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Expression and function of utrophin associated protein complex in stretched endothelial cells: dissociation and activation of eNOS

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1. ABSTRACT

Several studies have emphasized the relevance of dystrophin-associated protein complex (DAPC) to maintain the vascular function. Previously we postulated the presence of an utrophin associated protein complex (UAPC) in endothelium from umbilical cord vessels. In the present work, we demonstrate that utrophin (UTR) indeed forms a complex, with beta-dystroglycan (DG), epsilon-sarcoglycan (SG), caveolin-1 (cav-1), and endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells (HUVEC) by co-immunoprecipitation analysis. Additionally, we observed an increment in the protein levels of epsilon-SG, beta-DG, UTR and cav-1 after mechanical stretching. Interestingly, this stimulus also induced eNOS up-regulation, activation and release from the UAPC, and led to a significant increase in nitric oxide (NO) production. Finally, we propose that UAPC in HUVECs may play an important role in the regulation of vascular tone.

2. INTRODUCTION

Cardiomyopathy is a multifactorial disease. Recent evidence shows that hereditary and acquired forms of cardiomyopathy can be caused by alterations within the dystrophin-associated protein complex (DAPC) (1, 2, 3, 4). Dystrophin gene mutations cause Duchenne- or Becker-type muscular dystrophy and dilated cardiomyopathy (DCM) (5, 6). Additionally, it is frequent that patients with mutations in α-, β-, γ- or δ-SG, which cause limb girdle muscular dystrophy (LGMD 2C-F, respectively), also present cardiomyopathy. In the case of δ-SG, there are documented cases of mutations in the sporadic and hereditary cardiomyopathy-gene related (7).

The DAPC is a multimeric array comprising membrane and cytoskeletal proteins that link the extracellular matrix with the cytoskeleton (8). This complex is composed of dystrophin, the syntrophins, the DG, the α, β, γ, δ, ε and ζ SG, and sarcospan (SSPN) (2, 6,

8). Under certain conditions, dystrophin is substituted in the protein complex by utrophin, the autosomal homologue protein of dystrophin (4); in this case the protein complex may be denominated the utrophin-associated protein complex (UAPC). Utrophin presents an ubiquitous distribution and is expressed in striated and smooth muscles, as well as in non-muscular tissues such as nervous system and endothelial cells (4).

Null mice models for β- and δ-SG develop muscular dystrophy accompanied by dilated cardiomyopathy, and it has been observed that these mice have coronary microvasculature constrictions, apparently caused by the SG-SSPN loss in coronary vascular smooth muscle (9, 10).

Despite of the importance of the DAPC in cardiomyopathy development, the study of this complex has been mainly focused on cardiomyocytes and vascular smooth muscle. There is a modest knowledge on the expression and function of these proteins in endothelial cells, which play a fundamental role in the vascular function. In a recent study performed by our research group in human umbilical cord vessels, we postulated the presence in endothelial cells of an UAPC, constituted by ε-SG, β-DG and UTR (11). We also postulated that this complex could be localized in endothelial caveolar transduction domains because of its probable association with cav-1 (11). These results prompted us to study the putative endothelial UAPC in greater detail and to explore its physiological relevance in human umbilical vein endothelial cells (HUVECs). Due to the importance of endothelial cells in cardiovascular function, we consider of high significance to analyze the composition and possible change in response to stretching of this protein complex in this tissue type.

Our results confirmed the association of utrophin with ε-SG, β-DG and cav-1, as well as its association with eNOS.

Additionally, we observed that under the equibiaxial stretching stimulus that resembles physiologic mechanical forces, UAPC components modified their expression pattern in HUVECs. Interestingly, the mechanical stimulus also induced the activation and release of eNOS from the complex, suggesting an active role of the UAPC in endothelial mechanoreception.

3. MATERIALS AND METHODS

The study was approved by the Institutional Research and Ethics Committees of the Hospital de Pediatria, Centro Medico Nacional Siglo XXI-IMSS.

