

Electrical Stimulation Induces Calcium-dependent Up-regulation of Neuregulin-1 β in Dystrophic Skeletal Muscle Cell Lines

Nevenka Juretić^{1,2}, Gonzalo Jorquera¹, Pablo Caviedes³, Enrique Jaimovich^{1,2} and Nora Riveros^{1,2}

¹Centro de Estudios Moleculares de la Célula, ²Programa de Biología Celular y Molecular and ³Programa de Farmacología Molecular y Clínica, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia 1027, Santiago, Chile

Key Words

Muscle gene expression • Excitation-transcription coupling • Inositol-1,4,5-trisphosphate receptors • Dystrophin

Abstract

Duchenne muscular dystrophy (DMD) is a neuromuscular disease originated by reduced or no expression of dystrophin, a cytoskeletal protein that provides structural integrity to muscle fibres. A promising pharmacological treatment for DMD aims to increase the level of a structural dystrophin homolog called utrophin. Neuregulin-1 (NRG-1), a growth factor that potentiates myogenesis, induces utrophin expression in skeletal muscle cells. Microarray analysis of total gene expression allowed us to determine that neuregulin-1 β (NRG-1 β) is one of 150 differentially expressed genes in electrically stimulated (400 pulses, 1 ms, 45 Hz) dystrophic human skeletal muscle cells (RCDMD). We investigated the effect of depolarization, and the involvement of intracellular Ca²⁺ and PKC isoforms on NRG-1 β expression in dystrophic myotubes. Electrical stimulation of RCDMD increased NRG-1 β

mRNA and protein levels, and mRNA enhancement was abolished by actinomycin D. NRG-1 β transcription was inhibited by BAPTA-AM, an intracellular Ca²⁺ chelator, and by inhibitors of IP₃-dependent slow Ca²⁺ transients, like 2-APB, Ly 294002 and Xestospongine B. Ryanodine, a fast Ca²⁺ signal inhibitor, had no effect on electrical stimulation-induced expression. BIM VI (general inhibitor of PKC isoforms) and Gö 6976 (specific inhibitor of Ca²⁺-dependent PKC isoforms) abolished NRG-1 β mRNA induction. Our results suggest that depolarization induced slow Ca²⁺ signals stimulate NRG-1 β transcription in RCDMD cells, and that Ca²⁺-dependent PKC isoforms are involved in this process. Based on utrophin's ability to partially compensate dystrophin dysfunction, knowledge on the mechanism involved on NRG-1 up-regulation could be important for new therapeutic strategies design.

Copyright © 2012 S. Karger AG, Basel

Introduction

Duchenne muscular dystrophy (DMD) is a devastating neuromuscular disease affecting 1/3,500 male births [1]. It is caused by several mutations in the dystrophin gene, that results in little or no production of a functional

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2012 S. Karger AG, Basel
1015-8987/12/0296-0919\$38.00/0

Accessible online at:
www.karger.com/cpb

Nevenka Juretić,
Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas,
Facultad de Medicina, Universidad de Chile
Casilla 70086 Santiago-7, Santiago (Chile)
Tel. +562 9786842, Fax +562 7355580, E-Mail njuretic@med.uchile.cl

cytoskeletal protein, normally expressed at the inner surface of the sarcolemma of muscle fibers [2].

Dystrophin associates with a large complex of proteins known as the dystrophin-associated proteins (DAPs). Dystrophin-DAPs complex (DAPC) serves as a link between the extracellular matrix and the intracellular actin cytoskeleton, providing structural integrity to muscle fibers [3, 4]. Failure in dystrophin expression results in loss of DAPs and impairment of plasma membrane stability [5] leading to degeneration and progressive weakness of heart and skeletal muscle, and causing disability and death in adolescence or young adulthood [6].

Although no therapy has been described to effectively slow or halt muscle degeneration in dystrophic patients, different strategies have been designed to correct the deleterious consequences of dystrophin absence and to alleviate the secondary effects of the disease [7]. One of them consists in expressing a protein that could functionally compensate for the lack of dystrophin. Utrophin, a protein that shares structural and functional motifs with dystrophin and also associates with members of the DAPC, is a good candidate for such a role.

It has been established that increased utrophin levels in muscle fibers of dystrophin-deficient *mdx* mice restores sarcolemmal expression of DAPs members and improves dystrophic phenotype. This observation has raised the possibility that up-regulation of utrophin expression might be able to counteract dystrophin deficiency in DMD patients. This promising pharmacological treatment requires the identification of signals and regulatory pathways that modulate utrophin expression in the muscle [reviewed in 8].

Neuregulin growth factor [NRG; also known as heregulin, neu differentiation factor, acetylcholine receptor (AChR)-inducing activity, glial growth factor II and sensory motor neuron-derived factor] is one of many factors that increase utrophin expression [reviewed in 8].

NRG-1 treatment leads to increased utrophin mRNA levels and transcriptional activity in mouse and human myotubes [9, 10]. Furthermore, Krag et al. (2004) described that intraperitoneal injection of a small peptide region of NRG-1 ectodomain increases utrophin expression in *mdx* mice. Observed increase was accompanied by a reduction in muscle degeneration and inflammation, and by decreased susceptibility to the damage induced by lengthening contractions. Improvement in muscle function was deemed to result specifically from the utrophin up-regulation because NRG-1 administration has no beneficial effect in dystrophin/utrophin double-knockout ani-

mals [11].

NRG-1 belongs to a family of proteins structurally related to the epidermal growth factor (EGF) that are synthesized in and secreted from motoneurons and muscle [12]. Four members of NRG proteins, NRG-1 to NRG-4, have been identified. The best studied and most characterized products are those encoded by NRG-1 gene. At least 15 different isoforms are generated by alternative splicing and differential promoter usage of NRG-1 gene. All identified NRGs feature an EGF-like motif, necessary and sufficient for biological activity, that binds to NRG receptors and activates downstream signals [13, reviewed in 14]. Variations in the EGF-like domain, give rise to α , β , and γ isoforms, with different affinities for NRG receptors [reviewed in 14].

NRG-1 has relevant effects on several other processes in the muscle [12, 15] such as myogenesis [16], muscle fiber survival [17], muscle spindle development [18], and in the differentiation of satellite cells during muscle regeneration [19]. In addition, NRG-1 contributes to the regulation of skeletal muscle mass by increasing protein synthesis [20], mitochondrial oxidative capacity and insulin sensitivity [21].

Regardless the evidences supporting such important roles for NRG-1 in skeletal muscle, the molecular mechanisms involved in its expression are still unclear.

The role of Ca^{2+} in the induction of specific transcriptional response in skeletal muscle has been a current subject of study in our laboratory. We have shown that depolarization of cultured muscle cells, either by high K^+ treatment or electrical stimulation, produces a biphasic increase in intracellular Ca^{2+} concentration [22-25]. The first increase or fast Ca^{2+} transient, localized in the cytoplasm, is related to the ryanodine receptor (RyR) and is associated to excitation-contraction (EC) coupling. The second one or slow Ca^{2+} transient, generated by Ca^{2+} release through inositol-1,4,5-trisphosphate (IP_3) regulated channels and associated to nuclei [22], has been involved in early steps leading to gene expression. We have already described that IP_3 -mediated Ca^{2+} signals induce an increase in mRNA levels of early and late genes in normal skeletal muscle cells after depolarization [26-29].

Several works suggest that alterations in IP_3 pathways are involved in DMD physiopathology [30, 31]. Both IP_3 total mass and the amount of IP_3 receptors (IP_3Rs) are largely increased in dystrophic lines compared to normal cells [30]. Furthermore, expression and localization of IP_3Rs are different in both cell lines, and IP_3 -dependent slow Ca^{2+} transients evoked by electrical stimulation are larger and faster in dystrophic (RCDMD) than in

normal (RCMH) myotubes [32].

