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Description of a utrophin associated protein complex in lipid raft domains of human artery smooth muscle cells

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ABSTRACT

The dystrophin-associated protein complex (DAPC) is a multimeric complex that links the extracellular matrix to the actin cytoskeleton, and in some cases dystrophin can be substituted by its autosomal homologue utrophin to form the utrophin-associated protein complex (UAPC). Both complexes maintain the stability of plasma membrane during contraction process and play an important role in transmembrane signaling. Mutations in members of the DAPC are associated with muscular dystrophy and dilated cardiomyopathy. In a previous study with human umbilical cord vessels, we observed that utrophin colocalize with caveolin-1 (Cav-1) which proposed the presence of UAPC in the plasma membrane of vascular smooth muscle (VSM). In the current study, we demonstrated by immunofluorescence analysis, co-immunoprecipitation assays, and subcellular fractionation by sucrose gradients, the existence of an UAPC in lipid raft domains of human umbilical artery smooth muscle cells (HUASMC). This complex is constituted by utrophin, β-DG, ε-SG, α-smooth muscle actin, Cav-1, endothelial nitric oxide synthase (eNOS) and cavin-1. It was also observed the presence of dystrophin, utrophin Dp71, β-SG, δ-SG, δ-SG3 and sarcospan in non-lipid raft fractions. Furthermore, the knockdown of α/β-DG was associated with the decrease in both the synthesis of nitric oxide (NO) and the presence of the phosphorylated (active) form of eNOS; and with a reduction in the downstream activation of some cGMP signaling transduction pathway components. Together these results show the presence of an UAPC complex in HUASMC that may participate in the activity regulation of eNOS and in the vascular function.

1. Introduction

Muscular dystrophies (MD) are caused by genetic alterations in several components of the DAPC [1]. This complex is known as a large multimeric complex of cytoskeletal and membrane-spanning proteins composed by dystrophin, syntrophins, dystrobrevin, dystroglycans (DG) (α- and β-), sarcoglycans (SG) (α-/ε-, β-, δ- and γ-/ζ-), and sarcospan (SSPN) [2–5]. The DAPC serves as a molecular scaffold in maintaining membrane stability, mechanotransduction signals during contraction and relaxation process, and Ca^{2+} homeostasis [6–9]. Mutation in the dystrophin gene causes Duchenne/Becker type muscular dystrophy (DMD/BMD), whereas defects in α-, β-, γ- and δ- SG cause limb girdle muscular dystrophy (LGMD 2D-F, respectively) [10–12].

Utrophin, the autosomic homologue of dystrophin, is an ubiquitous protein found in striated and smooth muscle [13,14] as well as in non-muscular tissues like brain, kidney and endothelial cells [15–17]. By interacting with some DAPC members, utrophin can replace its homologue dystrophin to form a UAPC [18]. In the dystrophin-deficient mdx mouse, a model of DMD, utrophin overexpression corrects the dystrophic phenotype, so that it has been proposed that utrophin may rescue the muscular functionality in DMD patients [19,20].

Several evidences have shown that patients with DMD/BMD and LGMD 2C–F are accompanied by dilated cardiomyopathy (DCM) [21–28]; which has been also observed in null mice models for β-, δ- and γ- SG [29–31]. Besides, the presence of coronary artery constrictions in these models has led to propose that these irregularities are one of the causes to develop DCM [29]. All these results show the importance that proteins of the DAPC/UAPC may have in the function of VSM.

VSM tissue contains a specific profile of DAPC proteins which consists of dystrophin, α- and β-DG, ε-, β-, δ- and γ- or ζ-SG and SSPN [32–34]. Although several reports describe the expression of utrophin in this tissue, there has been little focus on its presence as a component

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of a potential protein complex. Biochemical evidences have reported in arteries of animal models the presence of long forms of dystrophin and utrophin, while small veins contained only long forms of utrophin [35]. In a recent study focused on the expression of DAPC/UAPC performed in arteries and veins of human umbilical cord by our research group, we found that SG proteins and Cav-1 either colocalize with utrophin or dystrophin in the smooth muscle tissue and with utrophin in endothelium [17]. We have also described a particular UAPC in endothelial cells which may have a mechano transduction role by its link with eNOS in caveolar domains [36]. Other studies have reported a DAPC in caveolar domains of airway smooth muscle (ASM) that is implicated in mediating intracellular Ca^{2+} release [37]. In the current study we describe an UAPC localized in lipid raft domains of HUASMC that partially participate in the NO/cGMP signaling pathway activity regulation and vascular function. Some other results point out the presence of a potential alternative UAPC/DAPC in non-caveolae membrane domains.

2. Materials and methods

The study was approved by the Institutional Research and Ethics Committees.

2.1. Tissues

The umbilical cords of mature newborns from normal full-term pregnancies were obtained immediately after birth, and 10–15 cm of tissue was placed in 0.9% NaCl plus 2% antibiotic–antimycotic mixture (Gibco-BRL Rockville MD, USA).

