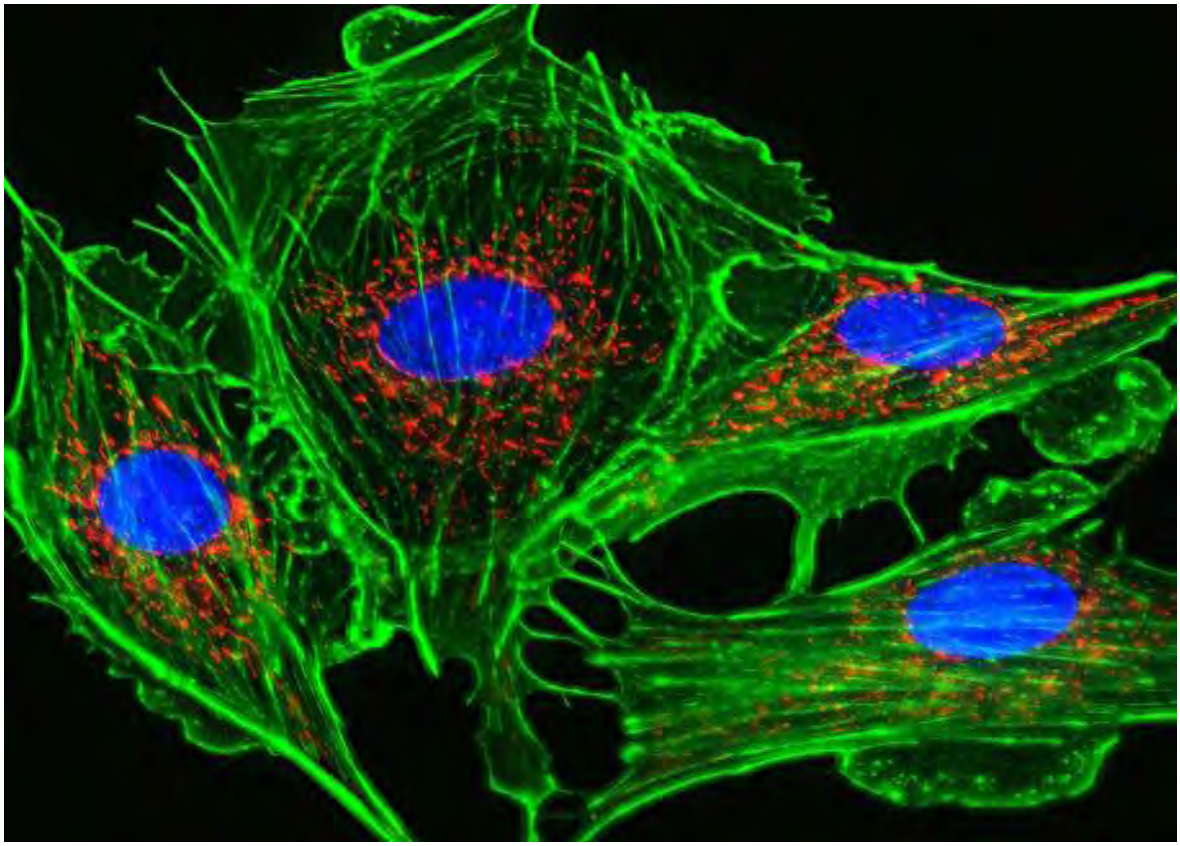
THE ROLE OF EXTRACELLULAR SUPEROXIDE DISMUTASE ON THE FUNCTION OF ENDOTHELIUM

Ο ρυθμιστικός ρόλος της εξωκυττάριας δισμουτάσης υπεροξειδίου στη λειτουργία του ενδοθηλίου



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ABSTRACT

Reactive oxygen species are responsible for the limitless neovascularization during the tumorigenesis. One of the most important enzymes that is responsible to detoxify the ROS is SOD3. SOD3 is mostly secreted in the extracellular matrix and act near the endothelium and the blood vessels. The endothelium is responsible for the connection between the tissues and tissues-vessels. The main structural protein of endothelium is VE-cadherin which is responsible for the adherent junctions of the endothelial cells and modulating the permeability of endothelial barrier. Previous studies of our laboratory focused on the role of SOD3 on the endothelium. SOD3 is related to the function of endothelium in variable manners. Among others is the potential correlation with VE-cadherin in RNA and protein levels. To address if this hypothesis is real we operated some experiments on BAEC cells that are bovine endothelial cells. Mainly we infected the cells with AdSOD3 intending the overexpression of SOD3 and with Adβ-GAL intending the overexpression of β-gal. We incubated various periods and we analyzed the levels of RNA and of protein. We also visualized the proteins of the cells intending to observe any correlation in the protein expression. Finally, according to previous findings we wanted to identify the localization of SOD3. There is a hypothesis that indicates that SOD3 is located also in the nucleus. This analysis happened by the fractionation of the infected cells, both BAEC and 1G11 (endothelial cells). Most of the results support the hypothesis that there is an overexpression of VE-cadherin at the RNA levels, there is a few overexpression at protein levels. Furthermore, results of the fractionation suggest that there is a possible localization of SOD3 in the nucleus in both cell types. Thus, our results indicate that SOD3 plays important role in endothelium affecting the RNA levels or VE-cadherin and SOD3 detoxify ROS in the nucleus.

ΠΕΡΙΛΗΨΗ

Οι ενεργές ελεύθερες ρίζες οξυγόνου είναι υπεύθυνες για την συνεχή αγγειογένεση κατά τον σχηματισμό όγκου. Ένα από τα πιο σημαντικά ενζυμικά συστήματα που είναι υπεύθυνα για την αποτοξίνωση των ελεύθερων ριζών είναι η εξωκυττάρια δισμουτάση υπεροξειδίου υπεροξειδική δισμουτάση SOD3, η οποία ανήκει στην ευρύτερη οικογένεια των υπεροξειδικών δισμουτασών. Το ένζυμο SOD3 εκκρίνεται στην εξωκυτταρική μήτρα και δρα στα ενδοθηλιακά κύτταρα και τα αγγεία. Το ενδοθήλιο είναι υπεύθυνο για την σύνδεση ανάμεσα στους ιστούς και τους ιστούς-αιμοφόρα αγγεία. Η κύρια δομική πρωτεΐνη του ενδοθηλίου είναι η VE-cadherin που είναι υπεύθυνη για την στενές συνδέσεις των ενδοθηλιακών κυττάρων και ρυθμίζει τη διαπερατότητα της ενδοθηλιακής ασπίδας. Προηγούμενες μελέτες του εργαστηρίου μας επικεντρώθηκαν στον ρόλο της SOD3 στα ενδοθηλιακά κύτταρα. Η SOD3 εμπλέκεται με διάφορους τρόπους στα ενδοθηλιακά κύτταρα. Μεταξύ άλλων είναι και μια πιθανή συσχέτιση των επιπέδων RNA και πρωτεΐνης με την VE-cadherin.Για να εξετάσουμε αν αυτή η υπόθεση είναι αληθής πραγματοποιήσαμε μια σειρά πειραμάτων με ενδοθηλιακά κύτταρα βόος (BAEC). Κυρίως μολύναμε τα κύτταρα με AdSOD3 με σκοπό την υπερέκφραση της SOD3 και με Adβ-GAL με σκοπό την

υπερέκφραση της β-gal ως κύτταρα μάρτυρες. Επωάσαμε για διαφορετικά χρονικά διαστήματα και αναλύσαμε τα επίπεδα RNA και πρωτεΐνης της VE-cadherin. Επιπλέον οπτικοποιήσαμε με χρήση φθοριζουσών χρωστικών της ενδοκυτταρικές πρωτεΐνες με σκοπό να παρατηρήσουμε κάποια πιθανή συσχέτιση σε επίπεδο πρωτεΐνης. Μια άλλη υπόθεση υποστηρίζει τον υποκυτταρικό εντοπισμό της SOD3 στον πυρήνα. Η ανάλυση αυτή έγινε εφαρμόζοντας πρωτόκολλο κλασμάτωσης και ανοσοαποτύπωση κατά Western σε δυο διαφορετικές ενδοθηλιακές σειρές(BAECκαι 1G11). Τα αποτελέσματα κυρίως υποστηρίζουν ότι η υπερέκφραση της SOD3 προκαλεί την αύξηση της VE-cadherin κυρίως σε επίπεδο RNA αλλά και σε πολύ μικρότερο ποσοστό σε επίπεδο πρωτεΐνης . Τέλος, τα αποτελέσματα της κλασμάτωσης προτείνουν ένα πιθανό υποκυτταρικό εντοπισμό του ενζύμου στον πυρήνα και στις δύο ενδοθηλιακές σειρές. Οπότε τα αποτελέσματα της παρούσης έρευνας υποδεικνύουν τον σημαντικό ρόλο της SOD3 στο ενδοθήλιο και την πιθανή δράση του ενζύμου για την αποτοξίνωση των ελεύθερων ριζών στον πυρήνα.

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INTRODUCTION

Extracellular superoxide dismutase or SOD3

Oxidative stress is a fundamental unbalance between the systematic production of reactive oxygen species (ROS) and the ability of cells to detoxify the ROS or at least to repair the damage that ROS may cause. This damage may occur in the nucleus (DNA), in the cytosol (protein, mRNA) , in the membranes (lipids) or in the organelles (mitochondria etc.). Disturbances in the balance between detoxification and production of ROS can cause toxic effects through the overproduction and accumulation of free radicals and peroxides. Some of the ROS are more effective and in contrast some of them are not so active. There are several reactive oxygen species but the most well studied are superoxide ($\bullet O^-_2$), hydrogen

hyperoxide (H_2O_2), hydroxyl radical (•OH) and peroxynitrite (ONOO-). Superoxide is the less reactive specie but interestingly can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage.

It is important for cells to retain the homeostasis of the reactive oxygen species in normal levels and avoid the cell death and other deregulations. For that reason, the cells produced some very important antioxidant systems. The systems best studied so far are the enzymes of the families of the superoxide dismutases, catalases and glutathione peroxidases. Dismutases and catalase act synergistically to detoxify the ROS. More specific, dismutases catalyze the detoxification of superoxide into hydrogen hyperoxide while catalases, which are located in peroxisomes, catalyze the decomposition of hydrogen hyperoxide to water and molecular oxygen.