The aim of this study was to investigate the effect of electrical stimulation on NRG-1 expression in normal and dystrophic cells, and to explore whether Ca^{2+} dependent mechanisms are involved in the process. Using microarrays technology, we showed that electrical stimulation up-regulates the expression of NRG-1 isoforms β and γ in RCDMD cells. By real time PCR, we confirmed that depolarization induces NRG-1 β gene expression in this cell line, whereas it has not a significant effect in RCMH cells in the same time period. Our results suggest that IP_3 -dependent slow Ca^{2+} signals evoked by electrical stimulation stimulate NRG-1 β transcription in dystrophic RCDMD cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium-F12 (DMEM-F12) and horse serum were from Sigma (Sigma Chemical, St. Louis, MO). Fetal bovine serum from GIBCO BRL (Carlsbad, CA). Antibiotics and antimycotics from Life Technologies (Burlington, Ontario). 2-aminoethoxydiphenyl borate (2-APB) was purchased from Aldrich. Ly 294002 and actinomycin D (Act D) were from Calbiochem (La Jolla, CA, USA). BAPTA-AM from Molecular Probes (Eugene, OR). Ryanodine (Rya) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Bisindolymaleimide VI (BIM VI) was from Sigma Aldrich and Gö 6976 from Alexis Biochemicals. Xestospongin B was kindly provided by Dr. Jordi Molgó (Institut de Neurobiologie Alfred Fessard, Centre National de la Recherche Scientifique, France).

Illumina 22K human oligonucleotide microarrays were prepared by National Cancer Institute, NCI (Frederick, Maryland, USA). Alexa 555-AHA-dUTP, Alexa 647-AHA-dUTP, human Cot-1 DNA, SuperScript II, Taq DNA polymerase, SYBR(R) Green Nucleic A and Platinum Taq DNA polymerase were from Invitrogen (Carlsbad, CA). Recombinant RNAsin from Promega. All other reagents were from Sigma or Life Technologies.

Antibody directed against Neuregulin-1 β /GGF2 goat polyclonal IgG (C-18; sc-1793), secondary antibody chicken anti-goat IgG-Horseradish peroxidase (HRP) (sc-2953) and antibody anti-actin rabbit polyclonal IgG (H-300, sc-10731) were obtained from Santa Cruz Biotechnology, Inc (Palo Alto, CA, EEUU). Secondary HRP-conjugated anti-mouse antibody and Coomassie Plus Protein Assay were from Pierce Biotechnology (Rockford, ILL, USA). Enhanced chemiluminescence (ECL) reagents were from Pierce Biotechnology or Amersham Biosciences (Piscataway, NJ, USA).

Cell culture

Dystrophic human myoblasts of the RCDMD cell line [33] and normal human myoblasts RCMH [34] were cultured in

DMEM-F12 (1:1) supplemented with 10% fetal bovine serum and 10% horse serum at 37°C and 5% CO_2 . Myogenic differentiation was accomplished by mere horse serum reduction to 1%.

Both cell lines have been well characterized previously [30, 33, 34]. In addition, immunofluorescence studies to detect dystrophin expression in RCMH and RCDMD cells have been performed to test possible reversions in either the phenotype or the genotype that could have occurred during several culture passages. RCMH cells retained full expression of dystrophin on the cell membrane region, whereas RCDMD cells did not show any staining [32].

Microarray analysis

Microarray analysis was used to identify up- or down-regulated genes in RCDMD cell lines after 4 h post-electrical stimulation.

Total RNA from RCDMD cells was obtained employing Trizol reagent (Invitrogen, Corp., Carlsbad, CA, USA) according to manufacturer's protocol. cDNA from control and stimulated samples was labeled with Alexa 555 or 647 dyes, respectively. Briefly, cDNA was obtained by priming 20 μ g of total RNA with 2 μ M of oligodT in the presence of either 0.02 mM Alexa 555 or Alexa 647-AHA-dUTP (Invitrogen), 0.5 mM of each dATP, dGTP, dCTP, 0.08 mM dTTP, 2 U RNAsin, 1X buffer RT in a total volume of 50 μ l. Reaction was started by addition of 1.5 μ l of SuperScript II Reverse Transcriptase. To suppress gene specific dye effects, reciprocal labeling was performed. After 3 h incubation at 37°C, reaction was stopped with 5 μ l 0.5 M EDTA. Template RNA was hydrolyzed with 1 μ l 10 M NaOH during 15 min at 70°C, and then 10 μ l 2 M Tris-HCl pH 7.5 was added. Cleanup of labeled cDNA was performed using MinBio-spin columns (Biorad, Laboratories, Hercules, CA, USA). Illumina 22K human oligonucleotide microarray was kindly provided by Dr. David J. Munroe [National Cancer Institute (NCI), Frederick, Maryland, USA]. Hybridization and scanning was performed as previously described [28].

Microarrays data processing

Images were analyzed with GenePix Pro III software (Axon instruments, Foster City, CA, USA). GenePix Pro files (gpr) and JPEG images were deposited at the NCI's Microarray Database ("mAdb"; <http://nciarray.nci.nih.gov>). Annotated files were retrieved from mAdb and print-tip loess normalized, scale was corrected by means of DNMA (<http://dnmad.bioinfo.cnio.es/>). Data set size was 3,226 clones after the elimination of genes found in less than 70% of arrays. Missing values were estimated by KNN-impute at the Preprocessor of GEPAS (<http://gepas.bioinfo.cipf.es/>). Mean and standard deviations were calculated for total data size (3,226 clones). Mean expression \pm 2 standard deviation was considered as the statistical criteria to state differential expression. Then, filtered data were averaged and expressed as mean \log_2 ratios.

Electrical stimulation

Myotubes were depolarized using electrical stimulation as previously described [25]. The electrical pulse train

stimulation consisted in 400 pulses of 1 ms-duration at 45 Hz. Then, cells were maintained in resting conditions for different times. When pharmacological inhibitors were used, myotubes were preincubated for 30 min and afterwards depolarized in the same medium. Inhibitors were not present during the subsequent incubation period.

All experiments were matched with vehicle-treated controls to discard pharmacological agent's non-specific effects. Both control and experimental cells were submitted to the same procedures.

Semiquantitative RT-PCR

Total RNA was prepared by TRIzol Reagent (Invitrogen, Carlsbad, CA) extraction and reverse transcribed by using SuperScrip II, RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA). cDNA was amplified using human specific NRG-1 β primers [35]. DNA concentration was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

The primers used were:

Neuregulin-1 β :

5'-GGT GAT CGC TGC CAA AAC TA-3' (sense)

5'-GAG TGA TGG GCT GTG GAA GT-3' (antisense)

GAPDH:

5'-TCC CAT CAC CAT CTT CCA-3' (sense)

5'-CAT CAC GCC ACA GTT TCC-3' (antisense)

PCR amplification was maintained in the exponential phase for each product. PCR conditions were: one cycle of 95°C for 2 min followed by 26 cycles at 95°C for 1 min, 55°C for 2 min, 72°C for 1 min and a final cycle of 10 min at 72°C. PCR products (NRG-1 β : 370 bp and GAPDH: 379 bp) were resolved by electrophoresis on 2% agarose gel and stained with ethidium bromide. Bands were quantified by densitometric analysis with the Scion Image program from NIH.

Real time PCR

cDNA was prepared by reverse transcription of 1 μ g of total RNA, using SuperScrip II according to manufacturer's protocol. Real time PCR was performed using a Stratagene Mx3000P (Stratagene, La Jolla, CA) as follows: the reaction mixture consisted of 2 μ l buffer PCR 10X, 0.2 μ l Platinum Taq DNA polymerase 5U/ μ l, 1.4 μ l MgCl₂ 50 mM, 1 μ l sense primer 10 pmol/ μ l, 1 μ l antisense primer 10 pmol/ μ l, 0.4 μ l dNTPs 10X, 2 μ l SYBR(R) Green Nucleic A (1/2000) and 1 μ l cDNA. The final volume of the mixture was adjusted to 20 μ l with the addition of DNase- and RNase-free H₂O. Thermocycling conditions were as follow: 95°C for 2 min and 45 cycles of 95°C for 20 sec, 55°C for 20 sec, 72°C for 20 sec and 90°C for 7 sec, and a final cycle of 7 sec at 72°C. PCR amplification of the housekeeping gene GAPDH, was performed as a control. mRNAs were quantified and expressed in nanograms. Results are showed as mean log₂ NRG-1 β / GAPDH ratio (of percentage of control).