2.2. Cell culture

Under sterile conditions, cord arteries were identified and excised from the umbilical cord. The vessels were rinsed 3 times in Hank's balanced salt solution containing 1% of antibiotic–antimycotic mixture (Gibco-BRL, Rockville MD, USA). Endothelial cells were separated from the vessels by enzymatic digestion [38]. Arteries were cut longitudinally and dissected into small pieces of tissue (3 to 5 mm). Vessel explants were placed in 100-mM cultures plates (approximately 20 per plate) with plated vessels lumens down. 6 mL of smooth muscle cell basal medium (SmBm, Lonza Walkersville, MD USA) supplemented with SmGM-2 SingleQuot® kit and 15% fetal bovine serum (Lonza) was added to culture plates and incubated at 37 °C with 5% CO_2. Cell culture media was gently removed and replaced with fresh medium twice a week. Cells started growing from explants within two weeks and reach confluence in approximately 4 weeks. Confluence cell cultures were harvested by trypsinization and centrifuged at 1000 ×*g* for 10 min. The pellet was resuspended in supplemented SmBm and incubated at 37 °C with 5% CO_2. Smooth muscle cells were characterized by α-SMA protein presence (clone αsm1 from Vector Laboratories, Burlingame, CA, USA); >99% of cells were positive for this smooth muscle marker. Also, protein extracts of HUASMC, analyzed by Western blot, showed the presence of α-SMA; but, endothelial and fibroblast markers were not detected, CD31 and fibronectin respectively (data not shown). Cell cultures were typically assayed on passages 8–10.

2.3. Detergent-resistant membrane isolation

Membrane enriched caveolae domains were isolated from HUASMC by detergent-resistant membrane method (DRMs) as previously described [39]. Briefly, about 10^7 cells were washed three times in ice-cold PBS, and subsequently lysed in 1.0 mL of cold TNE buffer (20 mmol/L Tris, pH 7.4, 140 mmol/L NaCl, 2 mmol/L EDTA) containing 0.05% Triton X-100 plus protease inhibitor cocktails (Roche Applied Sciences). Cell lysate was homogenized by passage through an insulin syringe 20 times and subsequently centrifuged at 2000 rpm for 5 min at 4 °C to remove the cell nuclei and debris. Supernatant was recovered and incubated at 4 °C for 45 min and then mixed with 1 mL of ice-cold 80% (w/v) sucrose in TNE buffer. This mixture was seeded at the bottom of a Beckman SW50.1 ultracentrifuge tube, and gently overlaid with 2 mL of ice-cold 35% (w/v) sucrose in TNE buffer, and 1 mL of ice-cold 5% (w/v) sucrose in TNE buffer. Then, the samples were centrifuged at 130,000 ×*g* at 4 °C for 17 h. After centrifugation, twelve fractions (Fx) were collected from the top to the bottom of the tube. Finally, an aliquot of each fraction was mixed at a 3:1 (v/v) ratio with 4× concentrated Laemli loading sample buffer, boiled and subjected to 3–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Co-immunoprecipitation assays

HUASMC were lysated with ice-cold lysis buffer (0.5%, Triton X-100, 50 mmol/L Tris–HCl, pH 7.4, 0.15 mol/L NaCl, 0.5 mmol/L EDTA) supplemented with protease inhibitor cocktail. We used the Immunoprecipitation Kit-Dynabeads Protein G for the co-IP. Fifty microliters of protein-G Dynabeads (Invitrogen) was washed three times in pH citrate–phosphate buffer. The volume of the bead suspension was adjusted with PBS plus 0.1% Tween-20 (PBS-T) to 50 μL and incubated with 2 μg of antibodies against utrophin, Cav-1, flotillin-1, or PKG 1with tilting/rotation. Antibodies bound to Dynabeads were washed three times in 500 μL of pH5 citrate–phosphate buffer and pelleted in a magnetic rack. 50 μL of cleared cell lysates was added and incubated by 2 h at 4 °C. Then, the mixture was washed three times (5 min) in 500 μL of PBS-T containing protease inhibitor cocktail and pelleted. Pelleted beads and supernatant were boiled in SDS-sample buffer for 5 min. Finally the co-immunoprecipitated proteins and those remaining in the supernatant were separated by 2% to 13% SDS-PAGE gradient and analyzed by immunoblot with antibodies against DAPC or UAPC members. Control experiments were included using irrelevant monoclonal antibodies, as well as precipitations with specific antibodies, and using lysis buffer alone instead of extracts.

Regarding the HUASMC knockdown with the specific α/β DG siRNA the precipitated proteins were analyzed by immunoblot with antibodies against the cGMP-dependent protein kinase 1 (PKG 1), the vasodilator-stimulated phosphoprotein (VASP) or the phospho-VASP (Ser239).

2.5. Western blot analysis

For Western blot analysis HUASMC were homogenized in 50 μL lysis buffer (1% Triton X-100, 20 mmol/L Tris, 140 mmol/L NaCl, 2 mmol/L EDTA, and 0.1% SDS) and supplemented with protease inhibitor cocktails. Then the cell lysates were passed through an insulin syringe five times, sonicated for 30 min at 4 °C and centrifuged at 12,000 ×*g* for 10 min. Total protein content was measured in the supernatant using Bradford assay (Bio-Rad). A total of 40 μg of protein was loaded onto 2% to 13% SDS-PAGE gradient, electrotransferred, and incubated for 1 h in blocking solution (5% nonfat dry milk in PBS plus 0.1% Tween 20 [PBS-T]) followed by overnight incubation at 4 °C with appropriate primary antibody. Primary antibodies were typically diluted in PBS-T plus 5% nonfat milk (NFM). Membranes were washed four times for 10 min in PBS-T and incubated 1 h at room temperature in the presence of a specific HRP-conjugated secondary antibody in PBS-T solution. Membranes were again washed four times in PBS-T, and finally were developed using a Plus-ECL chemiluminescence detection kit (Thermo scientific, Rockford, Illinois, USA).

2.6. Dot-blot

A volume of 10 μL of each sucrose gradient fraction were dropped onto a nitrocellulose membrane (Amersham™ Hybond™-ECL). After air-drying, the membrane was blocked with 5% nonfat milk (NFM) in PBS-T at room temperature for 2 h. The nitrocellulose membrane was subsequently overnight incubated at 4 °C with a Cholera Toxin Subunit B-peroxidase conjugate (CT-B), (used as GM1 marker) (Sigma) at

0.25 mg/mL in blocking solution. Followed by three times washing with PBS-T, the nitrocellulose membrane was developed using a Plus-ECL chemiluminescence detection kit as described above.