1) $\mathbf{M^{n+}\text{-}SOD + O_2^- + 2H^+ \rightarrow M^{(n+1)+}\text{-}SOD + H_2O_2}$ **(catalyzed by SOD enzymes)**
2) $\mathbf{2\ H_2O_2 \rightarrow 2\ H_2O + O_2}$ **(catalyzed by calatases)**

The major cellular system is the family of SOD enzymes. There have been reported three different active isoforms of superoxide dismutases in cells. The first isoform is SOD1 or CuZnSOD mainly located and active in the cytosol. The second isoform of dismutases detoxify free radicals in the mitochondria and is called SOD2 or MnSOD. These two isoforms are well studied the last years in contrast to the third isoform SOD3 or extracellular SOD (ecSOD). Curiously SOD3 is mainly secreted in the extracellular space and is responsible for maintaining the oxidative homeostasis in extracellular matrix. [1]

SOD3 is a slightly hydrophobic glycoprotein of 135 kDa, which mainly secreted by smooth muscle cells and macrophages. Normally SOD3 exists as a tetramer (hetero - or homotetramers) or sometimes as a dimer. Tetramer formation involve the interaction between N-terminal domains of the enzymes .Also, it is possible sometimes that larger multimers of extracellular superoxide dismutase formed. Furthermore, the N-terminal is necessary for the translocation of the enzyme in the endoplasmatic reticulum, where the enzyme is glycosylated in the N-terminal site; after the glycosylation the enzyme goes to the Golgi apparatus and then secreted to the extracellular matrix. It was reported that C-terminal domain reacts with heparin and heparan-sulfate (HS) of the endothelial cells thus anchoring SOD3 to the surface. The HBD consists of positively charged amino acids (Arg, Lys) , thus binds to negatively charged proteoglycans in an electrostatic manner. In addition, the enzymatic activity of SOD3 it depends on the presence of one copper and one zinc atom per subunit. It worth to note also that SOD3 is 60 % homologue to CuZnSOD, but the homology to MnSOD is less [2] .

The gene is located in the locus 4q21 of human's chromosome and it consists of 3 exons and 2 introns (5900bp). The promoter of the gene contains various regulatory elements such as ARE (antioxidant response element), AP-1 binding site, xenobiotic response element and NF-κB motifs. The most important regulators is ARE, which is located in the 5' untranslated region of the gene. When the levels of the ROS are elevated NRF2 activates ARE that promotes the transcription of the gene. It is also important to refer that SOD3 exists in 3 different clusters: ecSOD-A, ecSOD-B and ecSOD-C. Obviously, it is possible

that they form different tetramers (heterotetrameres) with different affinity to heparins and heparan sulfates. SOD3 is mostly synthesized and secreted as type C and binds to heparin sulfate proteoglycans of endothelial cells [3]. It is reported also that proteolysis of HBD control the localization of SOD3, because some researches support that the cleaved enzyme enter easily into the vasculature through capillaries and lymph flow. Proteolysis could occur from trypsin or furin-like protease and it is important because it increase tissue half-life from 7 to 85h [4].

Findings support that a portion of the SOD3 isolated into the nucleus. This hypothesis is really important because it means that SOD3 maintains the oxidative homeostasis in the nucleus [5]. Ookawara et al, showed that SOD3 can be localized in the nucleus by immunohistochemical and western blot analysis, thus suggesting that SOD3 potentially exists in vivo in two forms, one secreted and one nuclear. Further experiments showed that the HBD in the C-terminal is primarily responsible for the nuclear translocation of SOD3 and potentially act as a NLS (nuclear localization signal). They observed that HBD deletion mutants does not localized in the nucleus supporting strongly the hypothesis. Also they observed that the secreted protein is larger than the protein that remains in the cytosol and furthermore the protein that is located to the nucleus had the same size as the secreted protein. These observations support that there is a possibility that the secreted protein is reuptaked from the cells and then is going directly to the nucleus. Other findings support also the importance of the HBD in the internalization of SOD3 in the endothelial cells nuclei and its importance for the binding of enzyme to the surface proteoglycans of the endothelial cells and also proved that the endocytosis of the enzyme mediated by clathrin-depedent pathway. The only big difference in these findings is that they did not observe localization of the enzyme to the nucleus and that the HBD does not work as a nuclear localization signal. Instead, they hypothesize that the internalized protein is degraded by lysosome-mediated proteolysis.[6]

SOD3 and vascular function

Other interesting findings support, the correlation between the protein levels of SOD3 and the bioavailability of NO (nitric oxide). NO is a very important cellular signaling molecule involved in many pathophysiological processes. Despite being a simple molecule with a short half-life of a few seconds in the blood, NO is an important biological regulator and is therefore a fundamental component in the fields of neuroscience, physiology, and immunology. Among other important functions, NO is a powerful vasodilator since the endothelium (innerlining) of blood vessels uses NO to signal to the surrounding smooth muscle, which causes its relaxation and results in vasodilation and increasing blood flow. Also, NO is a gaseous mediator of a big spectrum of pathophysiological processes in tumors, including angiogenesis. NO is produced in different tissues by different isoenzymes of the NOS (nitric oxide synthase) family. NOS has three differentially expressed and regulated isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Nitric oxide may be a crucial regulator of the normalization of vasculature of tumors, which it will be described further below. Some researchers hypothesized that if NO could be localized

selectively around blood vessels, the morphology and function of the tumor vasculature would be improved [7].

As it is obvious SOD3 is localized in the area of vascular wall through which NO must pass on its way from the endothelium to the muscle cells, so it would be possible that are correlated somehow. Indeed, some interesting studies proved that the absence of SOD3 is strongly associated with attenuated endothelium-dependent relaxation and reduced NO [8]. In other words, SOD3 controls the bioavailability of NO in the vascular wall. The mechanism might be related to the regulation of superoxide anion levels by SOD3. It is well known that high levels of superoxide anions limit the available bioactive NO, because of the formation of peroxynitrate anions. The reduction of the levels of NO leads to different serious pathophysiological damages, such as attenuated endothelium-dependent relaxation and increased hypertensive responses.

SOD3 in tumor biology

It is proposed that SOD3 contribute in different ways to the decrease the growth and limit the metastasis of invasive cancer cells. One interesting theory support that the overexpression of extracellular superoxide dismutase attenuates the heparanase expression. Heparanase is a glycouronidase that is involved in the degradation of heparan sulfate (HS). As a key component of cell surface proteoglycans and extracellular matrix (ECM), HS participates in the self-assembly, insolubility and barrier properties of the basement membrane. Cleavage of HS by heparanase releases signaling molecules, which can then be activated through binding to their corresponding receptors, thereby promoting cancer growth, angiogenesis and metastasis. Furthermore, heparanase-mediated degradation of the constituents of basement membranes and ECM promotes cancer cell invasion into the underlying stroma and metastasis to distal sites via vascular and lymphatic routes. In addition, heparanase has been shown to simulate angiogenesis by inducing vascular endothelial growth factor (VEGF). Indeed, SOD3 overexpression significantly inhibited heparanase expression and activity, and enhanced the inhibitory effects of heparin/LMWH in two aggressive and invasive breast cancer cell lines. In addition, exogenous SOD3 inhibited the invasion of breast cancer cells, thus suggesting that systemic application of SOD3 may be a promising therapeutic intervention for breast cancer treatment. Interestingly, it has also observed that the SOD3 lacking the HBD is a better inhibitor of tumor cell growth, clogenic survival and invasion [9]. This observation could means that the bioavailability of the truncated enzyme is greater than that of full enzyme. As indicated above, the HBD might be involved in the internalization, endocytosis and subsequent lysosomal degradation of the enzyme. As a conclusion, an increase in truncated SOD3 alone or in combination with heparin/LMWH, may be a therapeutically benefit for invasive tumors [9]

A number of studies support that the reactive oxygen species such as superoxide, H_2O_2 and peroxynitrite are responsible for the upregulation of angiogenetic factors, mainly of VEGF [10]. Indeed some researchers tried to prove that the ovexpression of SOD3 and a following scavenging of ROS are related to the downregulation of VEGF. The downregulation

of VEGF is followed by reduced neovascularization and furthermore reduced tumorigenesis and metastasis. Indeed, they observed that overexpression of SOD3 is resulted in reduction of oxidant-dependent activation of NF-κB, the main transcription factor that promotes the expression of angiogenic factors (VEGF).[11]The reduction of VEGF caused reduction of tumor growth and invasion in breast cancer cells. Also, it is worth to note that they proposed that increased circulating SOD3 is really promising for successful normalization of tumor vasculature, a method that it would be analyzed furthermore below.

To sum up, SOD3 is a really important enzyme that affects the normal function either of the cells or the extracellular matrix, by detoxifying the supeoxide anion and impeding the generation of other reactive oxygen and nitrogen species. More studies are needed to clearly establish the kinetics and the role of SOD3 in the extracellular space and into the cells.

The role of VE-cadherin in the endothelial barrier

Endothelial cells form the vasculature and are the major barrier between the blood and the rest of the body. These specialized cells regulate the exchange of solutes and fluids between the blood and tissue and control entry of leukocytes into the surrounding tissue. The endothelium is also the site for angiogenesis, which involves extension and remodeling of blood vessels. Essential for all these functions is the ability of endothelial cells to properly regulate cell-cell adhesions between themselves and neighboring cells. Endothelial dysfunction is often a result of altered permeability of the endothelial cell monolayer and is a hallmark of many pathological and disease states, including atherosclerosis, diabetes, hypertension, inflammation, and tumor metastasis

Furthermore endothelial cells present a special challenge for the regulation of cell-cell adhesion. On the one hand, the integrity of cell-cell adhesion within the monolayer must be maintained for proper barrier function. On the other hand, cell-cell adhesion must be sufficiently plastic to allow passage of leukocytes as well as growth and development of blood vessels. Endothelial cells utilize two types of adhesion complexes to mediate cell-cell interactions, adherens and tight junctions. [12] Adherens junctions participate in multiple functions, including establishment and maintenance of cell-cell adhesion, actin cytoskeleton remodeling, intracellular signaling, and transcriptional regulation. Tight junctions regulate monolayer permeability, and play a greater role in endothelial cells that maintain stringent barriers, such as those that constitute the blood-brain barrier. Adherens junction assembly and organization precedes formation of tight junctions. In endothelial cells, adherens and tight junctions are intermingled, whereas in epithelial cells the tight junction is localized apical to the adherens junction [13].