3.1. Tissues

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

3.2. Cell Culture

HUVECs were isolated from several umbilical cord segments. Endothelial cells were separated from the umbilical vein by enzymatic digestion of the extracellular matrix (12). Briefly, the umbilical vein was cannulated and perfused until turgent with 0.125% trypsin in Hank's solution (Gibco-BRL Rockville MD, USA). The tissue was incubated 15 min at 37ºC; the total volume of the trypsin solution inside the umbilical vein was recovered, and a 0.1 volumes fetal bovine serum (FBS) was added. Cells were harvested and centrifuged at 2700 rpm/15 min (1000 x g), and the pellet was resuspended in M199 supplemented with 10% FBS, 2mM L-glutamine, 1% antibiotic-antimycotic mixture (all reagents were obtained from Gibco-BRL Rockville MD, USA), 1% heparin (Sigma Chemical Co. St. Louis, MO, USA) and 5 mg/ml brain bovine endothelial mitogen (13). Endothelial cells were incubated at 37ºC and 5% CO_2, the latter characterized by von Willebrand factor expression (anti-Von Willebrand factor from Sta. Cruz Biotechnologies, Sta Cruz CA, USA), >99% of cells being positive for this endothelial marker.

3.3. Mechanical Stress on HUVECs

An equibiaxial strain device was used to stretch HUVECs as described by Lee et al. (14). Cells were first cultured onto collagen-coated silicon membranes (Dow Corning Co. SD CA, USA), previously treated with 0.01% rat tail-collagen type 1 (Sigma Chemical Co. St. Louis, MO, USA). After a 24-h stabilization period in culture, HUVECs were subjected to a mechanical stimulus of approximately 1.4 N/cm^2, which was obtained after turning the nut of the device 540º. This force represents an increase of 9% in the cellular size from basal conditions. HUVECs were stretched for 0 (basal), and 8 h.

3.4. Immunoblot Assays

To determine variations of the protein expression levels of utrophin, cav-1, β-DG, ε-SG, and eNOS in stretched HUVECs compared to control non-stretched HUVECs, 10^7 non-stretched and stretched cells, were homogenized in 50 µl RIPA buffer (1% triton X-100, 0.5% sodium deoxycholate, all in PBS 1X with mini complete protease inhibitors (Roche Diagnostics, Mannheim Germany), supplemented with 1mM PMSF, 2 mM Na_3VO_4 and 1mM NaF. Homogenates were centrifuged for 10 min at 10,000 x *g* to pellet debris. Total protein content was measured in the supernatant with the DC assay kit (Bio-Rad Laboratories, Hercules CA, USA). A total of 40-60 µg of proteins were applied to a 3-12.5% SDS-PAGE gradient, electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules CA, USA), incubated 1 h at 37ºC in blocking buffer (5% nonfat dry milk in PBS plus 0.1% Tween 20 [PBS-T]) and then incubated 3 h at room temperature with the following different primary antibodies: ε-SG (C-17), Cav-1 (C-97), Utrophin (H300), β-DG (C-20), endothelial nitric oxide synthase (eNOS) (NOS3 N -20), and eNOS $pSer^{1177}$ ($eNOS^p$ ALX-804-396). Membranes were simultaneously incubated with anti-GAPDH (V-19) as the internal control used to normalize the densitometric analysis. All the primary antibodies were purchase from Santa Cruz Biotechnologies (Sta. Cruz, CA, USA), except $eNOS^p$ ALX-804-396 which was obtained

from Alexis Biochemicals (Carlsbad CA, USA). All primary antibodies were diluted 1:100 in PBS-T. Membranes were washed three times during 5 min in PBS-T and incubated 1 h at room temperature in the presence of horseradish peroxidase-conjugated secondary antibodies diluted 1:1000 in PBS-T. Membranes were washed three times in PBS-T and then the immunoblots were developed with a diamine benzidine (DAB) colorimetric method (Bio-Rad Laboratories, Hercules CA, USA). Band intensity was directly quantified from nitrocellulose membrane using Labworks software and the EpiChemic Darkroom image analyzer (UVP Bioimaging Systems, Upland CA, USA).