2.7. Immunofluorescence

Co-localization of GM1 marker with utrophin or dystrophin was performed by previously established method [40]. HUASM cells attached to coverslips were washed with PBS buffer and incubated with CT-B Alexa Fluor-conjugated 594 (5 μg/mL) dissolved in bicarbonate-free DMEM/Hepes, 0.5% BSA, for 30 min on ice to label GM1. Cells were then washed three times in cold DMEM/Hepes/BSA and fixed with methanol for 10 min at −20 °C. Cells were permeabilized with 0.2% Triton X-100 for 5 min and blocked in PBS buffer containing 2.5% BSA and 0.5% gelatin for 1 h. For antibody binding, cells were incubated overnight at 4 °C with 1/100 dilution of primary antibody, washed 3 times with PBS, and incubated with 1/200 dilution of secondary fluorescent antibodies for 1 h at room temperature. After a final washing step with PBS the coverslips were mounted with immunofluorescence mounting medium (Vector Laboratories, Burlingame, CA) and analyzed by a confocal laser scanning microscope Pascal-LSM 510 (Carl Zeiss, Oberkochen, Germany), using Plan-Apochromat 63×/1.4 oil objective. Digital images were processed using Advanced Imaging Microscopy Release 4.0 (SP1) software (Carl Zeiss, Oberkochen, Germany). Negative control labeling was performed with non-immune IgGs instead of the primary antibodies.

2.8. α/β-DG knockdown

α/β-DG precursor was knockdown using a α/β DG siRNA reagent system (Santa Cruz Biotechnology, sc-45064, 43488) according to the manufacturer's instruction. Aliquots of 2×10^5 cells were seeded onto 6 well plate and grown to approximately 80–90% confluency. Then the cells were transfected with a pool of 3 target-specific siRNAs against α/β-DG, or with an irrelevant siRNA [(−) siRNA] using transfection media reduced in fetal bovine serum. After 7 h of incubation the transfection mixture was replaced with DMEM (Gibco-BRL, Rockville MD, USA), supplemented with 10% of fetal bovine serum plus 1% of antibiotic–antimycotic mixture and incubated for 72 h [41]. Later, the transfected cells were incubated for 6 h in DMEM without phenol red deprived of fetal bovine serum and finally stimulated with bradykinin 1 μM for 15 min. Culture supernatants were used to measure nitric oxide (NO) levels, and the cell proteins obtained with RIPA buffer were analyzed by Western blot.

2.9. Nitrite assay

Evaluation of NO levels produced by HUASM cells was measured indirectly by the Greiss method [36]. The supernatants obtained from intact control of HUASMC, as well as transfected cells, either (−) siRNA or α/β DG siRNA under bradykinin stimulus were prepared for nitrite detection with an equal volume of Griess reagent (0.8% sulfanilamide and 0.05% naphthylethylenediamine dihydrochloride in 1 N of acetic acid) and then incubated at room temperature for 30 min. Using $NaNO_2$ to generate a standard curve, nitrite production was determinated spectrophotometrically on ELISA plated reader (Labsystems Multiskan Plus, Conquer Scientific) reading at 540 nm.

2.10. Antibodies

Most of the primary and secondary antibodies used in this study were purchased from commercial sources, then used and stored according to the manufacturer's recommendations. Utrophin (UTR) (H300), β-DG (C-20), ε-SG (C-17), endothelial nitric oxide synthase (eNOS) (NOS3 N-20) and cGMP-dependent protein kinase 1 (cGKIα/β, E-1) were obtained from Santa Cruz Biotechnology, Inc.; phospho (ser1177) eNOS (9572), flotillin-1 (3253), vasodilator-stimulated phosphoprotein (VASP, 9A2), and the phospho-VASP (Ser239) from Cell Signaling; dystrophin (Dys) (Rod domain), β-SG (βSARC/5B1) and δ-SG (δSarc3/12C1) from Vector Laboratories; Cav-1 (2297/Caveolin-1) and PTRF (polymerase I and transcript release factor; cavin-1) (4/PTRF) from Transduction Laboratories BD Biosciences. In the cases of SSPN and Dp71 it was a generous gift from Dr. Rachelle H. Crosbie and Dr. Bulmaro Cisneros, respectively, whereas δ-SG3 was obtained as referred [42]. Alexa Flour-conjugated secondary antibodies and CT-B Alexa Fluor-conjugated 594 (C-34777) were obtained from Molecular Probes.

3. Results

3.1. Utrophin exhibits partial co-localization with lipid raft and caveolae markers in HUASMC

In order to investigate the localization of utrophin/dystrophin regarding lipid raft domains, we performed a set of double immunofluorescence assays employing a GMI marker and antibodies against dystrophin or utrophin in HUASMC. Since it is well known that lipid raft domains are enriched in the ganglioside GM1, which is detected specifically with the CT-B [40,43], we used this marker to identify utrophin or dystrophin co-localization. Double immunofluorescence analysis and confocal microscopy (Fig. 1) revealed an extensive co-localization of utrophin with GM1 (Fig. 1A Utr/CT-B). In contrast, dystrophin showed no co-localization with GM1 (Fig. 1A Dys/CT-B). Image amplification more clearly demonstrated that utrophin, but not dystrophin, co-localizes with GM1 marker (Fig. 1A * Utr and * Dys). We also observed a partial co-localization of β-DG, ε-SG and α-SMA with GM1 marker (Fig. 1B). Amplification of squares showed with more detail the partial co-localization of these proteins in spots along the cell membrane (Fig 1B * β-DG, * ε-SG and * α-SMA). Furthermore, since GM1 is a marker for caveolae and noncaveolae lipid raft domains we also tested the colocalization of utrophin with cavin-1 and cav-1, two caveolae domain markers. By this analysis, the colocalization of utrophin with cavin-1 and cav-1 in different areas of the cell membrane (Fig. 2 merge Utr/Cavin-1 and Utr/Cav-1) was evident. These outcomes suggest that utrophin and some UAPC members may be forming a complex in caveolae and non-caveolae-related domains.