The transmembrane component of endothelial junctions is VE-cadherin (vascular epithelium) or cadherin5 or CD144, especially produced by endothelial cells *[14]*. VE-cadherin is a structural protein important for maturation, extension and remodeling of vessels and plays a key role in the angiogenesis and neovascularization. VE-cadherin belongs to the superfamily of structural proteins. The cadherin superfamily includes cadherins, protocadherins, desmogleins, and desmocollins, and more. In structure, they share cadherin repeats, which are the extracellular Ca^{2+}-binding domains. There are multiple classes of cadherin molecule; each designated with a prefix depends on the tissue that is secreted. The most studied protein of the superfamily of cadherins is E-cadherin (epithelial), which is really important for the structure of epithelium. At the gene level, the expression of VE-cadherin is

regulated by several transcriptional factors, such as ETS transcription factors, which are expressed early in development and are important for vasculogenesis and angiogenesis. Mostly, Erg and Ets-1 bind to the promoter of VE-cadherin and induce the expression of the gene.

Tight junctions are composed of transmembrane proteins that include claudins, occludins, and junctional adhesion molecules (JAMs). These membrane proteins associate with cytoplasmic proteins including, zonula occludens (ZO), AF-6/afadin, and PAR-3. Of the transmembrane components, claudin-5 is specifically expressed in endothelial cells .Disruption of claudin-5 causes increased monolayer permeability in tissue culture cells, but does not alter VE-cadherin localization. On the other hand, VE-cadherin regulates expression of claudin-5. VE-cadherin clustering at cell-cell contacts attenuates activity of the forkhead transcriptional repressor FoxO1, resulting in upregulation of claudin-5 mRNA. FoxO1 regulates expression of genes involved in vascular development and remodeling, and FoxO1 null embryos die due to defects in these processes. Downregulation of FoxO1 in response to VE-cadherin clustering occurs through two mechanisms: 1) activation of phosphatidylinositol-3 kinase (PI3K)-Akt signaling cascade, which induces phosphorylation and inactivation FoxO1, and 2) reduction of nuclear β-catenin levels, a binding partner and enhancer of FoxO1 activity .Thus, in addition to adhesive properties, the role of VE-cadherin is a transducer of intracellular signals critical to maintain endothelial cell function. [15]

As it is mentioned VE-cadherin is a transmembrane protein. It has an extracellular portion that engages in homophilic interactions in *cis* to form dimers. Those dimers interact in *trans* and stabilize adherens junctions between adjacent endothelial cells. The cytoplasmic tail of VE-cadherin is associated with the cell cytoskeleton via proteins of the catenin family. Both the VE-cadherin homophilic interaction and its association with catenins have considerable importance for the integrity of the endothelium barrier. Catenins are members of the armadillo repeat family of proteins such as p120-catenin, β-catenin and plakoglobin or even more binds indirectly to α-catenin via β-catenin or plakoglobin. P120-catenin and β-catenin can also shuttle in the nucleus to regulate gene expression. The complex of VE-cadherin with these proteins is crucial for the permeability of the vessels as it would be described below. It is generally accepted that phosphorylation of VE-cadherin leads to destabilization of the adherens junction complex and increased monolayer permeability, although which residues are phosphorylated is under big controversy. The phosphorylation of VE-cadherin is a result of the action of different soluble factors that mainly promote angiogenesis and increase the permeability of vessels. Most of these factors are VEGF, Tumor Necrosis Factor α (TNFα) , platelet-activating factor (PAF) , thrombin and histamine. Mostly, these factors induce the disassociation of the complex by phosphorylating either VE-cadherin or β-catenin, p120-catenin and plakoglobin increasing the vascular permeability. Additionally, other proteins such as IL-8, matrix metalloproteinases, semaphorins can change the VE-cadherin change resulting in different barrier permeability.

The phosphorylation of VE-cadherin is a crucial step

There have been described several different pathways of the phosphorylation of the complex. The most studied and strongest theory is the phosphorylation that is induced by the VEGF, which is a major regulatory pathway that modifies the structural integrity of cell-cell contacts [16].When VEGF stimulation results in Src tyrosine kinase-mediated phosphorylation of Y685 and in consequence resulting in high permeability of vessels [17]. VEGF also induces tyrosine phosphorylation of additional residues on VE-cadherin. VEGF

stimulation promoted Rac1-dependent production of reactive oxygen species, resulting in phosphorylation of VE-cadherin on Y658 and Y731. These phosphorylations results in the disorganization of cell-cell contacts and increased monolayer permeability. TNF-α stimulation also leads to tyrosine phosphorylation of Y658 and Y731 mediated by a signaling cascade initiated by PI3K p100α, including activation of proline -rich tyrosine kinase 2 (Pyk2) and Rac1/Tiam1. Y658 or Y731 phosphorylation disrupts VE-cadherin association with p120-catenin or β- catenin, respectively *[18].*

Several studies focused on the different results of the different residues of VE-cadherin that phosphorylated. Wessel et al, proved something really interesting about the phosphorylation of VE-cadherin. They believed that endothelial junctions can be opened differently for different purposes.[19] The induction of vascular permeability as well as leukocyte extravasation both require weakening of the adhesive function of VE-cadherin. However, it is evident that the passage of leukocytes requires larger gaps at endothelial junctions than does the passage of plasma proteins. Their results suggest that those different ways of opening junctions are addressed by the phosphorylation or dephosphorylation of distinct tyrosine residues of VE-cadherin. Tyr685 is induced under inflammatory conditions by VEGF, histamine resulting vascular permeability. On the other hand, Tyr731 phosphorylated under resting conditions and exclusively involved in leukocyte extravasation. Leukocyte-triggered dephosphorylation of Tyr731 required SHP-2, which enabled binding of the adaptin complex AP-2, which in turn stimulated endocytosis of VE-cadherin and as a consequence opening larger endothelial junctions for the passage of the leukocytes. Also, it worth to note that SHP-2 act in different phases, one early and one late phase. In the early phase, leukocyte emigration could induce activation of SHP-2, leading to dephosphorylation of VE-cadherin at Tyr731 and destabilization of adherens junctions, while during later phases, SHP-2 would dephosphorylate the catenins and restore barrier integrity. Further investigation is needed to confirm such a dual function for SHP-2 during leukocyte extravasation. Alternatively, other studies support that leukocyte docking to endothelial cells induce Src kinase that activate ICAM-1 which phosphorylates the VE-cadherin in other residues of tyrosine Tyr645, Tyr731 and Tyr733. [20]

Obviously, the phosphorylation of VE-cadherin can be reversed in different conditions by phosphatases resulting the closing of the gaps in the endothelial cells. Several protein tyrosine phosphatases (PTPs) associate with and dephosphorylate VE-cadherin. These PTPs include vascular endothelial receptor-type PTP (VE-PTP), whose expression is restricted to endothelial cells. One interesting theory supports, that the association of phosphorylated VE-cadherin with the vascular endothelial phosphatases is induced when Ang-1 is binds to its receptor [21, 22]. VE-PTP activity enhances VE-cadherin mediated cell-cell adhesion, which results in a decrease in endothelial barrier permeability. The balance between PTP and tyrosine kinase activities is important to regulate the level of VE-cadherin phosphorylation and thereby the degree of endothelial permeability. Interestingly, leukocytes trigger the dissociation of VE-PTP from VE-cadherin via VEGF, further supporting the idea that leukocytes induce changes in the phosphorylation state of VE-cadherin to enhance their transmigration [23]. Last but not least pathway of phosphorylation is induced by interleukin-8 (IL-8). IL-8 activates Rac1 through CXC chemokine receptor 2 (CXCR2) and activation of PI3Kγ , which unexpectedly phosphorylates a serine residue of VE-cadherin (Ser665) which results in increased barrier permeability due to internalization of VE-cadherin [24]. In conclusion to phosphorylation of VE- cadherin, it is necessary a balance between phosphatases and phosphorylases to maintain the balance in the endothelial barrier.

The permeability of endothelial barrier depends on the action of different factors

Metalloproteinases are a large family of proteinases that include MMP and ADAM . MMPs are belonging to a family of zinc-containing endopeptidases that degrade various components of the extracellular matrix and it has been reported that their overexpression is related to cancer progression, cell invasion and metastasis. MMPs promote tumor progression by rearrangement of the ECM. Indeed, they trim cell adhesion molecules and degrade matrix proteins, favoring cell proliferation and angiogenesis. MMP-7 is responsible for degradating VE-cadherin, while MMP-2 and MMP-9 are involved in occluding proteolysis, thus enhancing endothelial permeability [25]. In addition, other families of metalloproteinases: ADAM10 and ADAM17 induce permeability by mediating the cleavage of adhesion molecules within cell-cell junctions, including VE-cadherin and JAM-A. Another example of proteins that are related somehow with VE-cadherin are semaphorins. Semaphorins correspond to a family of membrane-bound and secreted proteins that can act as both attractive and repulsive guidance molecules, playing a role in endothelial plasticity. Indeed, semaphoring plays a role in endothelial migration and tumor angiogenesis, which would be analyzed further below. More specific S3A and VEGF can also cooperate to induce angiogenesis. Indeed, S3A induces Akt phosphorylation through PI3K signaling, thus enhancing vascular permeability. In this context, S3A mediates endothelial cell–cell junction destabilization and elevates endothelial permeability. On a molecular level, S3A disrupts the VE-cadherin/PP2Acomplex, allowing VE- cadherin serine phosphorylation and subsequent internalization. [26, 27]

VE-cadherin binds to different proteins in the cytosol, but the most important binding is with p120-catenin. The interaction of VE-cadherin with p120-catenin prevents the endocytosis of VE-cadherin in a clathrin-mediated pathway, thus maintaining the endothelial barrier. Also, the key tyrosine residue Y658 is located in the binding site for p120-catenin and the phosphorylation prevents the association of the complex. . On the one hand, permeability factors such as VEGF or IL-8 promote the β-arrestin-mediated internalization into clathrin-coated vesicles of VE-cadherin, inducing the phosphorylation of Ser665 and association of β-arrestin to VE-cadherin. On the other hand, antipermeability factors such as Fibroblast growth factor (FGF) is proposed to be responsible for increasing the association of VE-cadherin and p120-catenin. Moreover, Angiopoietin-1 (Ang1), a proangiogenic factor known to promote stabilization of the vasculature, also prevents VEGF-induced phosphorylation of S665, when it binds to its receptor Tie2 which located near the VEGF-R2 and internalization of VE-cadherin through the inhibition of Src activity [28].Thus, Ang1 represents another signaling mechanism to counteract the increase in endothelial permeability by VEGF. Another one anti-angiogenic molecule is S1P (phosphorylated sphingosine), which binds to its receptor S1P1R which located near the VE-cadherin. When S1P1R is activated inhibits VEGF-induced VE-cadherin destabilization and internalization, and thereby enhances cell-cell adhesion [29, 30]. Thus, changes in the balance between these pathways may determine permeability properties of the endothelial monolayer.