Western blot analysis

Stimulated cells were lysed in 60 μ l of ice-cold lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 5 mM Na₃VO₄, 20 mM NaF, 10 mM sodium

pyrophosphate, and a protease inhibitor mixture (Calbiochem). Cell lysates were sonicated for 1 min, incubated on ice for 20 min, and centrifuged to remove debris. Protein concentration of the supernatants was determined using bovine serum albumin as standard. 50 μ g of lysate were suspended in Laemmli buffer, separated in 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked at room temperature for 1 h in Tris-buffered saline containing 3% fat-free milk and 0.2% Tween 20, and then incubated overnight with the appropriate primary antibody anti-NRG-1 β (dilution 1:1000). After washing with Tris-buffered saline, the membranes were incubated with the secondary antibody HRP-conjugated anti-goat dilution (1:2000) at room temperature for 2 h. Immunoreactive proteins were detected using ECL reagents according to the manufacturer's instructions. Films were scanned, and Image J program was employed for densitometric analysis of the bands. To correct for loading, the membranes were stripped in buffer containing 30% trichloroacetic acid, 30% sulfosalicylic acid and 2% ponceau solution, at room temperature for 60 min and reprobed with the corresponding control antibodies: primary antibody anti-actin (dilution 1:1000) and secondary antibody HRP-conjugated anti-mouse (dilution 1:1500) [36].

Statistics

Results are expressed as a mean \pm S.E., and the significance of differences was evaluated using Student's t test for paired data, ANOVA followed by Dunnett's multiple comparison test or Bonferroni's test for comparison between groups.

Results

Differential gene expression in dystrophic human skeletal muscle cell lines induced by membrane depolarization

To identify differentially expressed genes in dystrophic human skeletal muscle cells (RCDMD) after electrical stimulation, we used an oligonucleotide microarray representing 21,920 different human genes (Illumina 22K). Total RNA from RCDMD samples was obtained 4 h after stimulation (400 pulses, 1 ms, 45 Hz). Assessment of differential gene expression was achieved by simultaneous hybridization of Alexa 555-labeled cDNA from control non-depolarized RCDMD cells and Alexa 647-labeled cDNA from stimulated RCDMD samples. Images obtained were analyzed with GenePix Pro III software. Ratio of Alexa 647 to Alexa 555 signals, calculated for each spot on the array, indicates relative gene expression. Mean and standard deviations were calculated for total data size (3,226 clones). Mean expression \pm 2 standard deviation was considered as the statistical criteria to state differential expression. This analysis resulted in total 150 differentially expressed

Table 1. Highest differentially expressed genes in RCDMD myotubes after electrical stimulation. Microarray analysis was used to identify up- or down-regulated genes in RCDMD cell lines after 4 h post-electrical stimulation. Results from 4 arrays (stimulated RCDMD cells vs control non-depolarized RCDMD cells). Mean expression \pm 2 standard deviation was considered as the statistical criteria to state differential expression. Mean \log_2 ratios was ranked from the highest positive (up-regulated genes) and highest negative (down-regulated genes). Analysis was performed on 150 differentially expressed genes. Further details in Materials and Methods.

Gene	Description	Mean \log_2 ratios
Up-regulated genes		
C17orf64	chromosome 17 open reading frame 64	2.187
RAD54B	RAD54 homolog B <i>S. cerevisiae</i>	1.230
PARP10	poly ADP-ribose polymerase family member 10	1.194
C6orf115	PREDICTED chromosome 6 open reading frame 115	1.082
NRG1-beta	neuregulin 1 transcript variant HRG-beta2	1.051
MAP4K5	mitogen-activated protein kinase kinase kinase kinase 5	1.020
NRG1-gamma	neuregulin 1 transcript variant HRG-gamma	0.991
FHL1	four and a half LIM domains 1	0.973
WBP4	WW domain binding protein 4 formin binding protein 21	0.970
TNFRSF10C	Tumor necrosis factor receptor superfamily member 10c decoy without an intracellular domain	0.943
Down-regulated genes		
LOC285141	PREDICTED similar to CG14853-PB LOC285141 mRNA	-1.358
SMAD6	SMAD family member 6	-1.357
SEPTIN5	septin 5	-1.078
TSC22D3	TSC22 domain family member 3	-0.955
MCFD2	multiple coagulation factor deficiency 2	-0.955
EFS	embryonal Fyn-associated substrate	-0.942
CD79A	CD79a molecule immunoglobulin-associated alpha	-0.931
NDST1	N-deacetylase/N-sulfotransferase heparan glucosaminyl 1	-0.927
GGTLA4	gamma-glutamyltransferase-like activity 4	-0.907
MAPK11	mitogen-activated protein kinase 11	-0.897

genes, in depolarized samples related to controls (data not shown).

Highest differentially expressed genes at 4 h post-electrical stimulation are shown in Table 1. Results were expressed as \log_2 of the average of relative expression intensity values of depolarized samples related to control. Differences were ranked separately from highest to lowest positive (up-regulated genes) or negative (down-regulated genes) values ($\log_2=1$ corresponds to two fold induction).

An interesting finding was the induction of neuregulin-1 transcript variant HRG-beta2 (NRG-1 β) and transcript variant HRG-gamma (NRG-1 γ), isoforms of NRG-1, a growth factor that stimulates utrophin expression [9, 10].

Increased NRG-1 gene expression from stimulated vs control RCDMD cells, were 2.07 fold (\log_2 scale 1.051) for NRG-1 β , and 1.99 fold (\log_2 scale 0.991) for NRG-1 γ . We validated NRG-1 up-regulation by real time PCR (QPCR) and focused our interest on NRG-1 β isoform that displays a higher affinity for NRG receptors (Fig. 1A).

