a regulatory system of gene transcription that results in a number of downstream reactions, protecting tissues against the consequences of hypoxia. However, the mechanism of HIF-1α regulation is complex also because it is mediated by prolyl hydroxylases that reduce its stability by an enzymatic hydroxylation of specific prolyl residues, causing a signaling cascade that promotes HIF-1α degradation by the proteasome (22).

The present work reveals that endogenous NEU3 sialidase expression and activity are up-regulated in murine skeletal muscle cells (C2C12) upon oxygen starvation, leading to a signaling cascade resulting in the activation of HIF-1α. Moreover, induced overexpression of NEU3 significantly increases HIF-1α expression and cell resistance to hypoxic stress, whereas NEU3 silencing causes the opposite effects and renders myoblasts more susceptible to apoptosis. These data substantiate the hypothesis that NEU3 sialidase, which was found to be activated under hypoxic conditions, can activate the EGFR pro-survival signaling pathway by controlling the content of ganglioside GM3. To test this hypothesis, the effects of NEU3 overexpression and silencing were studied on the EGFR signaling pathway. As anticipated, NEU3 overexpression causes a reduction of ganglioside GM3, which is known to block

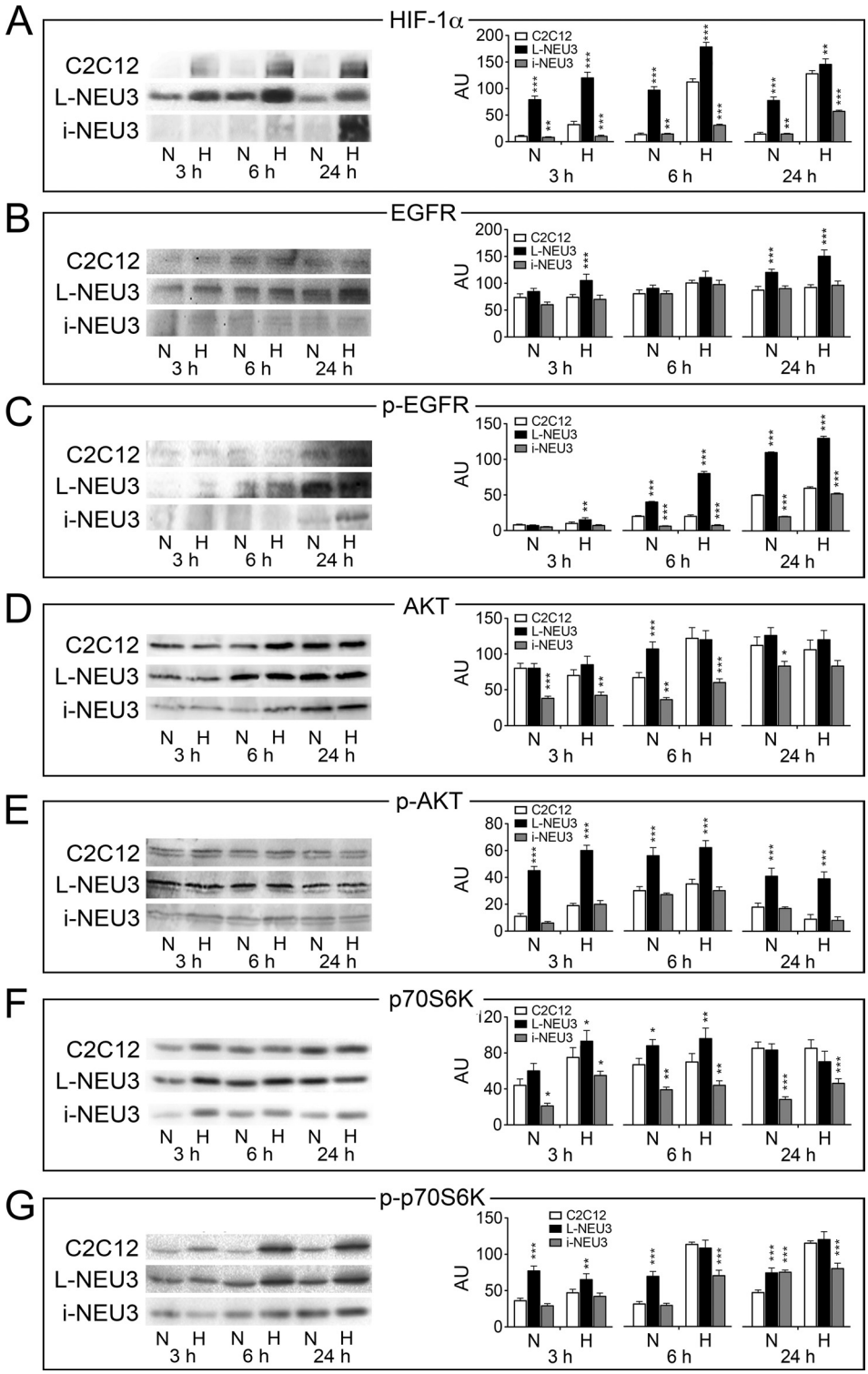