Additionally, it is described the important role of VE-cadherin in the cell signaling inducing a lot of different signaling pathways. Signaling via VE-cadherin influences endothelial cell behavior by modulating activity of growth factor receptors, intracellular messengers, and proteins that regulate gene transcription. VE-cadherin associates with two receptors of growth factors, either VEGF-R2 or TGF-β receptor. When , VE-cadherin binds indirectly to the VEGF-R2 induce the reduction of the MAPK activation and proliferation signaling , but parallel binds to TGF-β receptor inducing the phosphorylation of Smad

transcriptional factors and following anti-proliferative , anti-migration signals, but both result in stabilization of the vasculature. As a conclusion, VE-cadherin plays a key role as a balancing molecule, keeping the proliferation levels of vasculature in balanced levels. Finally, it is worth to note the correlation between the VE-cadherin and the cytoskeleton. Some studies proved that VE-cadherin signals via RhoC to activate ROCK and myosin light-chain 2 (MLC-2) phosphorylation, thus promoting the actomyosin contractility. In contrast, some other studies suggest that VE-cadherin activates Rac1/Tiam1 instead of RhoC , furthermore inhibit RhoC. It is possible that the different activation of different GTPases depends on the upstreaming signal [30].

The role of VE-cadherin in the neovascularization

Endothelial cells, pericytes, smooth muscle cells and the basal membrane collectively form the blood vascular wall, which ensures selective exchanges between plasma and tissues. The passage of macromolecules, fluids and cells through the endothelial barrier can occur either through (transcellular) or between the cells (paracellular). Endothelium is at the origin of neovessel formation through the extension of existing vasculature. For that reason, vessels are existing in two completely different states: 1) quiescent vessels and 2) angiogenic vessels [31]. It is widely accepted that the VE-cadherin is different in each state. In the quiescent vessel, VE-cadherin is normally located in the membrane of endothelial cells maintaining the junctions between the cells. Contrariwise, VE-cadherin in angiogenic vessels is differing a lot than the quiescent cells. As it is analyzed above, VE-cadherin is making a lot of different complexes with proteins and enzymes. On an angiogenic point of view, the most important complex is formed between VE-cadherin and Src-kinase. When VEGF binds to its receptor VEGF-R2 induce the activation of the receptor which is located near the complex. Furthermore, Src kinase is activated by phosphorylation of the receptor and phosphorylates the Y658 residue of VE-cadherin. So this key step of phosphorylation is important for endothelial cells to switch from quiescent phenotype to angiogenic phenotype [31]. In this angiogenic phenotype, VE-cadherin disappears from the adherens junctions or disassociate with the other VE-cadherins of the neighbor endothelial cells, resulting increased permeability. Other interesting theories support that VE-cadherin develops heterophilic adhesion reactions fibrin, thus inducing cell migration [32]. Finally, the presence of VEGF has other results related to the phosphorylated VE-cadherin, for example cell proliferation is stimulated by ERK/MAPK pathway activation, and membrane protrusions are induced through Cdc42 activation. β-Catenin may potentially translocate to the nucleus and subsequently activate cyclin D1 and myc, which induce cell cycle entry.

It is widely accepted the fact that VE-cadherin plays important roles in angiogenesis. One of the most studied disease is cancer and tumorigenesis which is the limitless proliferation of cells, that they are immortal. Except the unlimited proliferation it is described also an unlimited neovascularization. In fact because of the rapid growth of tumors, tumors need oxygen and nutrients in high level. Weirdly, tumor vessels are structurally abnormal resulting absolutely opposite conditions. More specific, tumor vessels are leaky, heterogeneous, tortuous and serpentine-like. Also, they branch irregular with abnormal stunts resulting in heterogeneous flow and poor drug delivery. In addition, it had been observed structural abnormalities in the endothelial cells causing the leakiness of the vessels. In details, abnormal endothelial cells lose their polarity, can detach from the basement membrane and stack upon each other or even more in other types of tumors endothelial cells die, establishing gateways for aggressive metastatic cancer cells. Vessels

with abnormal endothelial cells could characterized as leaky and have wide junctions and also contain multiple fenestrations and other trans-endothelial channels , resulting in hemorrhage and limited perfusion These high abnormalities in the vascular wall are responsible for characteristic tumor conditions such as hypoxia and acidity [33]

There have been described several causes for limitless angiogenesis. The most important are the conditions that are dominating in the tumors. More specific, abnormal and limitless angiogenesis is induced by hypoxia, acidity, hypoxia-reoxygenation cycles, accumulation of ROS and high glycolytic metabolism. These conditions also are responsible for tumor progression and metastasis. It is described that these conditions cause angiogenesis mostly via the upregulation of VEGF. As it is analyzed above, VEGF is responsible for the opening of the endothelial cells causing angiogenesis while the phosphorylation of VE-cadherin is induced. To conclude with the vessel abnormalization in tumors, is needed to highlighted the fact of a vicious circle. In this vicious circle, tumor cells instead of uptaking huge amounts of nutrients and oxygen , form a totally abnormal vasculature which function poorly causing hypoxia and acidity , which in turn these conditions stimuli a limitless neovascularization.[33]

The abnormal angiogenesis is widely studied and a lot of different groups tried to address the right way to solve this problem. For this problem there have been proposed two different strategies to address the solution. On the one hand, it is suggested the destruction of abnormal vessels. More specific it is suggested the destruction on purpose to starve the primary tumors from oxygen and induce tumor shrinkage. However, this way it was not completely successful. On the other hand, because of the partial failure of the destruction of abnormal vessels it was suggested a potential normalization of tumor vessels. The normalization presupposes that the tumor vessels should be normalized to reduce metastatic dissemination from oxygen – enriched tumors and to improve the response to conventional anticancer therapies while inducing the delivery of anticancer drugs. The theory of vascular normalization it was supported from some researchers or not accepted of other groups [34]. VEGF is playing a key role in angiogenesis as it is described many times, therefore VEGF is the key factor that is the first target of blockage. VEGF is responsible for the endothelial cell growth, migration and lumen formation and survival. A lot of different studies tried to inhibit the VEGF in the level or genes (silencing) or in the level of protein (inhibitors) [35]. These methods resulted in the recruitment of pericytes and normalizing of basement membrane. Pericytes are stellate-shaped cells that engulf the endothelium and provide mechanical stability to fragile endothelial cell channels, furthermore have a role of safeguard of endothelial cells. Also, these contractile cells regulate the microvascular flow and permeability. Furthermore, normalization it is succeeded by the increase of VEGF-PIGF instead of VEGF-VEGF or by VEGF blockade which results the upregulation of Angioprotein-1 which results in endothelial cells tightening and pericyte recruitment [36, 37] .Interestingly, the blockade of VEGF could have different results in different tumors. Either it could reduce oedema, increase perfusion, oxygenation and drug deliver or it could destroy vessels reducing oxygenation and drug deliver. For these reasons, it is needed more studies to address the right approach to reduce the limitless angiogenesis and normalize the tumor vasculature. Also, logically the normalization it expected sometimes to feed properly the cancer cells resulting rapid tumor growth, instead of diminishing the growth. Thus it is necessary to avoid feeding the tumor but only to reduce the tumor growth by normalizing the vasculature.

In addition, it is suggested as a normalization factor the PHD2 (propyl hydroxylase domain-containing protein 2). PHD2 is a detector of oxygen levels in vessels, which hydroxylates the hypoxia-inducible factors (HIFs) when sufficient oxygen is available: once hydroxylated, HIFs are targeted for proteosomal degradation.HIF1α and HIF2α differ from each other, not only by their downstream target specificity but also by their regulation by

oxygen levels. Specifically, HIF1α only becomes activated when oxygen levels are substantially reduced, while HIF2α can be activated by less severe hypoxia.PHD2 becomes inactive and HIFs initiate a range of response to increase the oxygen supply, partly via angiogenesis. The haplodeficiency of PHD2 leads to the upregulation of VE-cadherin, which tightens the endothelial cells and increase the levels of soluble FLT, which is a receptor of VEGF that trap the VEGF [38] The haplodeficiency also results in mature pericytes, lower vessel density and decrease of the metastasis and invasion. Thus, it seems that it works well as normalization factor, while reducing leakage, tortuosity and remodeling whereas endothelial cells quiescence, barrier tightening and vessel maturation are increased.

Previous work on our laboratory focused on the role of SOD3 in the tumor vasculature. Mainly, focused on the mechanism of action of statins, a group of compounds with anti-inflammatory and anti-oxidant activities with clear cardiovascular benefits. It was described the immunomodulatory effect of lovastatin in a DTH model of immune response [39]. Statins are inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A reductase, the key enzyme that regulates cholesterol synthesis .In the cholesterol synthesis pathway certain intermediate compounds are synthesized and they are the responsible of the pleiotropic effects produced by statins that are independent of the cholesterol synthesis. [40] Between the beneficial effects of statins it has been described the improvement of endothelial function, stability of atherosclerotic plaques, decrease of oxidative stress and inflammation, and inhibition of thrombogenic response. Concerning endothelial function, it is also known that lovastatin improves vascularoxidative stress, normalizes endothelial NO synthase and CuZn superoxide dismutase expression, showing antioxidant properties.

To conclude with VE-cadherin is key molecule in the process of angiogenesis and neovascularization. Also, is responsible for keeping the endothelial cells together and maintain the endothelial barrier tight. In the molecular level, it was described that VE-cadherin interacts with a lot of different proteins either receptors or enzymes. Furthermore, it is implicated in the paracellular migration of cells from the endothelial barrier to the vessels or the opposite direction. This opening is caused from different soluble factors favoring the transmigration of lymphocytes and other cell types such invasive cancer cell, affecting metastasis. Thus, it is necessary to clarify completely the complicated role of this structure molecule and how is related with different conditions in the organism.

AIM OF THE PROJECT

The main goal of the research is the understanding of the role of SOD3 on the endothelium and how is potentially related with the normalization of vessels in tumors. Endothelial cells are producing SOD3, but its expression is reduced in the tumor-associated endothelium. Our first hypothesis indicates a possible upregulation of VE-cadherin caused by the presence of SOD3. In order to address this hypothesis, previously our laboratory worked *in vitro* on 1G11 cell line, which is a typical endothelial cell line. Unpublished results that they obtained supported the hypothesis mostly in the levels of mRNA. However, at the protein levels the evidence was not strong, thus suggesting a potential correlation mainly at RNA levels. More studies are necessary to further support the hypothesis strongly.