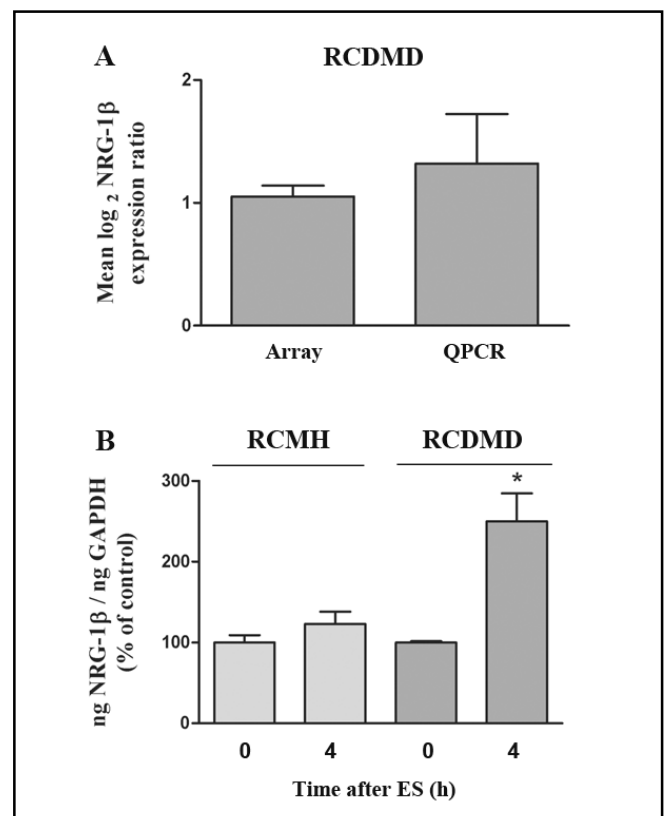
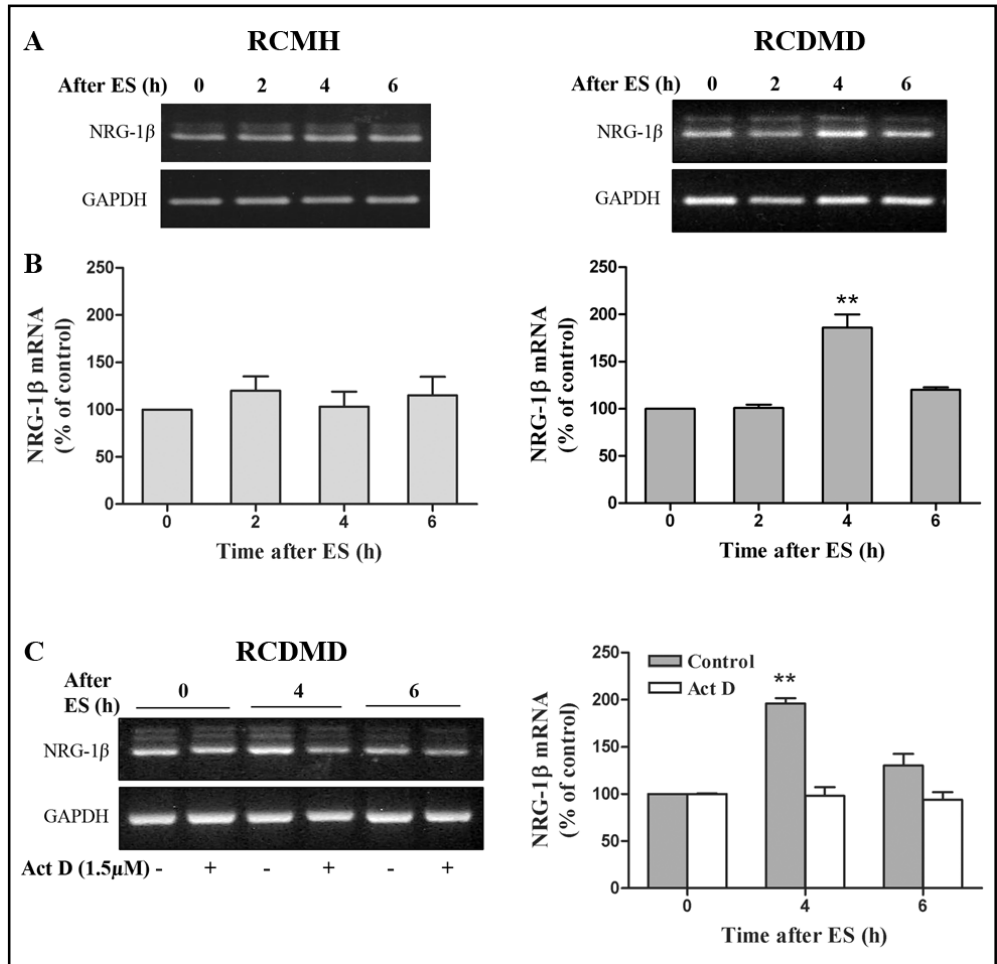


Fig. 1. NRG-1 β gene expression in RCMH and RCDMD cells after electrical stimulation. (A) Microarray (n=4) and QPCR (n=3) analysis of NRG-1 β gene expression in RCDMD cells. Results are expressed as mean \log_2 ratios. (B) NRG-1 β gene expression in RCMH and RCDMD cells submitted to electrical stimulation. Results, normalized to GAPDH, are expressed as percentage of non-depolarized control (100%, n=3). ES: electrical stimulation.

Fig. 2. Electrical stimulation increases NRG-1 β gene expression in RCDMD cell line. Total RNA was isolated from electrically stimulated RCMH and RCDMD myotubes and maintained in resting medium for the times indicated. NRG-1 β mRNA levels were analyzed by semiquantitative RT-PCR. (A) Representative agarose gel of products from NRG-1 β and GAPDH mRNA amplification. (B) Results were normalized to GAPDH expression and expressed as a percentage of corresponding non-depolarized control (100%). Bars represent mean \pm S.E (n=3). **P<0.01 ANOVA followed by Dunnett's multiple comparison test. (C) Characteristic agarose gel of RT-PCR products from RCDMD cells depolarized in the presence of either 1.5 μ M actinomycin D (Act D) or vehicle (DMSO). Bars represent mean \pm S.E (n=3). **P<0.01 vs corresponding control, evaluated by Bonferroni's test. ES: electrical stimulation.



Effect of electrical stimulation in NRG-1 β gene expression in normal and dystrophic human skeletal muscle cell lines

Given the NRG-1 β gene up-regulation in depolarized dystrophic cell line and the important role of NRG-1 protein in muscle function, we decided to contrast its expression in normal and dystrophic cells after electrical stimulation. We used normal RCMH and dystrophic RCDMD human skeletal muscle cell lines as experimental models, and QPCR for quantitative analysis.

Total RNA from RCMH and RCDMD myotubes was obtained 4 h post-electrical stimulation. Our results, normalized to GAPDH expression, indicate that electrical stimulation induces a significant increase in NRG-1 β mRNA level in RCDMD cells ($100 \pm 3\%$ to $250 \pm 60\%$, n=3), but has no effect on RCMH cells at this time, in which basal expression remains unchanged ($100 \pm 16\%$ to $123 \pm 26\%$, n=3) [Fig. 1B].

In order to establish the time course of NRG-1 β gene expression evoked by depolarization, RCMH and RCDMD cells were electrically stimulated, changed to

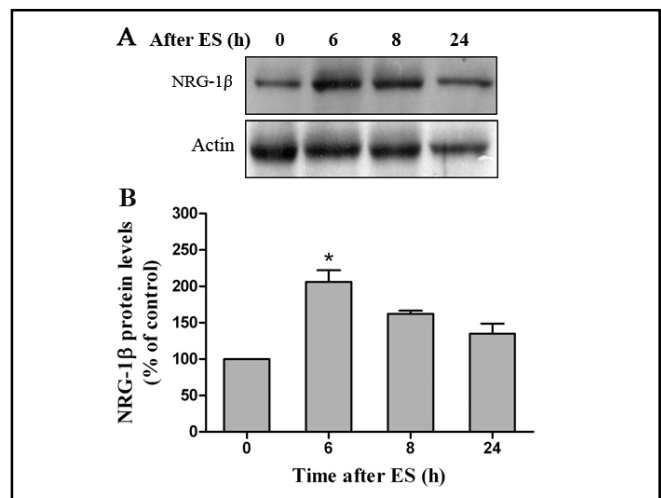
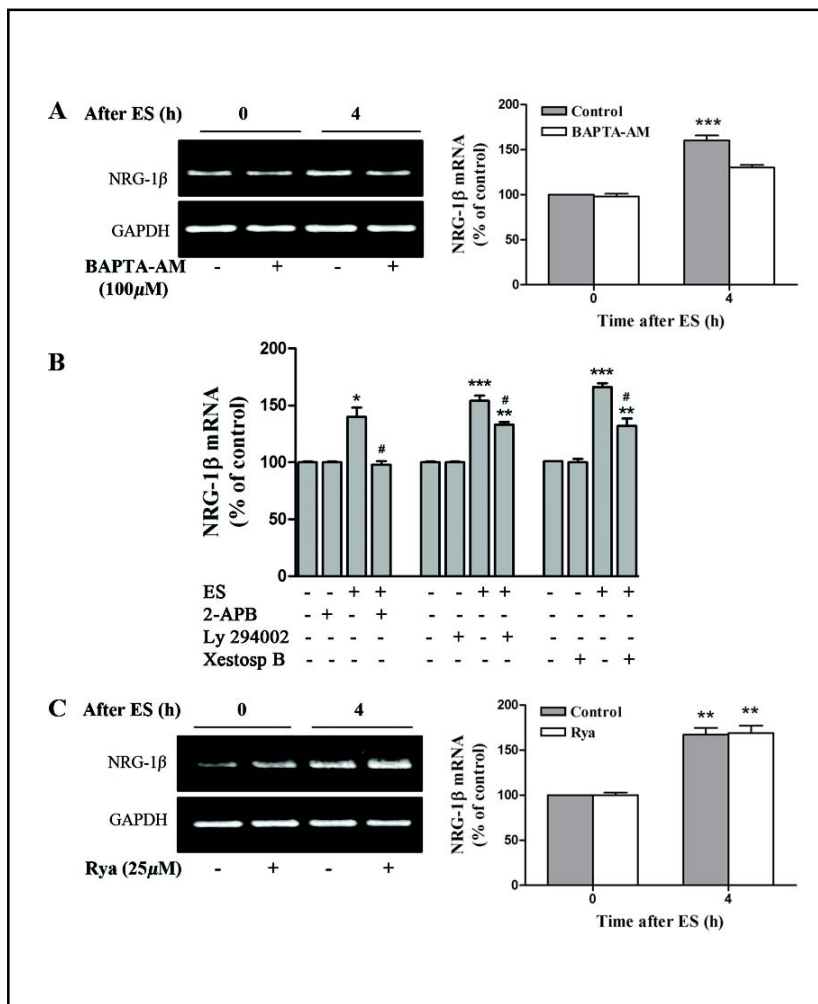