3.2. UAPC members partially reside in lipid raft domains of HUASMC

Cav-1 and cavin-1 are two essential protein components of caveolae lipid raft domains, which are plasma membrane invaginations enriched in cholesterol and glycosphingolipids [44,45]. These domains may play a role in transport, signaling, mechanosensing and lipid regulation [46]. Moreover, flotillins are proteins not constituents of caveolae domains, but that are present in distinct lipid raft domains of the plasma membrane [47]. To determine whether UAPC proteins are located in lipid raft domains of HUASMC, we isolated Triton X-100 insoluble cholesterol and sphingolipid membrane fractions by sucrose density gradient assay. Initially, we tested the purity of all the gradient fractions by dot blot using CT-B-HRP coupled, which binds ganglioside GM1, a sphingolipid found mostly in lipid rafts. Through this assay an enrichment of GM1 was observed in low-density fractions (Fx 3–5) (Fig. 3). Fractions 1 and 2 were discarded because they showed no evidence of proteins.

The presence of UAPC/DAPC proteins in the sucrose gradient fractions was determined by Western blot analysis. Utrophin, β-DG, ε-SG, α-SMA and eNOS coincided with Cav-1, flotillin and cavin-1, as well as the ganglioside GM1 in Fx 3–5 (Fig. 3). These data are consistent with those obtained in the double immunofluorescence assay using Cav-1 and/or cavin-1 and together confirm the presence of a UAPC in caveolae domains.

Conversely, dystrophin, Dp71, β-SG, δ-SG, δ-SG3, and SSPN were primarily evident in denser fractions (Fig. 3 Fx 8–12) along with utrophin,