FIGURE 5. **Analysis of EGFR-mediated signaling pathway by Western blotting.** Total proteins from C2C12, L-NEU3, and i-NEU3 cells cultured for 3, 6, and 24 h in 1% O_2 anoxic conditions were stained with anti-HIF-1α (*A*), anti-EGFR antibody (*B*), anti-phospho-EGFR antibody (*C*), anti-AKT antibody (*D*), anti-phospho-AKT antibody (*E*), anti-p70S6K (*F*), and anti-phospho-p70S6K (*G*). Changes in protein content were quantified by densitometry and are represented in columns. *N*, normoxic; *H*, hypoxic. All data are the means ± S.D. (*error bars*) of three different experiments. Statistical differences were determined by two-way ANOVA. *, $p < 0.05$; **, $p < 0.001$; and ***, $p < 0.0001$ compared with C2C12 cells.

EGFR autophosphorylation. Therefore, upon NEU3 up-regulation, the net effect is that EGFR signaling cascade is activated, as demonstrated by the activation of pro-survival and anti-apoptotic signaling molecules down-stream of EGFR, including AKT, p70S6K, and ultimately HIF-1α. This causes an increased resistance of myoblasts to hypoxia, ultimately opposing apoptotic cell death. Notably, upon NEU3 overexpression, HIF-1α mRNA and protein levels were also up-regulated. Analysis of NEU3-activated EGFR signaling revealed the activation of p70, which is known to promote HIF-1α transcription. These find-