Fig. 3. Electrical stimulation increases NRG-1 β protein level in RCDMD cell lines. RCDMD myotubes were electrically stimulated and maintained in resting medium for the times indicated. Proteins were isolated and analyzed by Western blot. (A) Representative gel of NRG-1 β and actin immunoblot. (B) Results were normalized to actin protein level and are presented as a percentage of untreated control cells (100%) (mean \pm S.E; n=3). *P<0.05 ANOVA followed by Dunnett's multiple comparison test.

Fig. 4. Slow calcium transient involvement in NRG-1 β mRNA increased levels, in RCDMD cell line. Dystrophic cells were depolarized in the presence of different pharmacological agents. After a 30 min preincubation period with or without these agents, myotubes were depolarized and then maintained in resting medium for 4 h. Inhibitors were present during the whole depolarization period. NRG-1 β mRNA levels were analyzed by semiquantitative RT-PCR after electrical stimulation (ES). (A) BAPTA-AM effect. Characteristic agarose gel of RT-PCR products from cells depolarized in the presence of either 100 μ M BAPTA-AM or vehicle (20% pluronic acid in DMSO). Results were normalized to GAPDH expression and expressed as a percentage of control (100%; mean \pm S.E, n=3). ***P<0.001 vs corresponding control, evaluated by Bonferroni's test. (B) IP₃ pathway inhibitors effects. Dystrophic cells were depolarized in the presence of either 50 μ M 2-APB, 40 μ M Ly 294002, 5 μ M Xestospong B (Xestosp B), or vehicle (ethanol for 2-APB, DMSO for Ly 294002 and Xestospong B). Bars represent mean \pm S.E, n=3. *P<0.05; **P<0.01; ***P<0.001 vs corresponding non-depolarized control; #P<0.05 vs depolarized conditions, evaluated by Bonferroni's test. (C) Ryanodine effect. Characteristic agarose gel of RT-PCR products from cells depolarized in the presence of either 25 μ M ryanodine or vehicle (ethanol). Bars represent mean \pm S.E, n=3. **P<0.01 vs corresponding control, evaluated by Bonferroni's test.



resting conditions and collected for semiquantitative RT-PCR analysis at different times. Results were normalized to GAPDH expression.

We observed that depolarization of RCDMD cells produces a transient increase on NRG-1 β mRNA levels, compared with non-depolarized controls, reaching a 1.8 fold maximal stimulation 4 h after treatment (186 \pm 24%, n=3). Electrical stimulation of RCMH cells has not effect on NRG-1 β mRNA levels at all times studied (Fig. 2A and B).

In addition, we observed that the effect on RCDMD cells was abolished by actinomycin D (Fig. 2C), suggesting that up-regulation occurs by stimulation of NRG-1 β transcriptional activity.

Electrical stimulation increases NRG-1 β protein level in RCDMD cells

Based on preceding results, we analyzed whether observed increase in NRG-1 β mRNA level in dystrophic

cell line goes together with a raise in protein quantity. RCDMD cells were electrically stimulated, changed to resting conditions and then collected at different times. Whole cells lysates were analyzed by Western blot using selective antibodies against NRG-1 β (40 kDa). Results were normalized to actin expression.

Our results revealed a significant increase in NRG-1 β protein level in depolarized cells, reaching a 2-fold maximal level (206 \pm 28%, n=3), at 6 h after stimulation (Fig. 3).

Calcium requirement for NRG-1 β induction: role of intracellular Ca²⁺

To investigate intracellular Ca²⁺ involvement in NRG-1 β gene expression, we made use of the cell-permeable Ca²⁺ chelator BAPTA-AM. Preloading of RCDMD cells with 100 μ M BAPTA-AM for 30 min under resting conditions, followed by electrical stimulation in the presence of BAPTA-AM, resulted in an inhibition

on NRG-1 β mRNA increased levels evoked by depolarization (Fig. 4A), suggesting intracellular Ca²⁺ participation in NRG-1 β gene expression.

Depolarization-induced slow Ca²⁺ transients increase NRG-1 β mRNA levels in RCDMD cells

Ca²⁺ increase induced by high K⁺ depolarization or electrical stimulation of skeletal muscle cells involves at least two components with different kinetics, the fast one is associated to RyR, and the slower transient is mediated by an intracellular IP₃ increase. Slow component of Ca²⁺ increase evoked by depolarization is blocked by compounds that interfere with the IP₃ system, such as 2-APB, inhibitor of IP₃-mediated Ca²⁺ release, Ly 294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, and Xestospongine B, an IP₃R blocker [23, 37], whereas high concentrations of Ryanodine (Rya) eliminate just the initial fast Ca²⁺ increase, while the slow Ca²⁺ transient is preserved [38].

In order to define which component participates on NRG-1 β induction in dystrophic muscle cells, we used specific inhibitors that selectively abolish one of them. Dystrophic myotubes were incubated separately with either 50 μ M 2-APB, 40 μ M Ly 294002, 5 μ M Xestospongine B or vehicle (Fig. 4B). Treatment of RCDMD myotubes with 2-APB resulted in complete inhibition of maximal induced NRG-1 β expression at 4 h (140 \pm 14% to 98 \pm 5%, n=3), whereas partial inhibition was observed after exposure to Ly 294002 (154 \pm 8% to 133 \pm 4%, n=3) or Xestospongine B (166 \pm 6% to 132 \pm 11%, n=3) [Fig. 4B].

Treatment of myotubes with 25 μ M Rya, did not prevent NRG-1 β mRNA induction observed in depolarized cultures (Fig. 4C).

These experiments, as a whole, suggest that IP₃-mediated Ca²⁺ release associated with the slow Ca²⁺ wave is needed for triggering the cascade leading to NRG-1 β expression, but other signalling pathways might also be involved in the process.

Ca²⁺-dependent PKC isoforms are involved in NRG-1 β -induced expression in depolarized RCDMD cells

We have previously reported that IP₃-dependent Ca²⁺ signals are involved in the activation and translocation of a Ca²⁺-dependent PKC isoform (PKC α) to the nucleus [36]. Although it has been demonstrated that PKC activation promotes NRG-1 cleavage and release of the extracellular EGF-like domain [39], their involvement in NRG-1 expression has not been described.