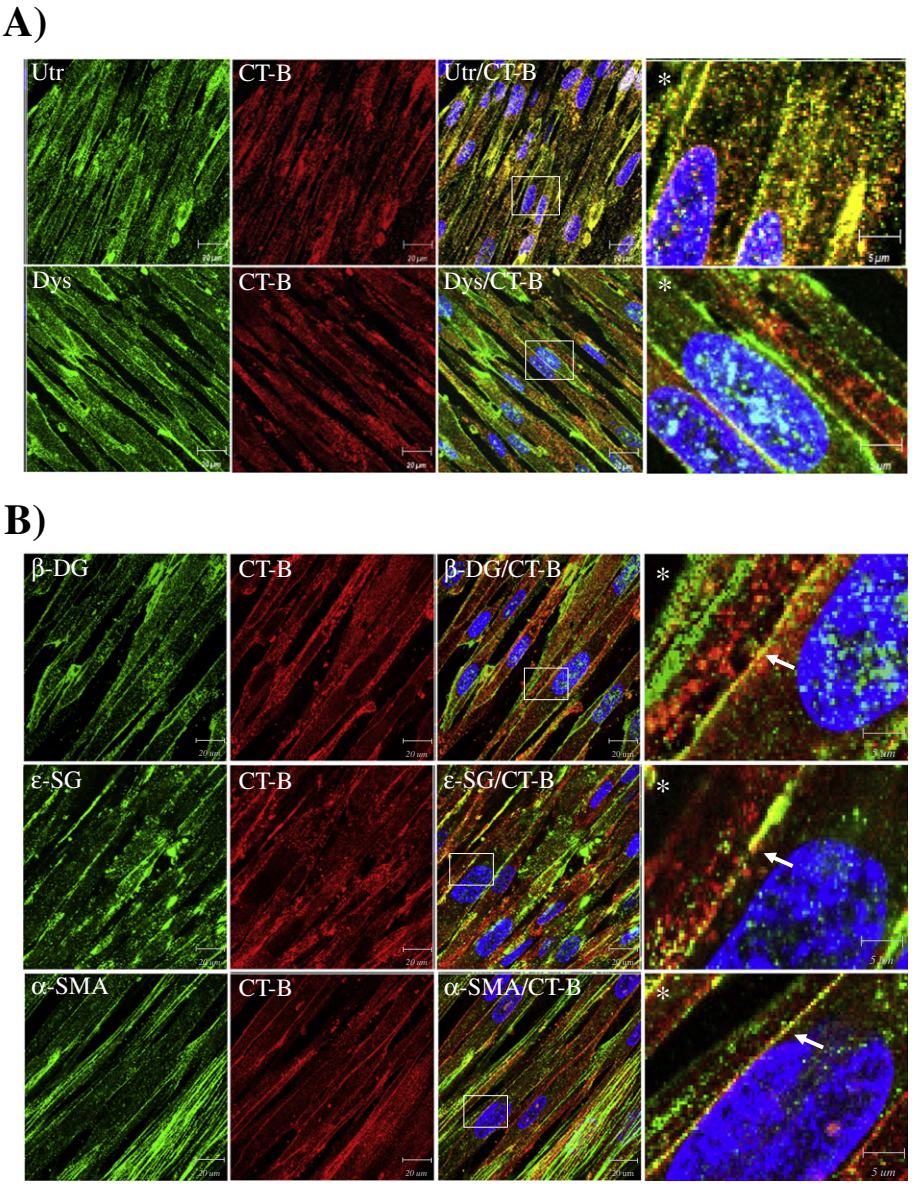

Fig. 1. Utrophin and members of the UAPC co-localizes with ganglioside GM1 in sarcolemma of HUASMC. Primary cultures of HUASMC were incubated with Cholera Toxin Subunit B (CT-B) Alexa Fluor 594-conjugate, which serves as a ganglioside GM1 marker. (A) Cells were then immunolabeled to either utrophin (Utr) or dystrophin (Dys). Merged confocal images reveal that Utr (Utr/CT-B), but not Dys (Dys/CT-B), co-localizes with the ganglioside GM1. Enlargements of the corresponding boxed areas (*) show clearer that Utr co-localizes with ganglioside GM1. (B) Cells marked with CT-B Alexa Fluor 594 conjugate were also immunolabeled with β-dystroglycan (β-DG), ε-sarcoglycan (ε-SG) and α-smooth muscle actin (α-SMA). Merged confocal images reveal that the three proteins co-localize with the ganglioside GM1. This was more evident in the enlargements of the corresponding boxes (*). Cell nuclei were marked with DAPI.

β-DG, ε-SG and cavin-1. These results suggest the possibility of the occurrence of alternative UAPC/DAPC in other membrane domains.

3.3. UAPC–Cav-1 interaction in HUASMC

In order to evaluate whether components of the UAPC interact with Cav-1 in HUASMC, a major structural component of the caveolae domains [48], we performed reciprocal co-immunoprecipitation assays using antibodies against utrophin or Cav-1 in total protein extracts obtained from cell cultures (Fig. 4). Western blot analysis of the immunoprecipitated proteins revealed the presence of utrophin, β-DG, ε-SG, α-SMA, eNOS and Cav-1 proteins (Fig. 4A). These results confirmed the interaction between UAPC components and Cav-1. Furthermore, the interaction of Cav-1 and/or utrophin with cavin-1 was also evident, another important component of the caveolae domains [49]. Regarding dystrophin, the protein was only evident in the input and supernatant of both co-immunoprecipitation assays (Fig. 4A). All these results suggest the presence of a UAPC conformed by utrophin, β-DG, ε-SG, eNOS, α-SMA, Cav-1 and cavin-1 proteins in caveolae domains of HUASMC. With respect to the presence of an UAPC in noncaveolae lipid raft domains, we did not observe a band of utrophin and β-DG in the Western blot of the proteins immunoprecipitated with an antibody against flotillin-1 (Fig. 4B). Negative control shows that there was no detectable protein binding to beads not conjugated with utrophin and Cav-1 antibody (data not shown).

3.4. α/β-DG deficiency reduce NO synthesis and eNOS phosphorylation, and affect cGMP signaling pathway

In a previous study our group proposed that UAPCs located in caveolae and non-caveolae lipid raft domains of human umbilical vein endothelial cells (HUVEC) may have a mechanosensory function that

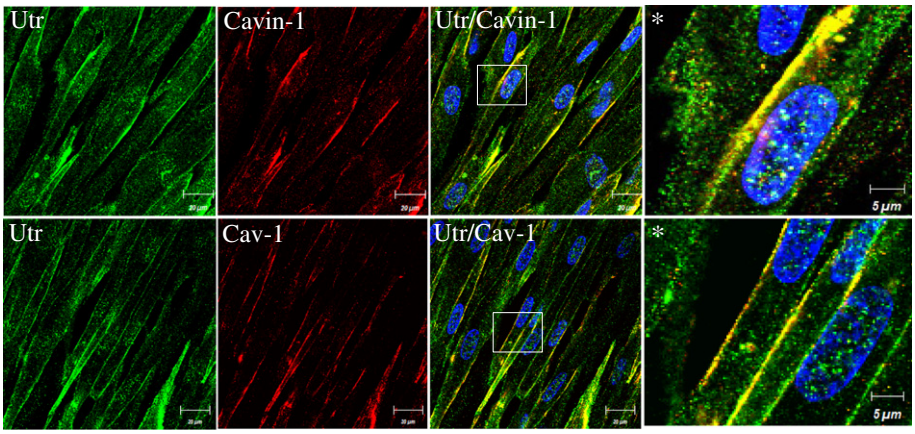

Fig. 2. Utrophin co-localizes with cavin-1 and cav-1 in sarcolemma of HUASMC. Primary cultures of HUASMC were double stained with antibodies against Utr/Cavin-1 and Utr/Cav-1. Merge images reveal that utrophin co-localize with both Cavin-1 and Cav-1. Enlargements (*) of the corresponding merged confocal images reveal clearer this co-localization. Cell nuclei were marked with DAPI.