According to those previous findings, the first aim of this project is to investigate whether the SOD3-induced upregulation of VE-cadherin observed in 1G11 cells is a general phenomenon occurring in endothelial cells of different species. To address this hypothesis in this study we worked with a different cell line; Bovine Aortic Endothelial Cells (BAEC). Mainly, we focused on the RNA and protein levels of VE-cadherin in cells that overexpressed SOD3. Also, based on previous findings on the subcellular localization, we tried to prove that SOD3 also located in the nucleus, although the enzyme is secreted to the extracellular

matrix. Therefore the main objective of this study is to understood further the role of SOD3 in the extracellular matrix and how is related with the endothelial barrier, especially with VE-cadherin and whether acts in the nucleus.

Materials and methods

Cell culture

For cell culture have been used the BAEC cell line, which are Bovine Aortic Endothelial Cells to approach the function of endothelial cells. First, before the incubation we prepared the plates (p60) overlaying them with gelatin 0,1 % (stock 1% gelatin). After two hours of incubation with gelatin at 37 ^{o} C, we washed the plates with PBS (Phosphate Buffered Phosphate). In this stage the plates are totally covered by gelatin. After that step we seed the cells, in a appropriate number, normally we seeded almost 500.000 cells per plate. Then we fill the plate with 4 ml of BAEC medium. BAEC medium consisted of 1gr/L low glucose (1%) Dulbecco-modified Eagle medium (DMEM) with 10% fetal bovine serum, 10 µg/mL penicillin, and 10 µg/mL streptomycin. The next step is to put the plates in the incubator with controlled conditions of temperature, CO_2 and O_2. When the cells are completely attached in the gelatin we can infect the cells.

Cell infections

If the cells are totally attached on the gelatin and are completely confluent, then we can infect the cells with Adenoviruses. We used two different types of Adenoviruses : 1) AdSOD3 MOI-100 (100 infective particles per cell) which can induce the overexpression of the SOD3 in the cells and 2) Ad-βgal MOI-100 which is responsible for the overexpression of β-gal in the cells using as a control of the infection. In the first place the concentration of AdSOD3 was 65,6 10^6 PFU/µl and the concentration of Adβgal was 218 10^6 PFU/µl . Then we incubated the infected cells at different times, depending on our experiment. In our experiments we incubated for 3-, 5- and 10 days.

qPCR analysis

a) RNA isolation. We extracted the RNA according to a specific protocol based on the TRI-reagent® protocol which is provided by Sigma-Aldrich. First , we lysated cells directly on the culture dish, using 1 ml of the TRI Reagent per 10 cm2 of glass culture plate surface area. After addition of the reagent, the cell lysate should be passed several times through a pipette to form a homogenous lysate. To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature. Then we added 0.1 ml of 1-bromo-3-chloropropane or 0.2 ml of chloroform per ml of TRI Reagent used and we covered the sample tightly, shaked vigorously for 15 seconds, and allowed to stand for 2–15 minutes at room temperature. After, we centrifuged the resulting mixture at 12,000 × g

for 15 minutes at 2–8 °C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). The next step was the transfer of the aqueous phase to a fresh tube and add 0.5 ml of 2-propanol per ml of TRI Reagent®. Then we allowed the sample to stand for 5–10 minutes at room temperature and centrifuged at 12,000 × g for 10 minutes at 2–8 °C. The RNA precipitate will form a pellet on the side and bottom of the tube. As a final step we removed the supernatant and washed the RNA pellet by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI Reagent. At the end we used vortex for the samples and then centrifuged at 7,500 × g for 5 minutes at 2–8 °C. Briefly dry the RNA pellet for 5–10 minutes by air-drying or under a vacuum.

b) Reverse transcription. First, in the spectrometer we calculated the concentration of RNA, because we wanted to know how many μg of RNA we will use for the reverse transcription. Then we prepared one mixture consisting of 2 μg of RNA diluted in RNAase free water and the mixture for the reverse transcription. This mixture is composed of Reverse Transcription Buffer (stock 10x), dNTP Mix (stock 25x), Random Primers (stock 10x) , Multiscribe RT. 50U/μl) , RNAase inhibidors (0,02U/μl) and RNAase free water. The conditions for the creation of cDNA were 10 minutes at 25 ^{o}C, 2 hours at 37 ^{o} C and finally for 5 minutes at 85 ^{o} C.

c) qPCR. The creation of cDNA is followed by the dilution of the mixture to 1/7 (triplicates) and 1/70 on purpose to proceed with quantitive PCR to evaluate the levels of mRNA of different genes. In the plate of quantitive PCR we use for every sample 3 wells of the dilution 1/7 and 1 well of 1/70. In each well we put 3 μl of the diluted cDNA and 5 μl of a mixture for the amplification of different genes. This mixture consist of 5x HOT FIREPol ®EvaGreen ® qPCR Mix Plus (ROX) which provided by Solis BioDyne , water and primers for every gene that we wanted to amplify . Concerning to the qPCR instrument we adjust the steps in : step 1 at 50 ^{o}C for 2 minutes , 1 cycle at 95 ^{o}C for 10 minutes and finally 40 cycles at 95 ^{o} C for 15 seconds and at 60 ^{o}C for 1 minute. We used the primers that demonstrated below (Table1).

NAME OF PRIMER	SEQUENCE
VE-CADHERIN FORWARD	5'-GAAGCCTCTGATTGGCTCAG-3'
VE-CADHERIN REVERSE	5'-GAAGAACTGGCCCTTGTCAC-3'
SOD3 FORWARD	5'-GGGGAGGCAACTCAGAGG-3'
SOD3 REVERSE	5'-CCAACATGGCTGAGGTTCTC-3'
β-ACTIN FORWARD	5'-CACAGGCCTCTCGCCTTC-3'
β-ACTIN REVERSE	5'-TATCATCATCCATGGCGAA-3'

Table 1. The sequences of the primers. In every experiment that we used the technique of qPCR , we used the primers that are showed above. For every gene that was amplificated, we prepared a mix of forward and reverse primers.

Western blot analysis

a) Protein extraction : Cultured BAEC cells were homogenized in lysis buffer containing 200μl of RIPA buffer which is composed of 150-mM NaCl2-mM EDTA, 1.5-mM MgCl2, 1% Triton X-100, 0.5-mM phenylmethylsulfonyl fluoride(PMSF) and also we added proteinases inhibitors 0.1-mM Na_3VO4, 10-Ag/ml leupeptin, and 10-mg/ml aprotinin. Then we quantified

the concentration of the protein extraction in every sample based on the Micro BCA ® method.

b) Conditioned medium. In the end of the incubation of every cell culture we collected the conditioned medium. In the conditioned medium is located the secreted SOD3, thus easily with a western blot analysis of all the conditioned medium we could detect the SOD3 and understand if the infection was succeeded or not.

c) Electrophoresis: The samples usually composed of the protein extraction or conditioned medium plus the loading buffer (6,25 µl) Samples were fractionated through 8% SDS–PAGE when we wanted to detect the VE-cadherin in the protein extractions and through 12% SDS-PAGE when we wanted to detect the SOD3 in the conditioned medium or in the protein extractions. If we wanted to detect both VE-cadherin and SOD3 we prepared a gel of 10 %. The gel that was prepared consisted of water, Tris buffer 1,5 M , 30 % Acrilamide/Bis Solution , APS and TEMED. Then the gel was running in 20-30 mA for about 2 hours. When the running of the proteins in the gel was finished we transferred the protein to a nitrocellulose membrane 0,45 µm (provided by BIORAD) for western blot analysis for about 90 minutes at 250 mA.

d) Hybridization with antibodies. We use 5% milk diluted in TBS-Tween® 1% to block the non-specific binding of the antibodies to the membranes for 1 hour. The primary antibodies were anti-pancadherin (produced in mouse) or anti-SOD3 (produced in rabbits) , depends on what we wanted to detect. After that we washed 3 times with a mixture of TBS-Tween 0,1% and finally we incubate the membrane overnight at 4 ^{o} C with the primary antibody diluted in milk. The next day we wash again with TBS-Tween and incubate for one hour with the secondary antibody diluted in milk. The secondary antibodies was polyclonal goat anti-rabbit Igs (1/2.000) to detect SOD3 or polyclonal goat anti-mouse Igs (1/1000) in order to detect VE-cadherin . The next step was the incubation with ECL to get signal. Final step was to develop the film (provided by KODAK, AGFA) to visualize the specific signal in the dark room.

Immunofluorescence analysis.

Because we wanted to ensure the overexpression of VE-cadherin in the protein levels we visualized the protein levels of SOD3 and VE-cadherin by immunofluorescence. First, we seeded 40.000 cells in each chamber in 200 µl of medium. When the cells are attached we aspirated the medium and we fixed the cells with 4% PFA (200 µl) for 10 min at RT. Then we added 0.25% TritonX-100 in PBS (200 µl) for 5 min at RT to permeabilize the cells. After that, we added 400 µl of PBS-staining (PBS-staining: 1%FCS; 0.5 % BSA; 0,065% AZIDA en PBS 1X) and incubated for 1 h at RT. The next step was to incubate the cells with the primary antibody: Anti-VE cadherin (LSBio ®; rabbit 1:400, stock 1mg/ml) , Monoclonal anti-Pan cadherin (mouse ascites fluid clone CH-19, mouse IgG1 isotype SIGMA ALDRICH ®; 1:500) and anti g/ml, Santa Cruz ®, M-106, sc-67089) SOD-3 (1:50) (rabbit, stock 200). The primary antibodies are diluted in 200µl of PBS-staining for 1 hour in RT. After washing the primary antibody we Incubate the cells with the secondary antibody (goat anti-rabbit Cy3 1:400 for SOD3 and VE-cadherin) or (goat anti-mouse Cy3 1:400for pan-cadherin) in PBS-staining (200 µl) for 1 h at RT. Finally, we mounted with Vectashield with DAPI and we analyzed the results using confocal microscopy.

Subcellular fractionation of BAEC and 1G11 cells

First, we seeded the cells as we did in every experiment and we infected them with same viruses. After 3 days of the infection we reinfected the cells until the 5 days of incubation. At the end of 5 days we kept the plates at -20 °C until the fractionation of the cells begin. The first step was the preparation of the buffers (Table 2).

Cell type	Adherent tissue culture cells
Cell amount	3 - 5 x 10^6 cells
Wash Buffer	2 x 2 ml
Extraction Buffer I	1 ml
Extraction Buffer II	1 ml
Extraction Buffer III	0.5 ml
Extraction Buffer IV	0.5 ml
Protease Inhibitor Cocktail	5 µl per fraction
Benzonase	1.5 µl

Table 2. At the table above are showed the appropriate amount of the buffers per plate that we used to carry out the fraction according to the protocol.