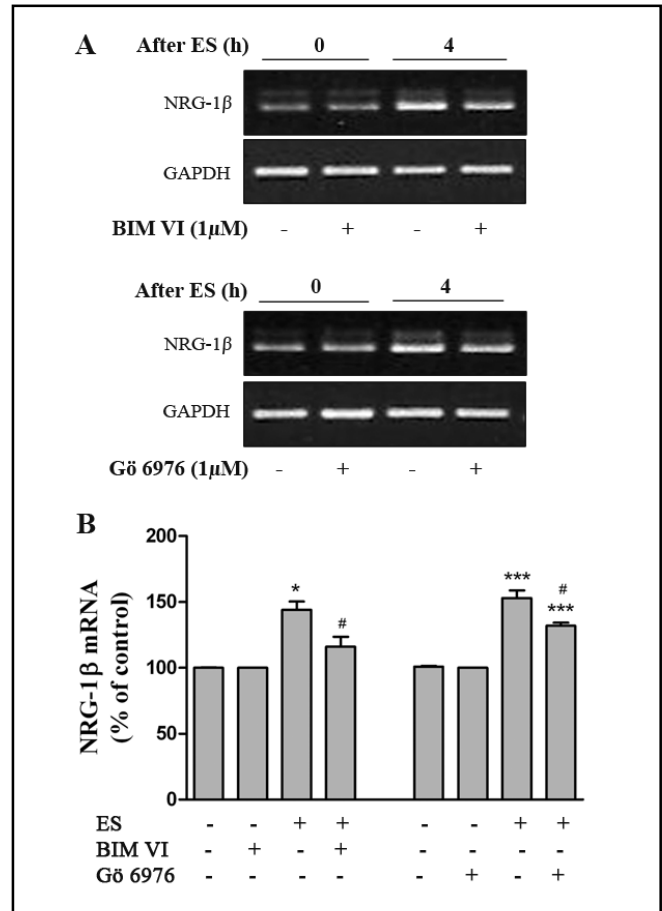


Fig. 5. Ca²⁺-dependent PKC isoforms are involved in NRG-1 β -induced expression in depolarized RCDMD cells. Cells were depolarized in the presence of PKC inhibitors. NRG-1 β mRNA levels were analyzed by semiquantitative RT-PCR at 4 h after electrical stimulation (ES). (A) Characteristic agarose gel of RT-PCR products from cells depolarized in the presence of either BIM VI, Gö 6976 or vehicle (DMSO) (n=3). (B) Results were normalized to GAPDH expression and expressed as a percentage of corresponding non-depolarized control (100%; mean \pm S.E, n=3). *P<0.05; ***P<0.001 vs correspondent non-depolarized control; #P<0.05 vs depolarized conditions, evaluated by Bonferroni's test.

To study the participation of PKC in NRG-1 β gene expression, dystrophic cells were depolarized in the presence of either 1 μ M BIM VI, a general inhibitor of PKC isoforms or 1 μ M Gö 6976, a specific inhibitor of Ca²⁺-dependent PKCs. Our results show a significant decrease in up-regulated NRG-1 β mRNA levels at 4 h post-stimulation with both inhibitors (144 \pm 11% to 116 \pm 13% for BIM VI, n=3; 153 \pm 10% to 132 \pm 4% for Gö 6976, n=3), suggesting that Ca²⁺-dependent PKC isoforms could be needed for activation of NRG-1 β expression evoked by depolarization [Fig. 5].

Discussion

Here we provide evidence that membrane potential changes, induced by electrical stimulation, result in significant up-regulation of genes encoding NRG-1 isoforms β and γ in dystrophic RCDMD cells. We demonstrated that depolarization increases NRG-1 β mRNA and protein levels in this cell line. We ascertain the role of depolarization induced slow Ca^{2+} transients as an early signal leading to NRG-1 β expression in dystrophic cells. Our results suggest that Ca^{2+} released from IP_3 -sensitive intracellular stores participates in NRG-1 β mRNA induction and that Ca^{2+} -dependent PKC isoforms are involved in the process.

Neuregulin-1 (NRG-1) growth factor was initially described as a neurotrophic factor involved in neuromuscular junction formation in skeletal muscle, but recently it has emerged as a myokine, with relevant effects on myogenesis, muscle metabolism and regeneration, and as a strong candidate to transduce muscle adaptation to chronic exercise [40, reviewed in 14]. Furthermore, NRG-1 is being presently explored as a therapy for DMD [41] due to its ability to activate utrophin promoter, increasing utrophin mRNA levels in cultured myotubes [9]. It has been reported that intraperitoneal injections of a small peptide region of the NRG-1 ectodomain up-regulates utrophin expression and improves dystrophic phenotype in *mdx* mice [11].

Initially we focused our study on the effect of membrane depolarization on global gene expression in dystrophic RCDMD cells. Electrical stimulation resulted in 150 differentially expressed genes compared to non-stimulated cells at 4 h. Among these it was particularly interesting to find that two NRG-1 isoforms (β and γ) appear within the ten highest up-regulated genes.

Taking into account the important biological effects of NRG-1 in the muscle and its potential clinical implication in DMD, we focused our study on the regulation of NRG-1 expression, specifically on NRG-1 β isoform, that displays a higher affinity for NRG receptor.

Time course experiments revealed that electrical stimulation induces a transient increase of NRG-1 β mRNA levels in RCDMD cells, with a maximum at 4 h post-stimuli. We observed that depolarization had no effect on NRG-1 β expression in RCMH cells treated with the same procedure. Western blot analysis of stimulated RCDMD cells demonstrates that observed increase in NRG-1 β mRNA levels was followed by actual enhancement of the corresponding protein.

Differential NRG-1 β expression in dystrophic vs nor-

mal cells could be explained by dissimilarities in IP_3 Rs and slow Ca^{2+} signals kinetic between both cell lines. We have previously demonstrated that IP_3 -induced Ca^{2+} signals, which appear most prominently in the nuclei, are involved in the regulation of several transcription-related events following membrane depolarization in normal muscle cells [23, 24, 26-28].

We have recently reported an abnormal expression and distribution of IP_3 Rs in human biopsies of DMD patients compared to normal human samples; these differences are also apparent when comparing RCDMD vs RCMH muscle cells. In addition, we described that IP_3 -dependent slow Ca^{2+} signals induced by tetanic stimulus are significantly larger and faster in RCDMD than in RCMH cells [32].

To further investigate the involvement of Ca^{2+} transients in NRG-1 β gene expression in dystrophic cells, RCDMD were electrically stimulated in the presence of specific inhibitors. We demonstrated that NRG-1 β gene expression was inhibited in the presence of BAPTA-AM, an intracellular Ca^{2+} chelator, whereas depolarization of myotubes performed in the presence of high concentrations of Rya, that eliminates just the initial fast Ca^{2+} increase, did not prevent NRG-1 β mRNA induction observed in depolarized cultures.

Concerning the effect of IP_3 -dependent slow Ca^{2+} transients inhibitors, we found that 2-APB treatment resulted in complete inhibition of maximal induced NRG-1 β expression at 4 h, whereas partial inhibition was observed after exposure of myotubes to either Ly 294002 or Xestospongine B. 2-APB inhibits IP_3 -mediated Ca^{2+} signals in various cell systems [42, 43], including depolarized myotubes [22, 38], but its specificity has been a matter of discussion [44, 45]. However, results obtained after Ly 294002 or Xestospongine B treatment, operating by a different mechanism, support 2-APB effect and give evidence for the involvement of Ca^{2+} released from IP_3 -sensitive intracellular stores in the regulation of NRG-1 β transcriptional activity. Taken together, our results suggest that IP_3 -dependent slow Ca^{2+} signals induced by depolarization stimulate NRG-1 β transcription in RCDMD cells, although other signalling pathways might be involved in the process.

Accumulating evidence suggests that integral DAPC components are implicated in signalling in DMD [46]. Thus, it is likely that loss of DAPC proteins in cell membrane affect in some way the regulation of Ca^{2+} transients and subsequently NRG-1 β gene expression in electrically stimulated dystrophic cells. In fact, experiments performed using SolC1(-) dystrophin deficient myotubes,

and SolD(+) transfected myotubes for mini-dystrophin expression, both originated from the same cell line, have demonstrated that Ca^{2+} rise evoked by depolarization is higher in SolC1(-) than in SolD(+) myotubes [31]. Analysis of the kinetics of Ca^{2+} rise, reveals that IP_3 -dependent slow Ca^{2+} release is increased in SolC1(-) compared to SolD(+), suggesting an inhibitory effect of mini-dystrophin on slow Ca^{2+} transients. Moreover, it has been demonstrated that IP_3 production after depolarization is significantly elevated in SolC1(-) and that the presence of mini-dystrophin under the membrane leads to reduced IP_3 production [31].

Our laboratory has reported that IP_3 -dependent Ca^{2+} signals regulate the activation and translocation of a Ca^{2+} -dependent PKC isoform (PKC α) to the nucleus, in depolarized myotubes [36]. PKCs are a family of serine/threonine kinases, classified into three main subgroups based on their amino acid sequence and lipid dependent activation requirements. Conventional PKC isoforms require Ca^{2+} and diacylglycerol (DAG) for activation, the novel PKC isoforms are Ca^{2+} -independent but DAG-dependent, and atypical PKC isoforms are unresponsive to DAG and/or Ca^{2+} [47].