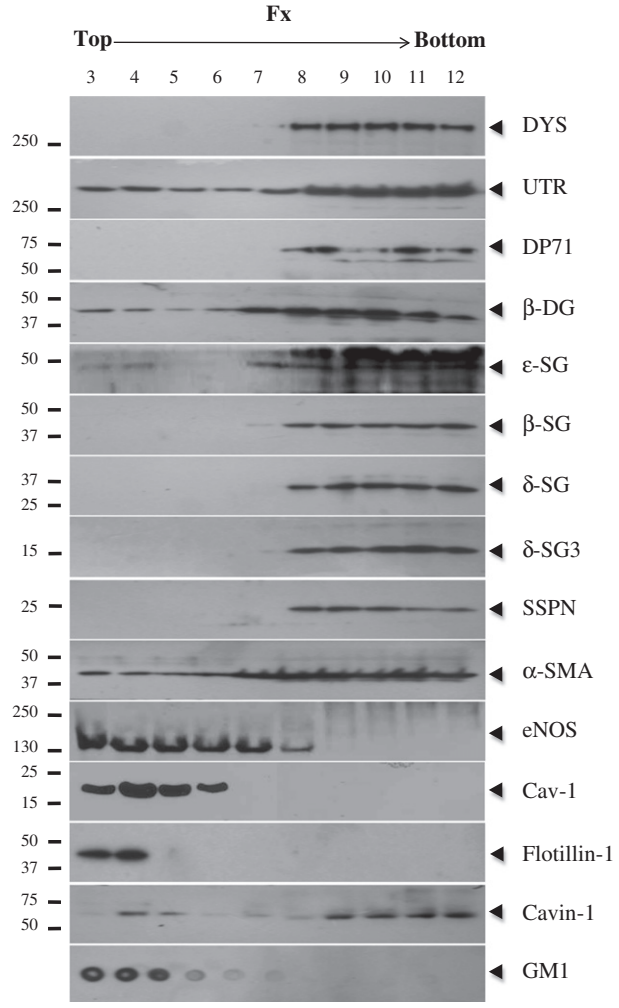

Fig. 3. UAPC proteins are present within caveolae domains. Primary culture of HUASMC were lysed, homogenized and subjected to membrane flotation assay to isolate detergent-resistant membranes (DRMs) that contain caveolae- and non-caveolae-related membranes as described in the Material and methods section. Immunoblotting of equal volumes of 12 fractions (Fx) was performed with antibodies against dystrophin (Dys), utrophin (Utr), 71-kDa dystrophin isoform (Dp71), beta dystroglycan (β-DG), epsilon, beta, delta sarcoglycan (ε-, β- and δ-SG), the 17-kDa delta sarcoglycan 3 isoform (δ-SG3), α-SMA, endothelial nitric oxide synthase (eNOS), caveolin-1 (Cav-1) and Cavin-1. An amount of utrophin, β-Dg, ε-SG, α-SMA and eNOS co-fractionated with Cav-1 and cavin-1. Dot blot analysis with CT-B conjugated HRP showed the presence of the ganglioside GM1 in the fractions that contained Cav-1. Fractions 1 and 2 were excluded because no proteins were detected.

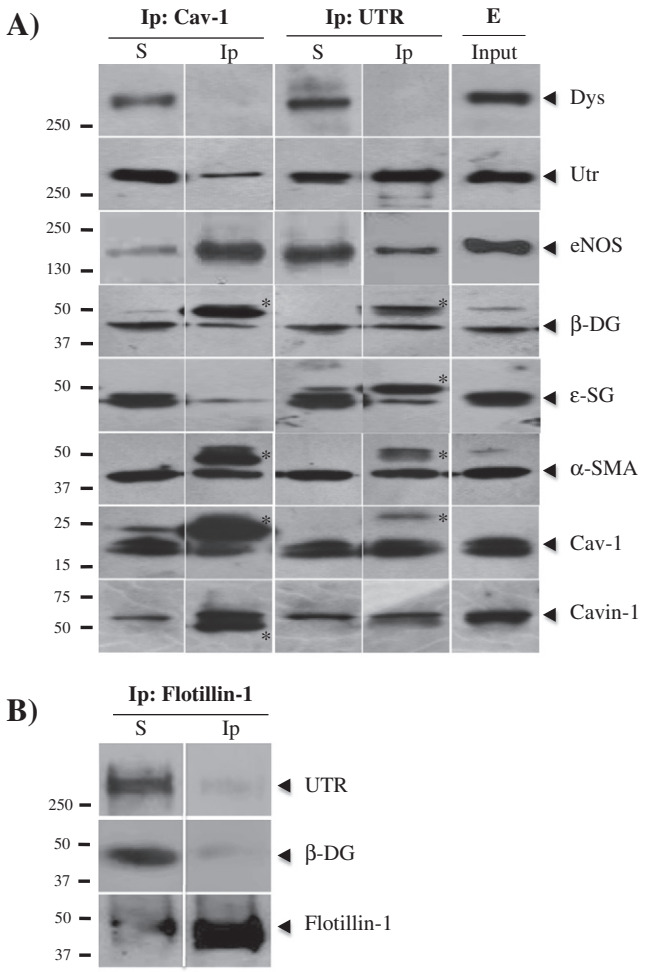

Fig. 4. UAPC proteins interact with caveolin-1 (Cav-1). Lysates of HUASMC (E) were co-immunoprecipitated reciprocally with antibodies against caveolin-1 (Cav-1) or utrophin (Utr). Cell lysate (Input), supernatant (S) and co-immunoprecipitated proteins (Ip) were analyzed by Western blot using antibodies against dystrophin (Dys), utrophin (Utr), beta-dystroglycan (β-DG), epsilon-sarcoglycan (ε-SG), α-smooth muscle actin (α-SMA), endothelial nitric oxide synthase (eNOS), caveolin-1 (Cav-1) and Cavin-1. Analysis of reciprocal co-immunoprecipitated proteins revealed the interaction of Utr, β-DG, ε-SG, α-SMA and eNOS with Cav-1 and Cavin-1. Dystrophin was observed only in the Input and supernatant. Bands indicated with (*) correspond to light and heavy chain of the antibodies used for the immunoprecipitation assays.

participates in the control of eNOS activity [50]. In order to evaluate the physiologic role of the UAPC, present in the HUASMC, on the activity of the eNOS, we knock down the α/β-DG proteins by a siRNA system. α/β-DG deficiency (≈55%, Fig. 5A) induced a reduction of the eNOS phosphorylated (eNOSpSer1177) by approximately 35% (Fig. 5B) as compared to (−) siRNA after the stimulation with bradykinin (BK) 1 μM. Besides, α/β-DG knockdown cells also presented a reduction in the NO synthesis (≈20%, Fig. 5C) under the same conditions.