First, we thawed the buffers before starting the extraction. Once thawed, we kept Extraction Buffers I, II, and III on ice. Then we washed the cells by carefully overlaying the cell monolayer with 2 ml ice cold Wash Buffer at 4°C for 5 min. After we mixed 1 ml ice-cold Extraction Buffer I with 5 µl Protease Inhibitor Cocktail and immediately added the mixture into the plates without disturbing the monolayer. Then we incubated for 10 min at 4°C with gentle agitation. At the end of ten minutes we transferred the supernatant (fraction 1) to a clean tube. Then we mixed 1 ml ice-cold Extraction buffer II with 5 µl Protease Inhibitor and we incubated for 30 min at 4°C with gentle agitation. Then we transferred the supernatant (fraction 2) to a clean tube without disturbing the cell monolayer. We continued by mixing 500 µl ice-cold Extraction buffer III with 5 µl Protease Inhibitor Cocktail and 1.5 µl (≥ 375 U) Benzonase® nuclease and we incubated with gentle agitation for 10 min at 4°C and transfered the supernatant (fraction 3) to a clean tube .Finally, we mixed 500 µl room temperature extraction Buffer IV with 5 µl Protease Inhibitor Cocktail. Thus, with this experiment we had 4 different fractions of the cells lines. The first fraction is the membrane, the second fraction is the cytosol, the third fraction is the nucleus and the final fraction is the cytoskeleton. All the details about the protocol of the fractionation are in the following reference [41]

RESULTS

Overexpression of SOD3 increase the RNA levels of VE-cadherin in BAEC cell line

As it is described above, we used BAEC cell line to carry out the *in vitro* experiments. More specific, as a first step we seeded almost 500.000 cells per plate until the cells attached to the gelatin and get over 80% confluent. The next step was the infection of the cells. For every experiment we prepared plates in 3 different conditions. The first condition was the uninfected cells to have a control of the natural function of the cells. The second condition was the BAEC cells infected by Adβ-gal (MOI 100) as a control of the infection of the cells as an irrelevant virus. The last condition is prepared by the infection of the cell lines by AdSOD3 (MOI 100), which will overexpress the extracellular superoxide dismutase. According to our hypothesis we expecting the overexpression of SOD3 is followed by the overexpression of VE-cadherin. The incubation with the Adenoviruses differs in the time and the conditions. BAEC were incubated for 3 days, 5days and 10 days. Also, in some conditions the cells after 3 days were re-infected with the same amount of virus, on purpose to identify the best conditions that the upregulation of SOD3 is responsible of the overexpression of VE-cadherin. In the next paragraphs will be analyzed the results of different days of infection separately.

A) Overexpression of SOD3 increase the RNA levels of VE-cadherin in BAEC cell line:3 days postinfection

In the first place, we wanted to address the appropriate days of infection that are sufficient for the overexpression of VE-cadherin. Thus, the first step was the experiments of 3 days of infection. We repeated those experiments 3 times in the same conditions. In the Figure 1 are demonstrated the results of the quantitive PCR. Actually, we took from qPCR ct values and after we calculated the RQ values that are represented in the graphics below. In the figure 1 a) are the RQ values of SOD3 after 3 days of infection. In this case we can conclude that the infection of the BAEC cells was successful, because the amount of SOD3 in AdSOD3–infected cells is about 5 to 6-fold higher in the control cells (uninfected, Adβ-gal). Because the infection was successful, then we wanted to quantify the RNA levels of VE-cadherin.

In the graphic in the figure 1 b) are represented the RQ values of VE-cadherin. It is worth to note that the cells that were infected by Adβ-gal were the control (RQ=1). Based on the control, the uninfected cells has a value almost RQ=1,7, whereas the RQ value of VE-cadherin in AdSOD3-infected cells is almost RQ=4,2. In other words, there is an increase at the RNA levels of VE-cadherin almost 4-fold, in the cells that overexpress SOD-3. Referring to the 3 days infection, we can conclude that there is an upregulation of RNA levels of VE-cadherin, while there is also an upregulation of SOD-3.In conclusion; it seems that 3 days of SOD3 overexpression are enough to observe upregulation of VE-cadherin in BAEC cells.

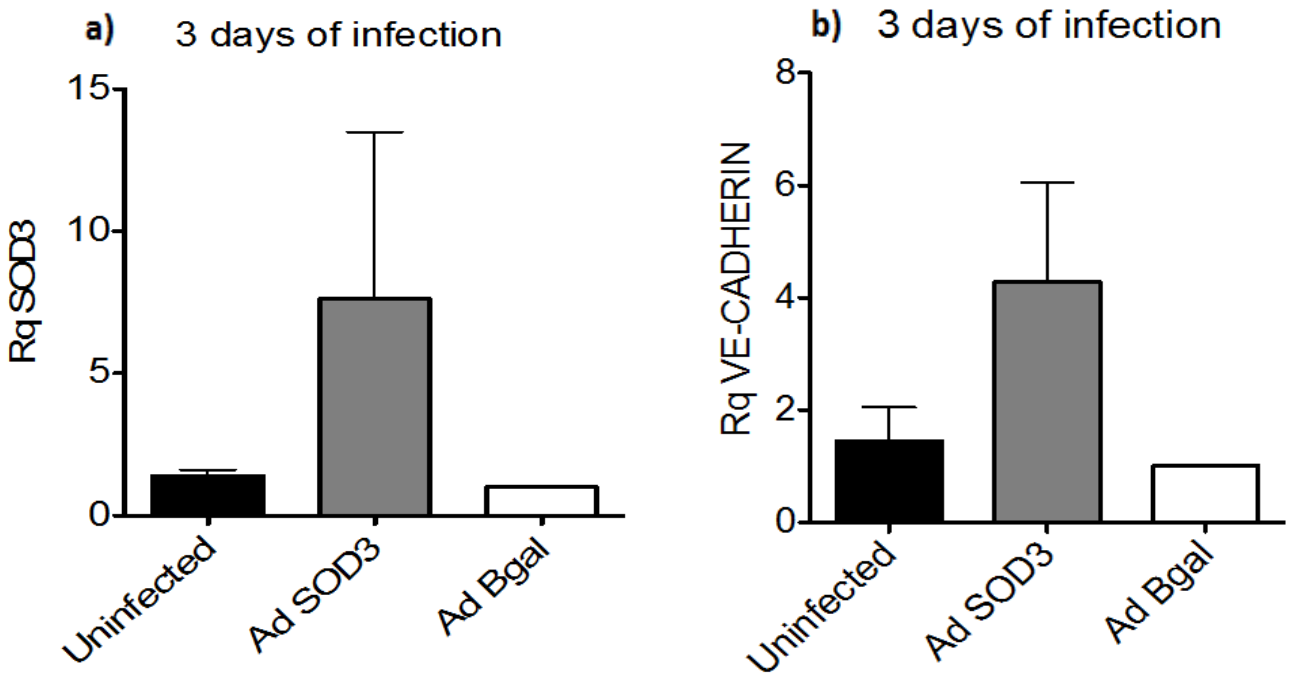


Figure1. Overexpression of SOD3 increase the RNA levels of VE-cadherin in BAEC cell line after 3 days of infection Analysis of the RNA levels of SOD3 and VE-cadherin in uninfected cells, AdSOD-3-and Adβ-gal infected cells. a) are represented the RQ values for the RNA levels of SOD3. b) are represented the RQ values for the RNA levels of VE-cadherin. Results expressed as mean±SEM (n=3/condition)

B) Overexpression of SOD3 increase the RNA levels of VE-cadherin in BAEC cell line:5 days postinfection

The results of the 3 days of infection suggest a potential correlation between the overexpression of SOD3 and the upregulation of VE-cadherin. For this reason, we wanted to operate more experiments to ensure the results. The next step of the experimental process was the infection of the BAEC cells for 5 days instead of 3 days. Our goal was to investigate if the infection for 2 days more was sufficient for a bigger increase in the RNA levels of VE-cadherin. We operated the experiments of 5 days of infection twice and our results are on Figure 2.

We collected the conditioned medium where the secreted SOD3 is located, on purpose to test if the infection was successful .In the Figure 2 *a)* we show the results of the western blot analysis. According to those results we can say that the infection of BAEC cells by AdSOD-3 was successful. SOD-3, based on the size of the protein (30kDa), was detected in the ADSOD3-infected cells (3rd, lane; a black arrow showing this band) and the positive control (1st lane).In addition, upper in the gel are some bands that refer to unspecific signal of the antibody. Unfortunately, the amplification of SOD3 in qPCR does not work properly and we did not have any ct values to demonstrate.

The results of RQ of VE-cadherin are shown in the Figure 2 *b).* We used also as control cells, the cells that was infected by Adβ-GAL. In this case we can observe that the overexpression of SOD3 is enough to increase the RNA levels of VE-cadherin. Indeed, according to the graphic we can say that the increase at RNA levels of VE-cadherin is a bit more than 5-fold compared with Adβ-gal cells, and 2.5-fold in comparison to the uninfected cells. Thus, we can conclude that 5 days of infection are enough to increase the RNA levels of VE-cadherin 5-fold than the Adβ-gal cells. In addition, in comparison to the cells that were infected for 3 days we can say that there is a small increase at the RNA levels of VE-cadherin induced by the overexpression of SOD3.