To investigate a possible involvement of PKC on NRG-1 β transcriptional activity in our experimental model, we treated RCDMD cells with a general inhibitor of PKC and with a specific inhibitor for Ca^{2+} -dependent isoforms. Our results suggest that Ca^{2+} -dependent PKC isoforms are needed for activation of NRG-1 β expression.

NRG-1 promoter region has been characterized in breast cancer cells, and a NF- κ B consensus sequence described as Ca^{2+} responsive regulatory element in other promoters has been identified [48]. It has been reported that membrane depolarization of skeletal muscle cells induces the activation of nuclear transcription factor NF- κ B by a mechanism that involves sequential changes in intracellular Ca^{2+} level, mediated by both RyR and IP_3 Rs [49]. Based on these antecedents, it is possible that NRG-1 β gene expression induced by electrical stimulation could be mediated by NF- κ B activation in the dystrophic cell line. Further studies are needed in order to assess a possible role of NF- κ B in NRG-1 β gene expression.

Our findings can be inserted in a mechanistic model, previously reported by our group, depicting the pathways that link membrane depolarization and intracellular Ca^{2+} increase to gene expression in skeletal muscle cells. This model has been described in normal skeletal muscle cells and includes receptors and pathways known to be

involved in IP_3 -generated Ca^{2+} signals. The signalling pathway begins in the dihydropyridine receptor, which by a mechanism that involves ATP release and purinergic receptors [50], via a G protein [51], activates PI3 kinase and PLC to produce IP_3 . It diffuses into the cytosol and reaches IP_3 Rs located both at the sarcoplasmic reticulum membrane and at the nuclear envelope, inducing Ca^{2+} release [24]. As a consequence several Ca^{2+} -dependent mechanisms, including different kinases and transcription factors [24, 26], are activated and lead to regulation of gene expression [26-28].

It has been postulated that the absence of dystrophin under the membrane increases IP_3 production in depolarized dystrophic cells [31], generating larger and faster slow Ca^{2+} transients [31, 32]. We propose that these signals stimulate Ca^{2+} -dependent PKC isoforms and its translocation to the nucleus [36], increasing NRG-1 β expression in dystrophic cells.

In summary, our results point out to an important role of slow Ca^{2+} transients evoked by electrical stimulation in the activation of pathways that couple excitation to NRG-1 β expression in dystrophin-deficient RCDMD cell lines. To our knowledge, this is the first report describing the involvement of IP_3 -mediated slow Ca^{2+} signals on NRG-1 transcriptional activity in dystrophic skeletal muscle cells. Further studies using IP_3 R over-expression, knockout or knockdown with interference RNA could provide additional proofs on the subject.

The strong similarities between dystrophin and utrophin encouraged the hypothesis that utrophin up-regulation could lead to the functional replacement of dystrophin in the sarcolemma. Since NRG-1 pathway induces utrophin expression, the understanding of the molecular events that regulate its expression will give new insights to the design of a therapeutic strategy in order to potentiate muscle survival and regeneration in DMD.

Acknowledgements

We are grateful to Mónica Silva for culture preparations and Dr. Ulises Urzúa for microarray laboratory's facilities. This work was supported by Project PSD24 Programa Bicentenario de Ciencia y Tecnología; Fondo de Investigación Avanzada en Áreas Prioritarias (FONDAP) Grant 15010006 and Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) Grant 11100267.

References

- 1 Monaco AP, Neve RL, Colletti-Feener C, Bertelson CJ, Kurnit DM, Kunkel LM: Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. *Nature* 1986;323:646-650.
- 2 Hoffman EP, Brown RH, Kunkel LM: Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Biotechnology* 1992;24:457-466.
- 3 Blake DJ, Weir A, Newey SE, Davies KE: Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol Rev* 2002;82:291-329.
- 4 Lapidus KA, Kakkar R, McNally EM: The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. *Circ Res* 2004;94:1023-1031.
- 5 Alderton JM, Steinhardt RA: How calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. *Trends Cardiovasc Med* 2000;10:268-272.
- 6 Bogdanovich S, Perkins KJ, Krag TO, Khurana TS: Therapeutics for Duchenne muscular dystrophy: Current approaches and future directions. *J Mol Med* 2004;82:102-115.
- 7 Kapsa R, Kornberg AJ, Byrne E: Novel therapies for Duchenne muscular dystrophy. *Lancet Neurol* 2003;2:299-310.
- 8 Miura P, Jasmin BJ: Utrophin up-regulation for treating Duchenne or Becker muscular dystrophy: how close are we? *Trends Mol Med* 2006;12:122-129.
- 9 Gramolini AO, Angus LM, Schaeffer L, Burton EA, Tinsley JM, Davies KE, Changeux JP, Jasmin BJ: Induction of utrophin gene expression by heregulin in skeletal muscle cells: role of the N-box motif and GA binding protein. *Proc Natl Acad Sci USA* 1999;96:3223-3227.
- 10 Khurana TS, Rosmarin AG, Shang J, Krag TO, Das S, Gammelfoft S: Activation of utrophin promoter by heregulin via the ets-related transcription factor complex GA-binding protein a/b. *Mol Biol Cell* 1999;10:2075-2086.
- 11 Krag TO, Bogdanovich S, Jensen CJ, Fischer MD, Hansen-Schwartz J, Javazon EH, Flake AW, Edvinsson L, Khurana TS: Heregulin ameliorates the dystrophic phenotype in *mdx* mice. *Proc Natl Acad Sci USA* 2004;101:13856-13860.
- 12 Falls DL: Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res* 2003;284:14-30.
- 13 Carraway KL III, Burden SJ: Neuregulins and their receptors. *Curr Opin Neurobiol* 1995;5:606-612.
- 14 Gumà A, Martínez-Redondo V, López-Soldado I, Cantó C, Zorzano A: Emerging role of neuregulin as a modulator of muscle metabolism. *Am J Physiol Endocrinol Metab* 2010;298:E742-E750.
- 15 Britsch S: The neuregulin-I/ErbB signaling system in development and disease. *Adv Anat Embryol Cell Biol* 2007;190:1-65.
- 16 Kim D, Chi S, Lee KH, Rhee S, Kwon YK, Chung CH, Kwon H, Kang MS: Neuregulin stimulates myogenic differentiation in an autocrine manner. *J Biol Chem* 1999;274:15395-15400.
- 17 Trachtenberg JT: Fiber apoptosis in developing rat muscles is regulated by activity, neuregulin. *Dev Biol* 1998;196:193-203.
- 18 Hippenmeyer S, Shneider NA, Birchmeier C, Burden SJ, Jessell TM, Arber S: A role for neuregulin1 signaling in muscle spindle differentiation. *Neuron* 2002;36:1035-1049.
- 19 Hirata M, Sakuma K, Okajima S, Fujiwara H, Inashima S, Yasuhara M, Kubo T: Increased expression of neuregulin-1 in differentiating muscle satellite cells and in motoneurons during muscle regeneration. *Acta Neuropathol* 2007;113:451-459.
- 20 Hellyer NJ, Mantilla CB, Park EW, Zhan WZ, Sieck GC: Neuregulin-dependent protein synthesis in C₂C₁₂ myotubes and rat diaphragm muscle. *Am J Physiol Cell Physiol* 2006;291:C1056-C1061.
- 21 Cantó C, Pich S, Paz JC, Sanchez R, Martínez V, Orpinell M, Palacín M, Zorzano A, Gumà A: Neuregulins increase mitochondrial oxidative capacity and insulin sensitivity in skeletal muscle cells. *Diabetes* 2007;56:2185-2193.
- 22 Jaimovich E, Reyes R, Liberona JL, Powell JA: IP₃ receptors, IP₃ transients, and nucleus-associated Ca²⁺ signals in cultured skeletal muscle. *Am J Physiol Cell Physiol* 2000;278:C998-C1010.
- 23 Powell JA, Carrasco MA, Adams DS, Drouet B, Rios J, Müller M, Estrada M, Jaimovich E: IP₃ receptor function and localization in myotubes: an unexplored Ca²⁺ signaling pathway in skeletal muscle. *J Cell Sci* 2001;114:3673-3683.
- 24 Araya R, Liberona JL, Cárdenas JC, Riveros N, Estrada M, Powell JA, Carrasco MA, Jaimovich E: Dihydropyridine receptors as voltage sensors for a depolarization-evoked, IP₃R-mediated, slow calcium signal in skeletal muscle cells. *J Gen Physiol* 2003;121:3-16.
- 25 Eltit JM, Hidalgo J, Liberona JL, Jaimovich E: Slow calcium signals after tetanic stimulation in skeletal myotubes. *Biophys J* 2004;86:3042-3051.
- 26 Carrasco MA, Riveros N, Rios J, Muller M, Torres F, Pineda J, Lantadilla S, Jaimovich E: Depolarization-induced show calcium transients activate early genes in skeletal muscle cells. *Am J Physiol Cell Physiol* 2003;284:C1438-C1447.
- 27 Juretic N, Garcia-Huidobro P, Iturrieta JA, Jaimovich E, Riveros N: Depolarization induced slow Ca²⁺ transients stimulate transcription of IL-6 gene in skeletal muscle cells. *Am J Physiol Cell Physiol* 2006;290:C1428-C1436.
- 28 Juretic N, Urzúa U, Munroe DJ, Jaimovich E, Riveros N: Differential gene expression in skeletal muscle cells after membrane depolarization. *J Cell Physiol* 2007;210:819-30.
- 29 Jorquera G, Juretic N, Jaimovich E, Riveros N: Membrane depolarization induces calcium-dependent up-regulation of Hsp70 and Hmox-1 in skeletal muscle cells. *Am J Physiol Cell Physiol* 2009;297:C581-C590.
- 30 Liberona JL, Powell JA, Shenoi S, Petherbridge L, Caviedes R, Jaimovich E: Differences in both inositol 1,4,5-trisphosphate mass and inositol 1,4,5-trisphosphate receptors between normal and dystrophic skeletal muscle cell lines. *Muscle Nerve* 1998;21:902-909.
- 31 Balghi H, Sebille S, Constantin B, Patri S, Thoreau V, Mondin L, Mok E, Kitzis A, Raymond G, Cognard C: Mini-dystrophin expression down-regulates overactivation of G protein mediated IP₃ signaling pathway in dystrophin-deficient muscle cells. *J Gen Physiol* 2006;127:171-182.
- 32 Cárdenas C, Juretic N, Bevilacqua JA, García I, Figueroa F, Hartley R, Taratuto AL, Gejman R, Riveros N, Molgó J, Jaimovich E: Abnormal distribution of inositol 1,4,5-trisphosphate receptors in human muscle can be related to altered calcium signals and gene expression in Duchenne dystrophy derived cells. *FASEB J* 2010;24:3210-3221.
- 33 Caviedes R, Caviedes P, Liberona JL, Jaimovich E: Ion channels in a skeletal muscle cell line from a Duchenne muscular dystrophy patient. *Muscle Nerve* 1994;17:1021-1028.
- 34 Caviedes R, Liberona JL, Hidalgo J, Tascon S, Salas K, Jaimovich E: A human skeletal muscle cell line obtained from an adult donor. *Biochim Biophys Acta* 1992;1134:247-255.
- 35 Brown DJ, Lin B, Holguin B: Expression of Neuregulin 1, a Member of the Epidermal Growth Factor Family, Is Expressed as Multiple Splice Variants in the Adult Human Cornea. *Invest Ophthalmol Vis Sci* 2004;45:3021-3029.
- 36 Cárdenas C, Müller M, Jaimovich E, Pérez F, Buchuk D, Quest AFG, Carrasco MA: Depolarization of skeletal muscle cells induces phosphorylation of cAMP response element binding protein via calcium and protein kinase Ca. *J Biol Chem* 2004;279:39122-39131.