NO activates soluble guanylil cyclases and the synthesis of cGMP [51], the increment of cellular cGMP activates the cGMP-dependent protein kinase (PKG) [52], which in turn phosphorylates at Ser-239 the vasodilator-stimulated phosphoprotein (VASP) as a vasodilation response [53]. In order to determine if the reduction in the synthesis of NO observed in the α/β-DG knockdown cells affected the cGMP signaling transduction pathway, we carried out immunoprecipitation assays with an antibody against the PKG-1 protein. Western blot analysis of the immunoprecipitated proteins showed a reduction of ≈39% in the interaction of this protein with VASP in cells treated with the α/β-DG siRNA (Fig. 5D). Consequently, it was also evident a decrement in the phosphorylation level of VASP (≈57%, Fig. 5E).

4. Discussion

In striated muscle, the DAPC is a multimeric complex consisting of subsarcolemmal (dystrophin and syntrophin) and transmembranal proteins (α-, β-, γ-, δ-SG and SSPN and the α- and β-DG) [54]. Utrophin, the orthologue of dystrophin [55], also anchors these proteins to form a UAPC [18,56]. Both complexes play a critical role in protecting muscle cells from damage by connecting the extracellular matrix to the actin cytoskeleton and in signal transduction pathways [57,58].

Patients with mutations in β-, δ-, and γ-SG develop muscular dystrophy that frequently is associated with DCM [59–62]. The presence of muscular dystrophy with cardiomyopathy has also been observed in SG mutant mice models [29–31]. Additional studies in β- and δ-SG null mice have shown the disturbance of the SG–SSPN complex in VSM, in addition to the presence of microvascular constrictions in arteries of the heart, diaphragm, and kidney [63]. These findings highlight the importance of the DAPC/UAPC in vascular function. Earlier studies by our group demonstrated the co-localization of some members of the UAPC/DAPC with Cav-1 in VSM [17].

In the present study we investigated the existence of a DAPC/UAPC in the caveolae domains from HUASMC. Initially, the presence of dystrophin and utrophin in HUASMC was identified by Western blot, along with the other members of the DAPC/UAPC together with Cav-1 and cavin-1. Although these results suggested the presence of DAPC or UAPC in caveolae domains of the plasma membrane, confocal immunofluorescence analysis showed that utrophin—instead of dystrophin—colocalized with the sphingolipid lipid raft marker GM1. It has been observed that caveolin-3 co-fractionates with dystrophin and certain members of the DAPC in the skeletal muscle [64,65].

Our co-immunoprecipitation results revealed the existence of a UAPC constituted by utrophin, β-DG, ε-SG, α-SMA, eNOS, Cav-1 and cavin-1 proteins. The latter two proteins are major structural components of the caveolae domains [49,66]. A previous co-localization study has shown the presence of dystrophin in the caveolae domains of nonvascular smooth muscle sarcolemma [67]. Additionally, Sharma et al. [37] revealed the presence of a DAPC or dystrophin in caveolae domains of nonvascular contractile smooth muscle cells and the direct interaction of Cav-1 with β-DG in concert with anchorage to the actin cytoskeleton. They also proposed implications of this caveolae DAPC in mediate intracellular Ca^{2+} release. In the current report we observed the presence of a UAPC in caveolae domains of VSM cells. Interestingly, this complex, conformed by utrophin, β-DG, ε-SG, α-SMA, eNOS, Cav-1 and cavin-1, does not include other major members of the UAPC/DAPC.