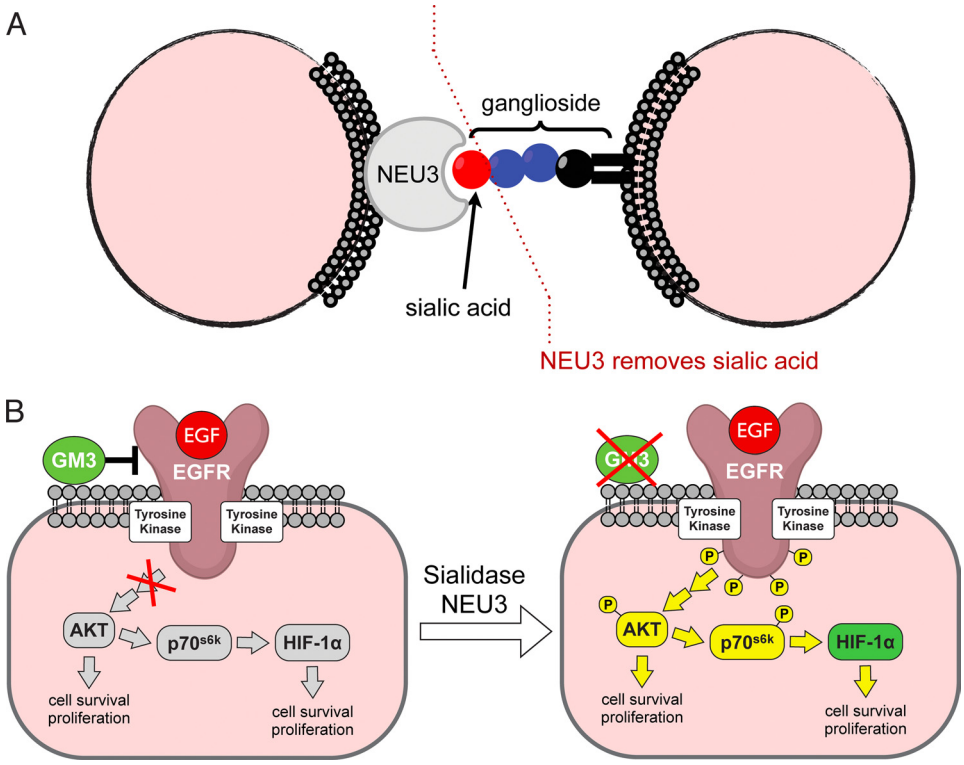

FIGURE 6. **Scheme of EGFR signaling pathway activated by NEU3-induced GM3 reduction.** *A*, NEU3 removing sialic acid from gangliosides exposed on the outer membrane of adjacent cells. *B*, mechanism of NEU3 activation of pro-survival signaling cascades, mediated by GM3 removal and consequent activation of EGFR signaling cascade, ultimately up-regulating HIF-1α.

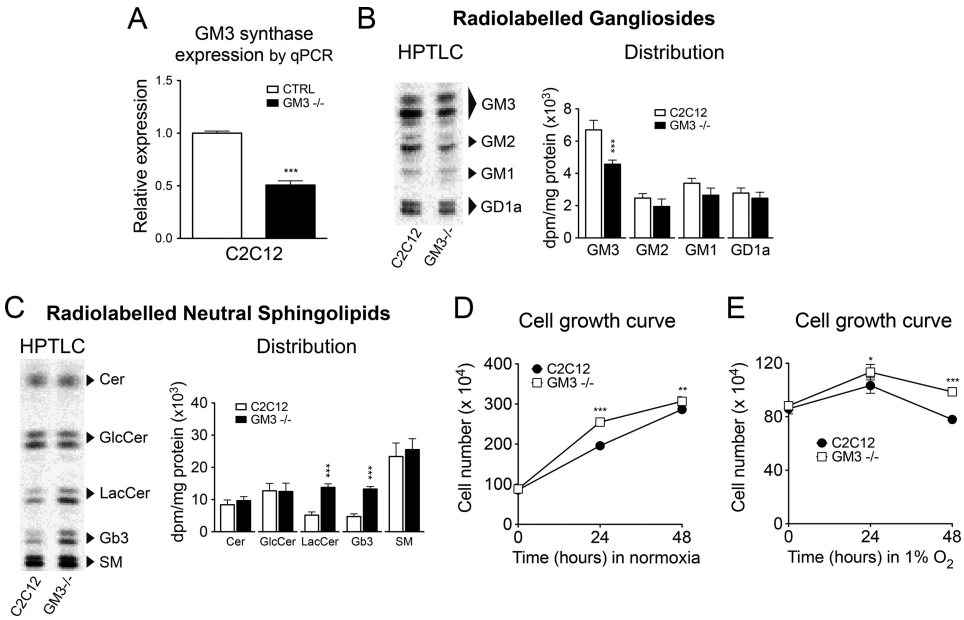

FIGURE 7. **Silencing of GM3 synthase increases C2C12 resistance to hypoxia.** *A*, qPCR analysis of GM3 synthase mRNA expression levels in control and GM3 synthase-silenced C2C12 cells. *B*, radiochromatoscanning images of HPTLC separation of gangliosides contained in the aqueous phase. *C*, radiochromatoscanning images of HPTLC separation of sphingolipids contained in the organic phase. Sphingolipid distribution is expressed as dpm/mg of protein. Data of *B* and *C* represent means ± S.D. (*error bars*) of three different experiments. Significance was determined according to two-way ANOVA. ***, $p < 0.0001$ compared with wild-type C2C12 cells. *D* and *E*, growth curves of cells cultured in normoxia (*D*) and in 1% oxygen (*E*). Data of *D* and *E* are means ± S.D. of three different experiments. Significance was determined according to two-way ANOVA. *, $p < 0.005$; **, $p < 0.001$; and ***, $p < 0.0001$ compared with wild-type C2C12 cells.