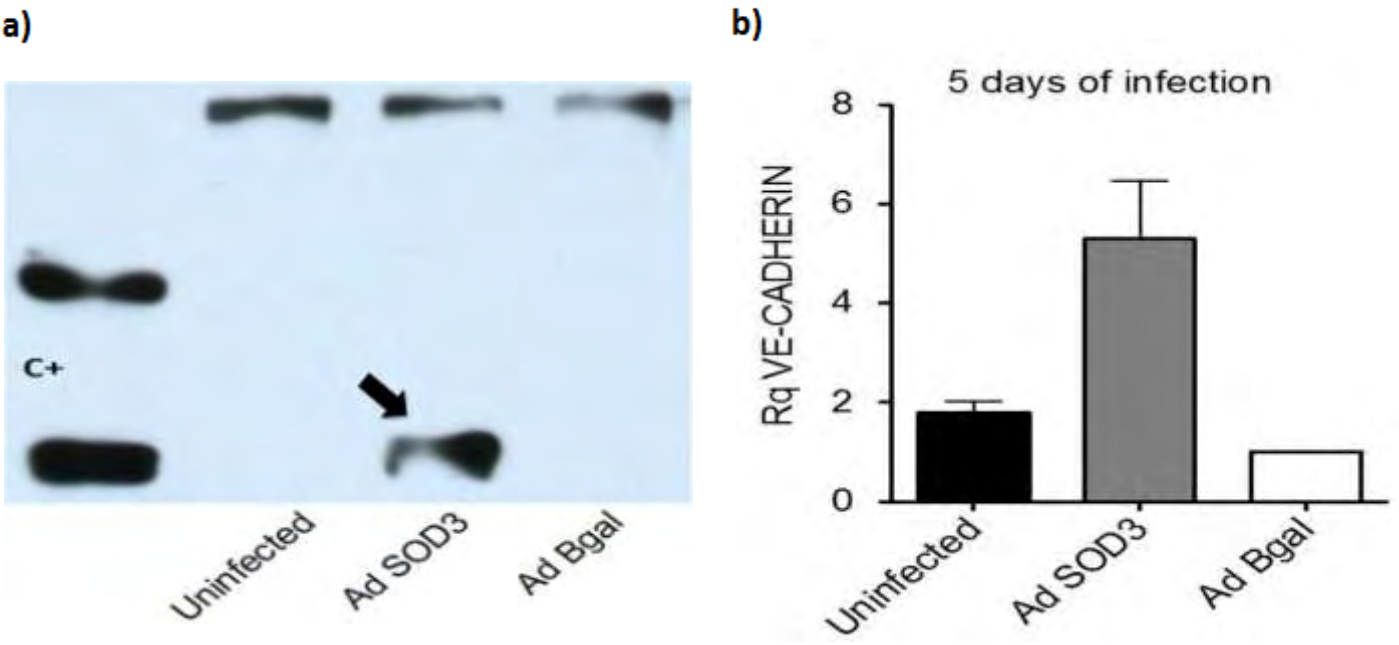


Figure 2 .Overexpression of SOD-3 increases the RNA levels of VE-cadherin after 5 days of infection. a) Western blot analysis of the conditioned medium that ensure our analysis that the infection was successful (band of SOD3 with a black arrow at 30 kDa). *b)* RQ values of VE-cadherin in the cells after 5days of infection. Results expressed as mean±SEM (n=2/condition).

C) Overexpression of SOD3 increase the RNA levels of VE-cadherin in BAEC cell line:10 days postinfection

Based on the results from the previous experiments, we decided to operate a final couple of experiments concerning the analysis of the RNA levels. The cell lines were the same (uninfected, Adβ-gal and AdSOD-3). However, the infection in those experiments lasted 10 days because we intended to see if the overexpression of SOD3 for extended periods of time enhances the expression of VE-cadherin.. After the incubation we collected the conditioned medium where is located the secreted SOD-3. Then, we operated a western blot analysis in case of to detect the protein levels of SOD-3. Thus, in the Figure 3 *a)* we show the results of the western blot analysis. Based, on those results we can say that the infection of BAEC cells by AdSOD-3 was successful and according to the size of the band the amount of SOD3 is higher than the other periods of infection. In the figure, is the positive control of western blot analysis and a big protein (30kDa). Moreover, upper in the gel are some bands that refer to unspecific signal of the antibody. Also, it worth to note that by the western blot is detected the monomer of the protein and not the polymers that form (135kDa).

Based on the success of the infection and overexpression of SOD-3 we analyzed VE-cadherin RNA in the samples. In the figure 3*b)* is represented the graph of the RQ values of VE-cadherin in the cells. The RQ value of uninfected cells is almost 1,9 and the RQ value of the cells that were infected by Adβ-gal is RQ=1. As we expected, the RQ value of VE-cadherin in the cells that overexpress SOD-3 is almost RQ=4,2 i.e. 4-fold higher in comparison to the Adβ-gal cells. Thus, also in this case we have an increase in the RNA levels of VE-cadherin in the cells that overexpress SOD-3 after 10 days of infection. Interestingly, the increase of RNA levels is not as high as we expected after 10 days of infection but is similar to the increase that was observed in the 3 days of infection and almost the same increase in the 5 days infection of the cells. These results suggest that there is a constant increase of RNA levels of VE-cadherin from the cells that overexpress SOD-3. Although the big increase of SOD3 levels, the RNA levels of VE-cadherin are increasing until one specific amount of RNA.

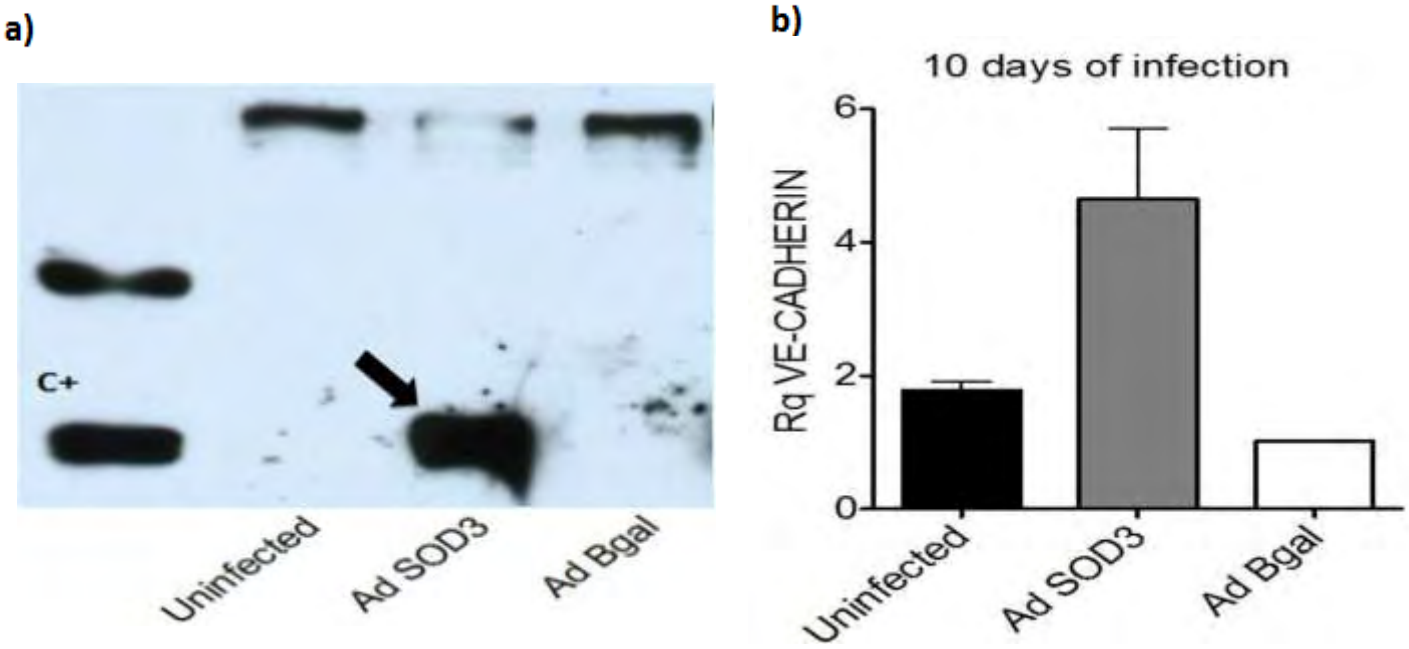


Figure 3.Overexpression of SOD-3 increases the RNA levels of VE-cadherin after 10 days of infection. a) western blot analysis of the conditioned medium that ensure our analysis that the infection was successful (band of SOD3 with a black arrow at 30 kDa).b) RQ values of VE-cadherin in the cells after 10 days of infection. Results expressed as mean±SEM (n=2/condition)

Overexpression of SOD3 in BAEC cell lines does not affect the protein levels of VE-cadherin

After the RNA analysis we wanted to quantify the protein levels of VE-cadherin in cells that overexpress SOD3 (Figure 4). As it is described in materials and method, first we extracted the protein from the cells and then we prepared the western blot analysis. In these two experiments we infected the BAEC with a different amount of virus (MOI-50). The results of protein analysis of both experiments are shown in the Figure 4. We observed that the infection of the cells was successful in both experiments. Although, the protein levels of VE-cadherin are not elevated as we expected. We used tubulin to normalize the amount of protein in cells and furthermore we used the ratio VE-cad/tubulin to quantify the levels of VE-cadherin in the cells. In the second experiment we could observe a slight VE-cadherin upregulation in the cells that were infected with MOI-50. After 3 days of infection the increase of VE-cadherin mRNA due to SOD3 overexpression do not translate in a clear upregulation of VE-cadherin at protein level, maybe because of the different sensitivity of the techniques that we used or by a mechanism that modulates and keeps stable the protein levels of VE-cadherin endothelial cells.