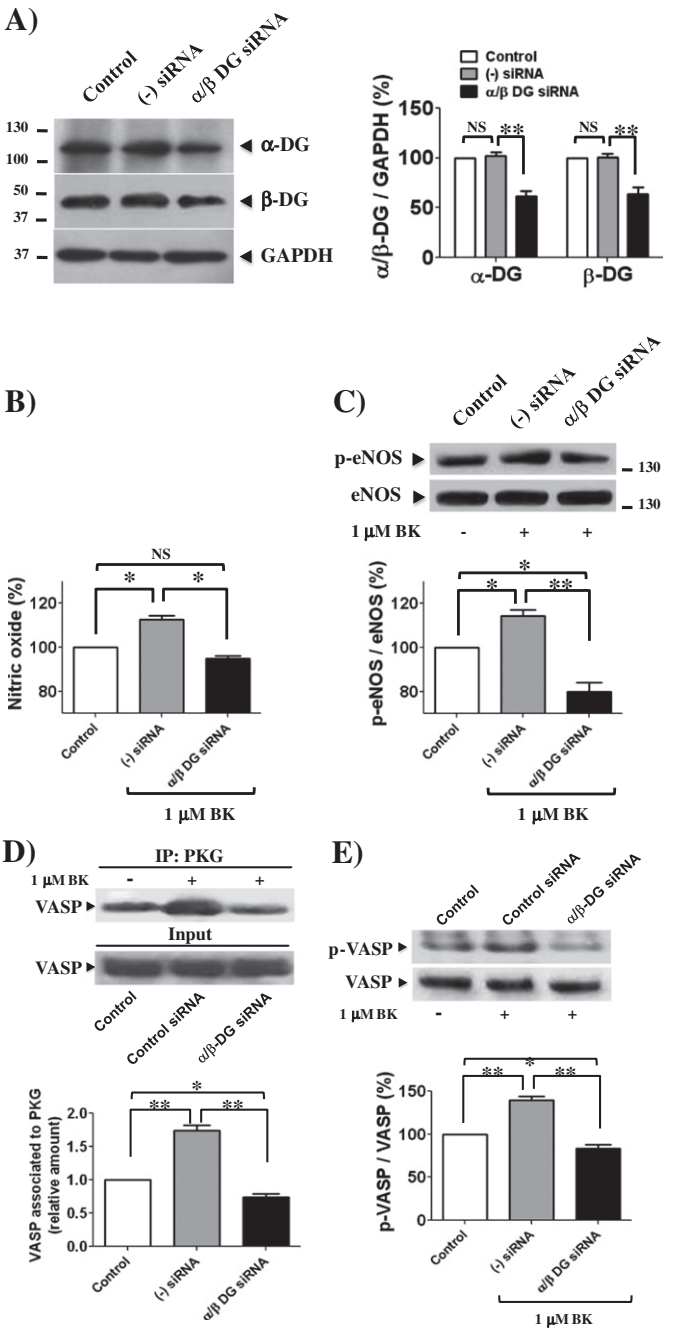

Fig. 5. Knockdown of α-DG reduce the NO synthesis, eNOS phosphorylation and the activation of PKG and VASP. A) Western blot and densitometry analysis demonstrating α/β-DG knockdown (50%) in cells transfected with a α/β-DG iRNA as compared with cells transfected with a control iRNA. B) NO amount in basal conditions (Control) or after 15 min stimulation with 1 μM bradikynin (BK) in HUASMC transfected with irrelevant siRNA or α/β-DG siRNA. C) Western blot analysis and densitometry analysis of eNOS nonphosphorylated and phosphorylated (p-eNOS) in protein extracts of cells transfected with irrelevant siRNA or α/β-DG and stimulated with 1 μM bradikynin. D) Lysates of HUASMC transfected with irrelevant siRNA or α/β-DG and stimulated with 1 μM bradikynin were immunoprecipitated with an antibody against PKG. The presence of VASP in the immunoprecipitated proteins was determined by Western blot and the intensity of the bands was analyzed by densitometry. E) Western blot and densitometry analysis of VASP nonphosphorylated and phosphorylated (p-VASP) in protein extracts of cells transfected with irrelevant siRNA or α/β-DG and stimulated with 1 μM bradikynin. Data are plotted as the average of three independent experiments standard error and analyzed with 'One Way Anova' and Tukey as a post hoc analysis. * = $P < 0.05$. ** = $P < 0.01$. NS = Not Significant.

Proteins such as β-SG, δ-SG, SSPN, as well as isoforms of dystrophin and δ-SG [42,68] (Dp71 and δ-SG3 respectively), were located in non-lipid raft domains. In addition, the research group of Dr. Crosbie-Watson

has observed that SSPN overexpression facilitates the transportation of the UAPC to the cell surface of skeletal muscle cells [69]. However, we did not detect SSPN in the UAPC located in the caveolae domains of VSM cell membrane; the protein was only evident in non-caveolae domains. It is possible to speculate that SSPN is a component of other DAPC/UAPC and that it may be important for the transport and location of these non-caveolae complexes. For instance, in normal skeletal muscle the DAPC displays a clear difference in its distribution pattern as compared with the UAPC; whereas the DAPC is found along the sarcolemma, the UAPC accumulates at the neuromuscular and myotendinous junctions [18,55,70]. Likewise, it has been reported the presence of Dp71 and members of the DAP in the nuclear envelope of C2C12 cells and a possible participation of these proteins in nuclear envelope associated functions [71]. Since our Western blot analysis of the gradient membrane fractions showed the presence of dystrophin, utrophin and Dp71 in non-lipid raft membrane domains, it is highly probable the existence of diverse DAPC/UAPC complexes with different combinations of DAP/UAP members in distinct external and internal cellular membranes of the HUASMC.

The caveolae UAPC revealed in this study was similar to the one previously determined by our group in caveolae- and non-caveolae-related domains of human umbilical endothelial cells (HUVECs) [50]. Remarkably, in both cases eNOS was a component of the UAPC. In regard to the complex of HUVECs, we previously disclosed that it may have a mechanosensory function regulating eNOS activity, which may partially control vascular function [50]. In this regard, interestingly we observed that the deficiency of α/β-DG in HUASMC induced the reduction of NO synthesis and the phosphorylated active form of eNOS after BK treatment. Likewise, this reduction was accompanied of a decrease on the cGMP signaling cascade that included the lessening of the interaction of PKG 1 with VASP, and the consequent decrease of the phosphorylated/active form of VASP (p-Ser269). All PKG family members are activated by cellular cGMP increase [52], and the active form of these proteins phosphorylate Ser-239 of the VASP [53]. Likewise, cellular cGMP increases through the activation of guanylil cyclases, a process known to occur in part through nitric oxide (NO) signaling [72]. In addition to well established roles in platelet activation and smooth muscle relaxation, PKG signaling is important in many biological processes including cardiac contractility, axon guidance, bone growth, contraction of intestinal smooth muscle and erectile dysfunction [73]. Besides, it has been demonstrated that the presence of eNOS and the NO production in VSM cells in vitro, inhibited cell proliferation which favors the maintenance of the blood vessel wall and vascular function [74,75], and that inhibition of eNOS in VSM augmented constriction of rat deep femoral artery [76]. Taking into account all these observations, we proposed that the UAPC present in the lipid raft domains of HUASMC plays an important role in the regulatory activity of the eNOS and in the function of the vascular smooth muscle.

In summary, our current results confirm the existence of a UAPC in lipid raft domains in VSM cells conformed by utrophin, β-DG, ε-SG, α-SMA, eNOS, Cav-1 and cavin-1 that may regulate the activity of eNOS which, in turn, may participate in the vascular function; whereas in non-lipid raft domains existence of several components of the DAPC/UAPC was evident, suggesting the hypothetical presence of alternative complexes in plasma membrane of HUASMC with different lipid compositions (caveolae and non-caveolae domains). Identification of DAPC/UAPC in sarcolemmal regions with different lipid compositions and caveolae and non-caveolae domains could contribute to the understanding of their role in the physiology of VSM and its possible participation in distinct cardiovascular pathologies.

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