3.5. Coimmunoprecipitaton Assays

10^7 stretched and non-stretched control HUVECs, respectively, were lysated with 50 µl of non-denaturing extraction buffer composed of 0.15 mM NaCl, 0.5 mM EDTA, 0.5% Triton X-100, 0.1%, 1 mM PMSF, 2 mM Na_3VO_4, 1 mM NaF (all these reagents were obtained from Sigma Chemical Co St. Louis, MO, USA.) and mini-complete protease inhibitor (Roche Diagnostics, Mannheim Germany) all diluted in PBS 1X (15). The homogenate was incubated 3 min on ice, and passed 5 times through an insulin syringe. Then, the homogenate was incubated on ice for additional 10 min, and the protein concentration was measured by the DC protein assay (Bio-Rad Laboratories, Hercules CA, USA). A total of 0.5-1 mg protein was incubated for 3 h at 4ºC, under mild orbital agitation with 3 µg of immunoprecipitating antibody (anti Cav-1 H-97 or anti utrophin H-300 Santa Cruz Biotechnologies, Sta. Cruz CA, USA). Next, 20 µl of protein G-sepharose (Sigma Chemical Co St. Louis, MO, USA.) were added and the mixture was incubated overnight at 4ºC. The immunoprecipitation reaction was centrifuged for 15 min at 12000 x g, and the supernatant was recovered and stored at 4ºC. The pellet, containing the immunoprecipitated proteins, was washed three times with extraction buffer 15 min at 12000 x g. Finally, the proteins immunoprecipitated in the pellet and those remaining in the supernatant were applied to a 3-12.5% SDS-PAGE gradient. Co-immunoprecipitation was performed at least three times with each immunoprecipitating antibody for confirmation of results. Western blots were performed according to the previously described procedure (15).

3.6. Nitric Oxide Quantification

Evaluation of the nitric oxide (NO) levels produced by HUVECs, under non-stretched and stretched conditions, was measured indirectly by the Greiss method (16, 17) using transgenic *E. coli* ATCC 1775 (ATCC Co. Rockville MD, USA) as nitrate reductase source. Briefly, HUVECs were cultured in the stretch device, changing M199 by DMEM without phenol red (Gibco BRL, Rockville MD, USA), then HUVECs in culture were stabilized overnight at 37ºC with 5% CO_2, and subsequently stretched as described previously. After the stimulus, 250 µl of cell culture medium from basal and stretched HUVECs were recovered and incubated for 1 h at 37ºC with 200 optic units of *E. coli* ATCC 1775 (ATCC Co. Rockville MD, USA) under shaking conditions at 100 rpm. The bacteria were separated from the liquid by centrifugation at 3000 rpm for 5 min, subsequently the supernatant was recovered and incubated 1 min with 250 µl of 1% sulfanilamide and 250 µl of 0.1% N-(1-naftil) ethylendiamine. We utilized HUVECs stimulated for 30 min with 6 µM bradykinin (Sigma Chemical Co St. Louis, MO, USA.), as positive control of oxide nitric synthesis. Finally, nitrites were measured in a spectrophotometer at 554 nm optical density (O.D.) interpolating with a standard curve.

4. RESULTS

4.1. Composition of the HUVEC utrophin associated proteins complex and

In a previous work, we postulated the presence, in endothelium derived from umbilical cord vessels, an utrophin associated protein complex that may be constituted by utrophin, ε-SG, Cav-1 and β-DG (11). To establish whether these proteins do in reality form a complex in HUVEC, we carried out immunoprecipitation assays with total protein extracts obtained from cultured cells (Figure 1). Total extracts (E) and the co-immunoprecipitated (Ip) proteins with anti-cav-1 or anti-utrophin antibodies were analyzed by Western blot assays using antibodies against β-DG, ε-SG, utrophin, eNOS, and cav-1 proteins. These results confirmed that there is a UAPC conformed at least by β-DG, ε-SG, utrophin, eNOS, and Cav-1 (Figure 1, panels NS, lane Ip) in HUVEC.