ings could also be useful for a better understanding of the NEU3 role in cancer, where sustained levels of HIF-1α are observed (23) and are necessary for malignant progression under hypoxic conditions, which is the most suitable environment for tumor proliferation and growth (24, 25). To further support the hypothesis of a key role played by ganglioside GM3 in activating the EGFR signaling cascade, we tried to mimic NEU3 overexpression effects on reducing GM3 content by silencing GM3 synthase. Remarkably, GM3 synthase down-regulation, although only partial, caused a reduction in GM3 content in

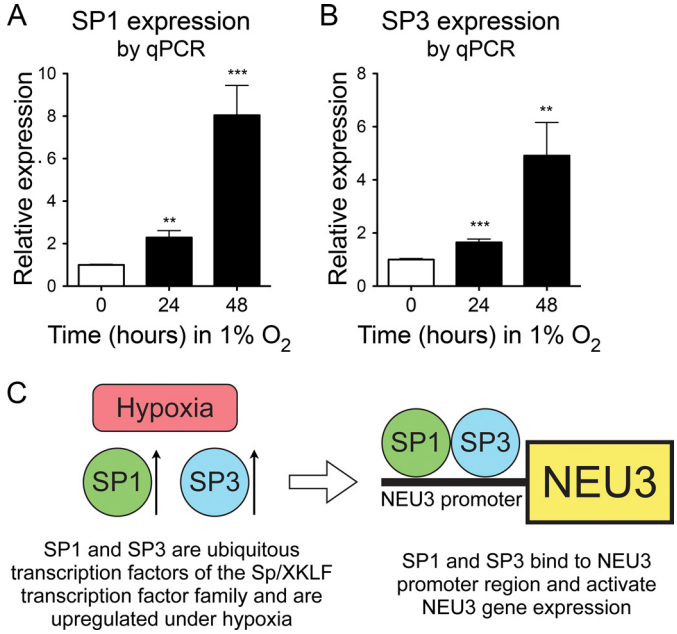

FIGURE 8. **Activation of SP1 and SP3 under hypoxia could lead to NEU3 up-regulation.** *A* and *B*, qPCR analysis of SP1 and SP3 mRNA expression levels in C2C12 cells cultured in 1% O_2 up to 48 h. *C*, schematic representation of SP1/SP3-mediated activation of NEU3 sialidase under hypoxia.

C2C12 cells which caused an increased cell survival upon subjection to hypoxia in 1% O_2. Noticeably, although GM3 is approximately 97% of the total ganglioside content in i-NEU3 cells, a significant reduction of gangliosides GD1a and GM2 could be observed upon NEU3 silencing. Therefore, at this stage, we cannot exclude an involvement of these sphingolipids in NEU3 activity.

Finally, whereas NEU3 effects and mechanism of action seem to be clarified, little is known about how and whether the enzyme is physiologically up-regulated under hypoxia. Interestingly, it has been recently reported the binding of transcription factor SP1 and SP3 to the NEU3 promoter region (20). These factors are ubiquitous transcription factors (of the Sp/XKLF transcription factor family) that are involved in basal transcription and housekeeping gene expression, and they are known to be activated under hypoxic conditions (26). Therefore, it is tempting to speculate that NEU3 response upon oxygen starvation might be regulated through SP1/SP3 activation. Interestingly, upon subjecting C2C12 cells to 1% O_2, both SP1 and SP3 mRNA levels were found up-regulated, thus supporting their involvement in activating NEU3 gene transcription under hypoxic stress. Further investigations to test this hypothesis are ongoing in our laboratories.

In a translational perspective, it would be very interesting to test whether NEU3 effects can be recognized also in other cells, in particular in those critically affected under hypoxic conditions in many pathologies, like cardiomyocytes and neural cells. Understanding the mechanism of action of NEU3 may lead to the development of drugs that can mimic these pro-survival effects, possibly in a controlled and reversible manner. For instance, GM3 synthase inhibitors, which could lead to an activation of the EGFR signaling cascade, could represent a possible new therapeutic approach for the hypoxia-related pathological conditions.

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