- 37 Jaimovich E, Mattei C, Liberona JL, Cardenas C, Estrada M, Barbier J, Debitus C, Laurent D, Molgó J: Xestospongins B, a competitive inhibitor of IP₃-mediated Ca²⁺ signalling in cultured rat myotubes, isolated myonuclei, and neuroblastoma (NG108-15) cells. *FEBS Lett* 2005;579:2051-2057.
- 38 Estrada M, Cárdenas C, Liberona JL, Carrasco MA, Mignery GA, Allen PD, Jaimovich E: Calcium transients in IB5 myotubes lacking ryanodine receptors are related to inositol trisphosphate receptors. *J Biol Chem* 2001;276:22868-22874.
- 39 Shamir A, Buonanno A: Molecular and cellular characterization of Neuregulin-1 type IV isoforms. *J Neurochem* 2010;113:1163-1176.
- 40 Lebrasseur NK, Cote GM, Miller TA, Fielding RA, Sawyer DB: Regulation of neuregulin/ErbB signaling by contractile activity in skeletal muscle. *Am J Physiol Cell Physiol* 2003;284:C1149-C1155.
- 41 Williams S, Ryan C, Jacobson C: Agrin and neuregulin, expanding roles and implications for therapeutics. *Biotechnol Adv* 2008;26:187-201.
- 42 Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K: 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P₃-induced Ca²⁺ release. *J Biochem (Tokyo)* 1997;122:498-505.
- 43 Ascher-Landsberg J, Saunders T, Elovitz M, Phillippe M: The effects of 2-aminoethoxydiphenyl borate, a novel inositol 1,4,5-trisphosphate receptor modulator on myometrial contractions. *Biochem Biophys Res Commun* 1999;264:979-982.
- 44 Gregory RB, Rychkov G, Barritt GJ: Evidence that 2-aminoethyl diphenylborate is a novel inhibitor of store-operated Ca²⁺ channels in liver cells, and acts through a mechanism which does not involve inositol trisphosphate receptors. *Biochem J* 2001;354:285-290.
- 45 Missiaen L, Callewaert G, De Smedt H, Parys JB: Aminoethoxydiphenyl borate affects the inositol 1,4,5-trisphosphate receptor, the intracellular Ca²⁺ pump and the non-specific Ca²⁺ leak from the non-mitochondrial Ca²⁺ stores in permeabilized A7r5 cells. *Cell Calcium* 2001;29:111-116.
- 46 Haslett JN, Sanoudou D, Kho AT, Bennett RR, Greenberg SA, Kohane IS, Beggs AH, Kunkel LM: Gene expression comparison of biopsies from Duchenne muscular dystrophy (DMD) and normal skeletal muscle. *Proc Natl Acad Sci USA* 2002;99:15000-15005.
- 47 Quest AFG: Multiple modes of protein kinase C regulation. *Recent Res Dev Biochem* 2000;2:171-190.
- 48 Frensing T, Kaltschmidt C, Schmitt-John T: Characterization of a neuregulin-1 gene promoter: Positive regulation of type I isoforms by NF-κB. *Biochim Biophys Acta* 2008;1779:139-144.
- 49 Valdés JA, Hidalgo J, Galaz JL, Puentes N, Silva M, Jaimovich E, Carrasco MA: NF-κB activation by depolarization of skeletal muscle cells depends on ryanodine and IP₃ receptor-mediated calcium signals. *Am J Physiol Cell Physiol* 2007;292:C1960-C1970.
- 50 Buvinic S, Almarza G, Bustamante M, Casas M, López J, Riquelme M, Sáez JC, Huidobro-Toro JP, Jaimovich E: ATP released by electrical stimuli elicits calcium transients and gene expression in skeletal muscle. *J Biol Chem* 2009;284:34490-34505.
- 51 Eltit JM, García AA, Hidalgo J, Liberona JL, Chiong M, Lavandero S, Maldonado E, Jaimovich E: Membrane electrical activity elicits inositol 1,4,5-trisphosphate-dependent slow Ca²⁺ signals through a G beta gamma/ phosphatidylinositol 3-kinase gamma pathway in skeletal myotubes. *J Biol Chem* 2006;281:12143-12154.