4.2. HUVEC-eNOS Becomes Activated Under Mechanical Stimulus

Because endothelial cells are usually subjected to hemodynamic forces, we decided to mimic the physiologic mechanical stimulus of blood vessels on HUVECs with an equibiaxial stretching device (14). This tool exerts a simultaneous equivalent stretching force in cells in all directions. Total protein extracts of non-stretched (NS) and stretched (S) HUVECs were co-immunoprecipitated with anti-cav-1 or anti-utrophin, while total extracts and precipitated proteins were analyzed by Western blot (Figure 1). By means of these studies, we observed that UAPC composition was the same between NS and S cells (Figure 1, lane Ip). Nonetheless, we observed an apparent reduction in the amount of eNOS immunoprecipitated from the cells submitted to the mechanical stimulus (Figure 1 panels S, lane Ip), as compared with eNOS detected in the immunoprecipitated fraction from NS cells (Figure 1 panels NS lane Ip). Since these experiments were conducted using an antibody that recognized both the phosphorylated and non-phosphorylated form of the protein, and with the knowledge that phosphorylation and release of eNOS from the membrane is associated with enzyme activation (18), we further analyzed eNOS employing the previously mentioned antibody and one that discriminates the phosphorylated form of the protein (Figure 2A and 2B).

Interestingly, with both antibodies we observed an eNOS increase in the supernatant fraction derived from the immunoprecipitated proteins of stretched HUVECs carried on with anti-cav-1 or anti-UTR (Figure 2A and 2B, panels S, lanes Sn). In parallel, immunoprecipitation assyas both with anti-cav-1 or anti-UTR revealed that the amount of eNOS associated to the complex is decreased after

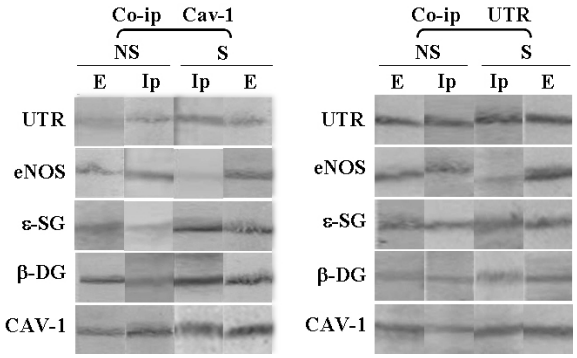

Figure 1. Composition of UAPC in stretched and control untreated HUVECs. Immunoprecipitation (Ip) and total protein extracts (E) analysis derived from non-stretched (NS) and 8-h equibiaxially stretched (S) HUVECs.. Total proteins were immunoprecipitated with anti-CAV-1 or anti-utrophin (UTR) antibodies. Total extracts (E), and co-immunoprecipitated proteins (Ip) were analyzed by Western blot using antibodies against utrophin (UTR), epsilon sarcoglycan and beta dystroglycan (ε-SG, β-DG), caveolin-1 (cav-1), or endothelial nitric oxide synthase (eNOS) that recognized the phosphorylated and no phosphorylated forms of the protein.

streatching, although this difference is more evident when immunoprecipitaing cav-1 (compare IP lanes in Figure 2A and 2B). All this information suggested that the mechanical stimulus induced eNOS release from the UAPC. Additionally, the phosphorylated form of eNOS ($eNOS^p$) was higher in the supernatant (Sn) of stretched (S) HUVECs (Figure 2A and 2B, panels S, lane Sn), as compared with the result obtained with the NS cells (Figure 2A and 2B, panels NS, lanes Sn). These results suggest that the stimulus liberates and activates the synthase. To determine whether the increase in $eNOS^p$ drives to an increment in NO production, we indirectly measured its concentration in the supernatant of the culture cells, by Greiss method before and after mechanical stimulus. An increase of up to six-fold was observed in NO production in stretched HUVECs in comparison with basal levels determined in NS cells (Figure 2C). Bradykinin-positive control of eNOS activation only increased NO levels up to three fold over basal levels (Figure 2C). Taken together these results suggested that mechanical stimulus not only liberates eNOS from utrophin complexes but also leads to its activation.

4.3. Over-Expression of HUEVEC eNOS During Mechanical Stimulus

Western blot analysis of eNOS in total extracts in Figure 2 A suggest a possible increase in the total endothelial content of this enzyme following stretching. To verify whether there was an increment in the amount of eNOS, we performed relative quantitation of the enzyme band obtained in protein immunobloting of proteins from non-stretched (NS), stretched (S), and bradykinin (BD) stimulated cells (Figure 3A). Band intensity analysis showed that the mechanical stimulus induced a two-fold increase of the enzyme, as compared with the basal level obtained with the NS and bradykinin-treated cells (Figure 3B). GAPDH was used as internal control for densitometric value normalization.

4.4. Mechanical Stress Induces Overexpression of UAPC Members

Due to the overexpression of eNOS in response to streatching, a component of the evidenced UAPC present in HUVECs, it was relevant to evaluate a possible increment in the expression of the remaining components of this complex. Expression of β-DG, utrophin, cav-1 and ε-SG proteins was analyzed by semiquantitative Western blot in non-stretched and stretched HUVECs (Figure 4A). Our results demonstrate that in contrast with the increase in eNOS, mechanical stress induces a more modest up-regulation in β-DG, utrophin, cav-1, and ε-SG to 0.39-, 0.3-, 0.8 and 0.79-fold respectively (Figure 4B). This result correlated with the ε-SG mRNA level increase (0.8 fold) exhibited by semicuantitative RT-PCR assays performed in total RNA from stretched HUVECs (unpublished data).

5. DISCUSSION

In skeletal muscle, utrophin and dystrophin serve as anchors for the DAPC , which is composed of isoforms of syntrophin, dystrobrevin, and dystroglycan, and sarcoglycan-sarcospan sub-complex (19). On the other hand, genetic defects of SGs and/or dystrophin may result in the development of cardiomyopathy and/or muscular dystrophies (20). Recently, several studies have shown the importance of the SG complex in the vascular smooth muscle function; notwithstanding this, there is a very restricted knowledge on DAPC in endothelial cells. Some reports have shown the presence of individual members of the complex such as utrophin and β-DG in endothelial cells from different sources (21, 22). In a previous study in endothelial cells from umbilical cord vessels *in situ,* we postulated the presence of an utrophin-associated protein complex conformed by β-DG, ε-SG, and cav-1 (12). To demonstrate the presence of this complex in endothelial cells, we analyzed total protein extracts from HUVECs by immunoprecipitation; this analysis confirmed the association of utrophin with β-DG, ε-SG, and cav-1. Interestingly, we also found the interaction of the unphosphorylated form of eNOS with the UAPC. To the best of our knowledge, this is the first time that an utrophin-associated protein complex in endothelial associated to caveolae has been described.

In endothelial cells, caveolin-1 is a principal protein of caveolae (1, 2, 23), and serves as an important regulator of the activity of many signaling molecules, including eNOS. Furthermore, endothelial cells are subject to physical deformations by two major types of mechanical forces: shear stress and mechanical strain (24), biomechanical stimuli that can be sense through caveolae (25). Therefore, it was interesting to determine whether the UAPC could respond to a mechanical stimulus. To evaluate this hypothesis, we cultured HUVECs under equibiaxial streching conditions utilizing a device (14) that mimics multidirectional hemodynamic stretching process in dilated vessels. Interestingly, this stimulus induced eNOS

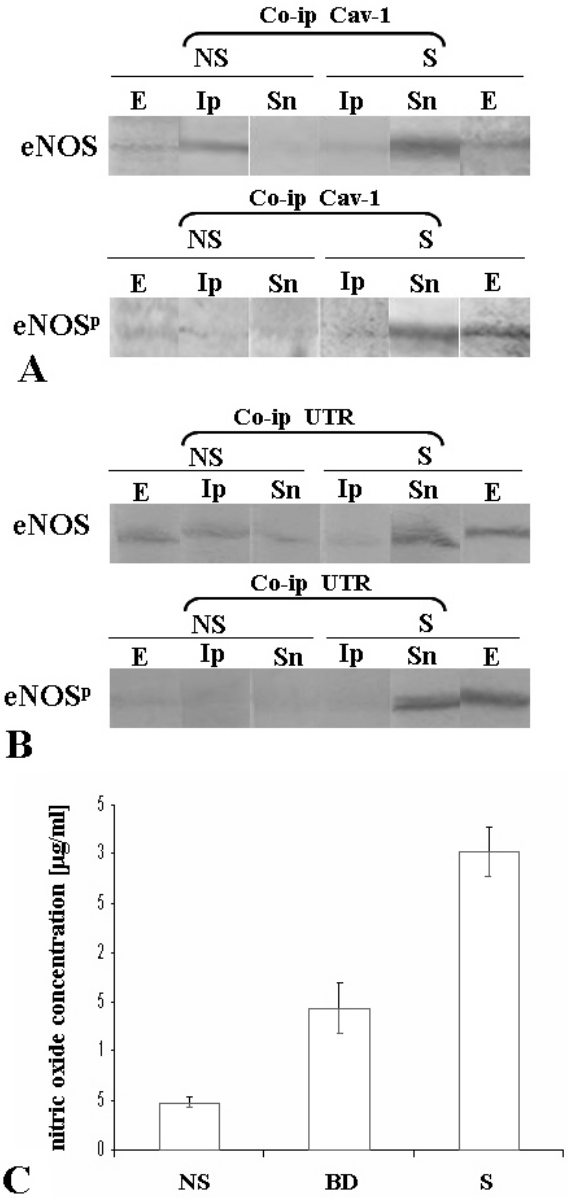

Figure 2. Release and activation of eNOS from UAPC in stretched HUVECs. Immunoprecipitation analysis of total protein extracts derived from non-stretched (NS) and 8-h equibiaxially stretched (S) HUVEC. Total extracts proteins were immunoprecipitated with anti-cav-1 (A) or anti-UTR (B) antibodies. Total extracts (E), co-immunoprecipitated proteins (Ip) and the supernatants (Sn) were analyzed by Western blot using antibodies against eNOS that discriminate between the phosphorylated ($eNOS^p$) and non-phosphorylated (eNOS) forms of the enzyme. (C) NO amount in basal conditions (NS) or after 30 min stimulation with 6 µM bradikynin (BD) or after 8 h of equibiaxiall stretched (S) HUVECs.

overexpression and activation of the enzyme, which was directly related with Ser1177 phosphorylation of and release from the UAPC. These results are in agreement with the information that eNOS is anchored and inhibited by cav-1's scaffolding domain in endothelial cells under basal conditions (26), thus regulating its enzymatic activity (27). Other reports have shown that during flow shear stress, eNOS is activated by Ser1177 phosphorylation and released from the caveolae (28). Likewise in the recent past, several reports have determined that up-regulation of eNOS gene expression after mechanical stimulus, enhanced NO production and improved endothelial function (29). In this sense and based on our results, we postulate that the UAPC might possess a function as a mechanical stimulus sensor in endothelial cells.

Additionally, we believe that NO concentration increase goes hand in hand with the increase in eNOS protein synthesis and the phosphorylation of residue Ser1177, contributing in a collaborative way to an enhanced enzymatic activity induced by the mechanical stimulus. Since in response to stretching we observe a one to one increase both in eNOS and cav-1, we interpret this as more of the protein complex, and consequently, more mechanosensor complex on the endothelial membrane which leads to a higher increase in eNOS enzymatic activity.

In addition to eNOS overexpression, the mechanical stimulus also induced the increase of β-DG, ε-SG, and cav-1 and UTR proteins. Although several reports indicate that cav-1 overexpression, markedly attenuates eNOS enzymatic activity (26, 30), in this study we observed that eNOS and cav-1 overexpression is accompanied by enhancement of eNOS enzyme activity. The increase of cav-1 in stretched HUVECs may be closer related to the increment of the caveolae density (25) and with the protein participation in the recruitment of several signaling molecules involved in cellular responses, such as the integration of focal adhesions triggered by mechanical stress (31).

In the case of β-DG and ε-SG, we suggested that their increase may be related with the participation of these proteins in a different cellular process and/or protein complex, since we had previously observed incomplete co-localization of these proteins with cav-1 in endothelial cells (11; Ramírez-Sánchez I, unpublished observations). On the other hand, the transcription-level of some genes has been reported to increase as consequence of augmented NO (32) or intracellular Ca^{2+} (33), induced by the mechanical stress. In this regard, it was also observed that the mechanical stress promoted a moderate increment in the expression level of β-, δ- and ε-SG mRNA (Ramírez-Sánchez I, unpublished observation). Nonetheless, in this context we have not been able to detect the β-, δ-SG proteins. These results confirm our previous observation that these two sarcoglycans are not present in endothelial cells (11), and suggest the existence of a post-transcriptional regulation process of their genes.

In summary, our results provide evidence for the presence of an UAP complex in HUVECs, comprising utrophin, β-DG, ε-SG, eNOS and cav-1. Furthermore, we propose that this complex may function as a mechanosensor that can contribute to the vasodilation

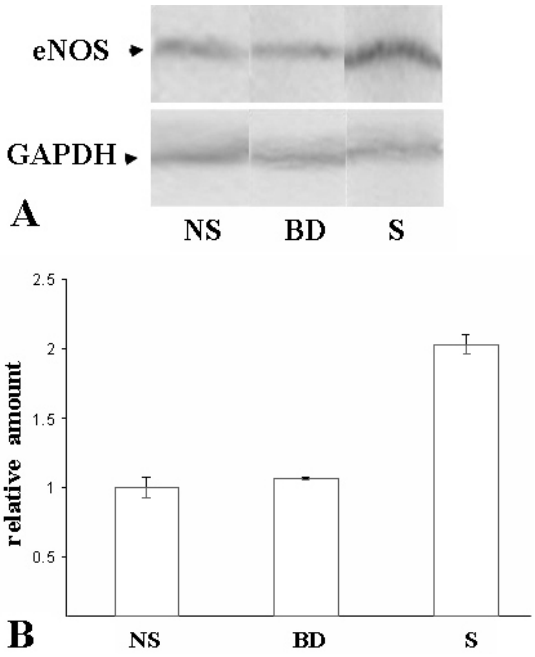

Figure 3. Mechanical stretch induces overexpression of eNOS in HUVECs. Effect of the mechanical strain on eNOS protein content in stretched HUVECs. (A) eNOS analyzed by Western blot of protein extracts obtained from HUVECs treated as follows: under basal conditions (NS); after 30 min stimulation with 6 μM bradikynin (BD), and after 8 h of equibiaxially stretched (S) HUVECs. eNOS relative protein amount was calculated by the ratio of eNOS densitometric units/GAPDH densitometric units. Densitometric analysis was performed using bands obtained in the Western blot assay (A).

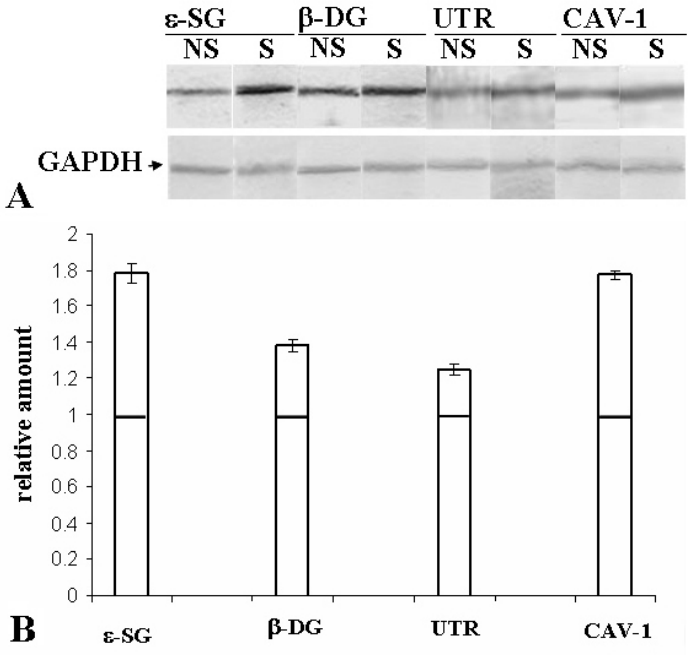

Figure 4. Equibiaxial strain induces overexpression of UAPC proteins. (A) UAPC was analyzed by Western blot using total protein cell lysates from non-stretched (NS) and 8 h equibiaxially stretched (S) HUVECs, and antibodies against ε-SG, β-DG, UTR, cav-1 and GAPDH. Immunodetection of GAPDH (35 kDa) was employed as internal control to evaluate amounts of the other proteins. (B) Effect of HUVEC equibiaxial stretching (9% form basal condition) on protein expression level of the members of UAPC. The graph shows densitometric values [(protein/GAPDH stretched cells)/(protein/GAPDH control cells)] from semi quantitative Western blot assays 1 represents basal values.

process through release and activation of the enzyme eNOS. Understanding these mechanosensors responses could address the implementation of potential genetic and pharmacologic therapeutic strategies, to prevent the development of cardiovascular diseases.

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