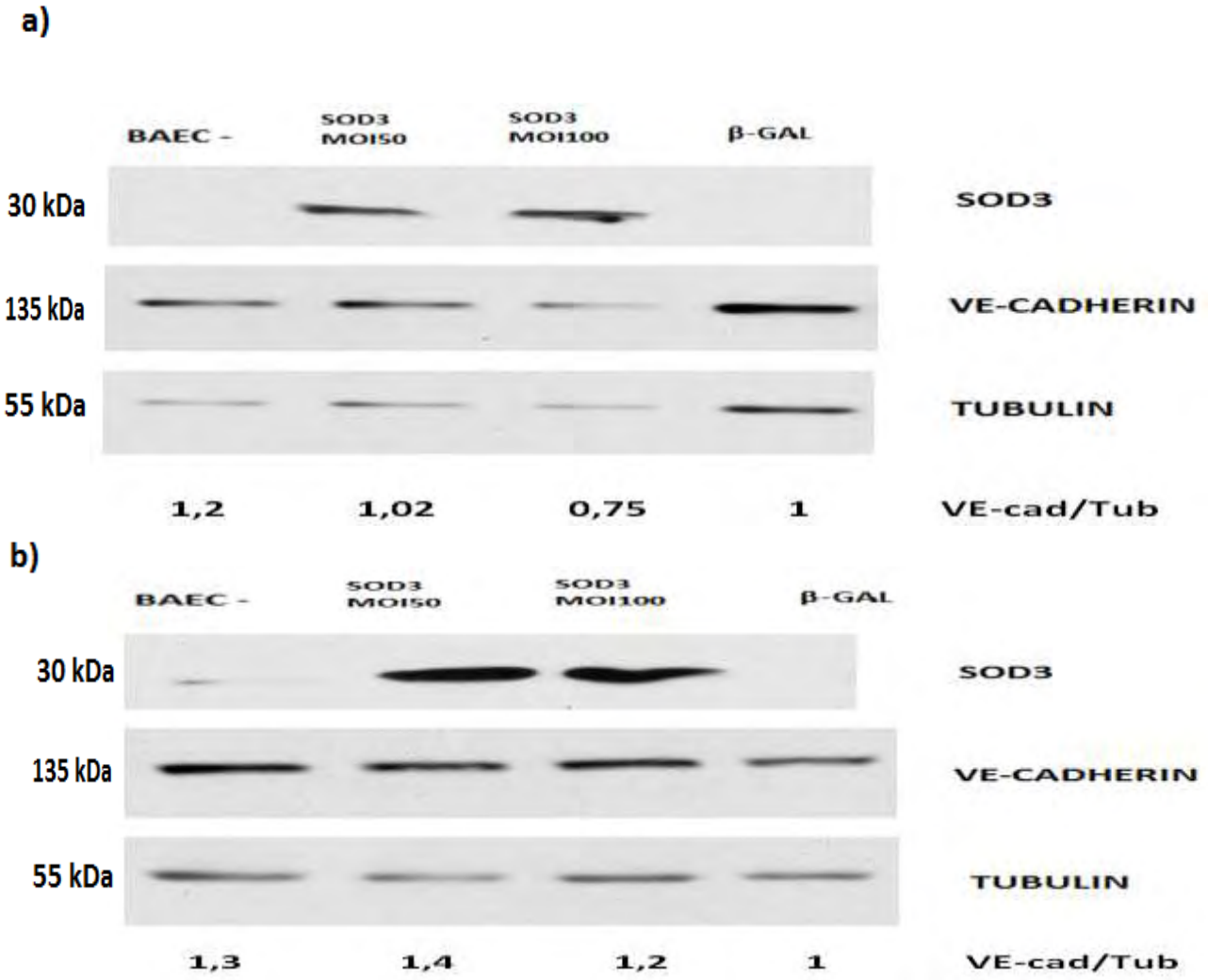


Figure 4. Protein levels of VE-cadherin in BAEC cells infected with Ad-SOD3. Both the figures *a)* and *b)* refer to 3 days postinfection and incubation. It is shown the western blots for SOD-3 (30kDa), VE-cadherin (135kDa) and α-tubulin (normalizing the protein levels in 55kDa). The ratio of VE-cadherin/α-tubulin is shown at the bottom. a) and *b)*represents independent experiments.(n=2)

SOD3 overexpression enhances VE-cadherin staining in BAEC cells

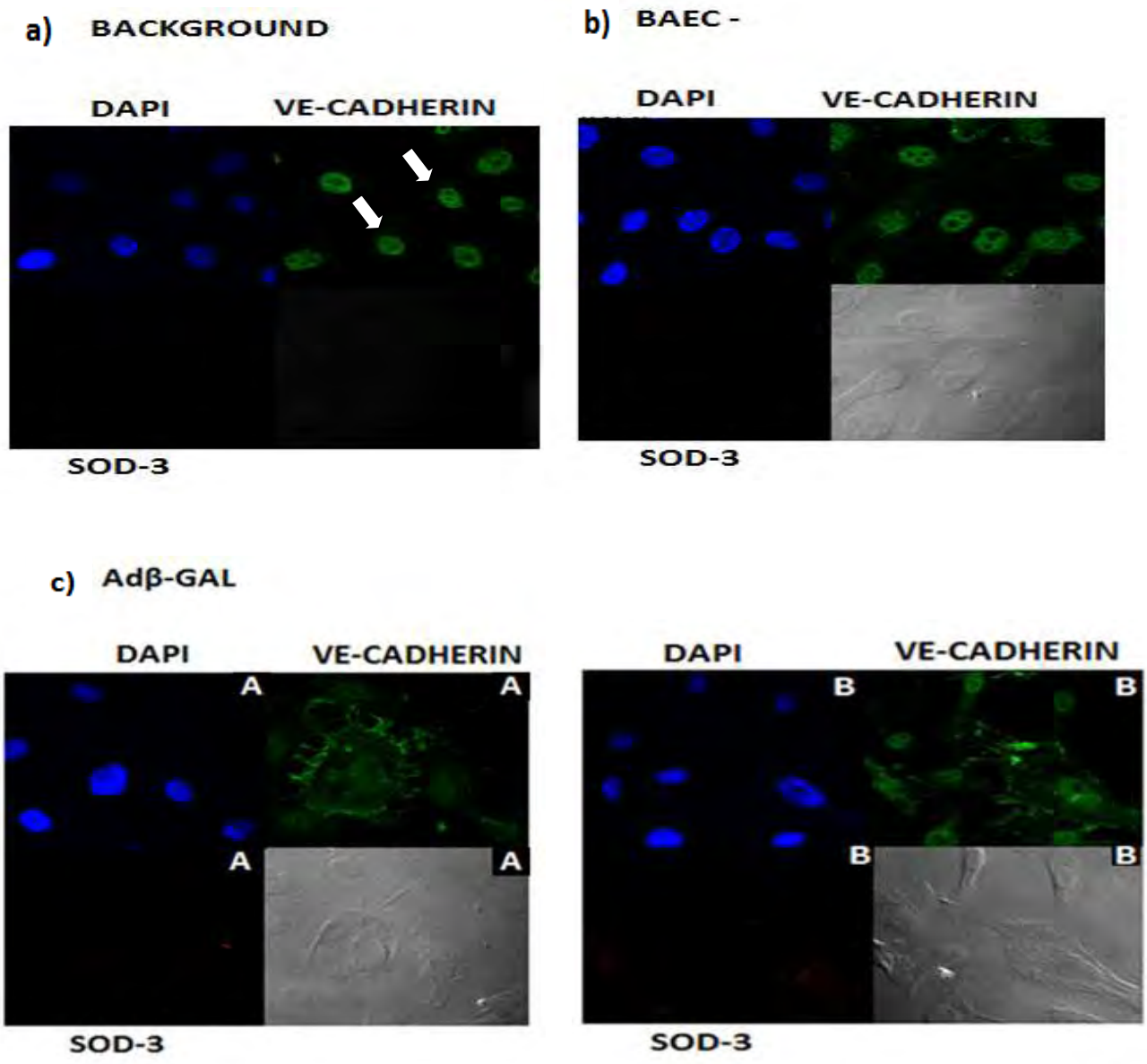
The next step of this investigation was an immunofluorescence analysis in order to analyze whether SOD3 increases VE-cadherin levels in the cells. In this analysis we hoped to visualize at the same cells both the VE-cadherin and SOD-3 at the same time. The immunofluorescence analysis of the cells was happened in specific plates-chambers. To these chambers were incubated BAEC cells (40.000 cells per chamber) and infected as it was described above. In these experiments we had the 3 same conditions of infection plus one condition of overexpression of SOD3 where we only added the secondary antibody to detect the background from the unspecific bindings. The incubation of the cells was lasted 3 days, 5 days and 10 days. Detection of both SOD3 (in red) and VE-cadherin (in green) was done simultaneously; DAPI staining(blue) indicates cell's nuclei (Figure 5).The staining of the proteins in 5 and 10 days was not worked successfully and for that reason the only results that are shown are those after the 3-days incubation (Figure 5).

In the Figure 5*a)* is shown the background of the unspecific binding of the antibodies. We can observe a lot of background staining in the nuclei (white arrows) using

the FITC-coupled secondary antibody, whereas the red staining was without background. In the Figure 5*b)* is shown the staining of the uninfected cells. In this figure, we can observe that there is no red staining because of the low levels of SOD-3 in the cells. Also, we can observe a small amount of VE-cadherin that is located in the membrane in addition to the nuclear background of the secondary antibody.

In the figure 5*c)* are the cells that are infected with Adβ-gal and in the figure 5*d)* are the cells that are infected AdSOD-3. The infection with Ad-SOD3 was successful because of the red staining of SOD-3 at cells that are infected with AdSOD3. Also we found that the green staining, corresponding to VE-cadherin, was brighter in ad-SOD3-infected cells than in those infected with Adβ-gal. This observation may suggest that overexpression of SOD3 causes enhancing of VE-cadherin staining in BAEC cells. In addition, it is worth to note that this technique is not quantitive.

In conclusion, we can support that the overexpression of SOD-3 may enhance the RNA levels of VE-cadherin, which translate in slightly higher levels of the protein according to immunofluorescence analyses.



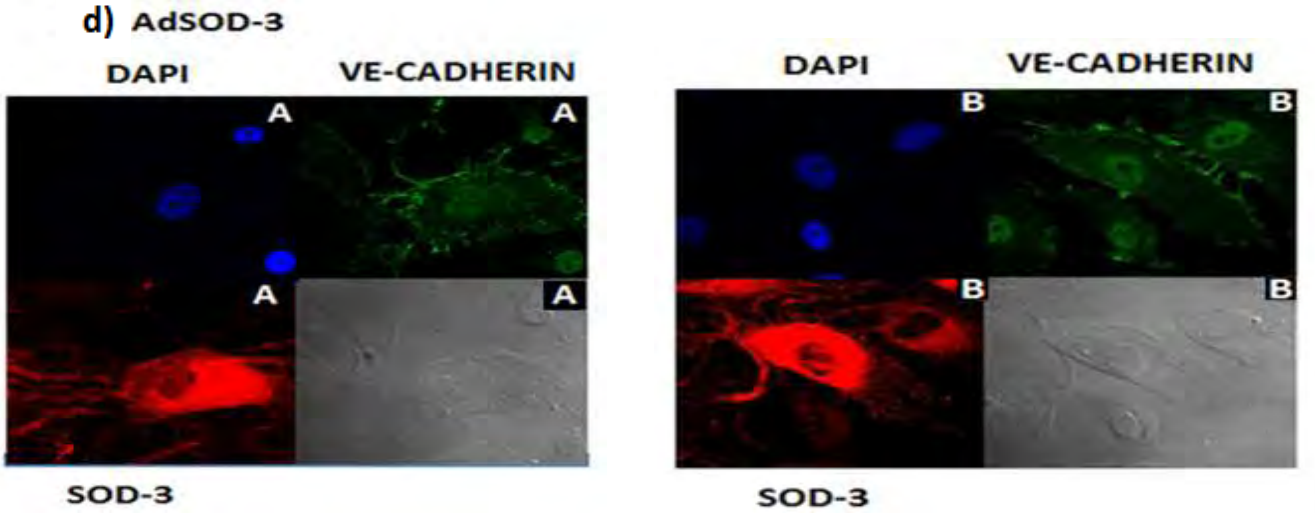


Figure 5 .VE-cadherin expression analysis of BAEC cells that overexpress SOD3 by Immunofluorescence. *a)*Background of the secondary antibodies used for staining. b*i)*uninfected cells stained for VE-cadherin, SOD-3 and DAPI; nuclear background in the nuclei was also observed.*c) A, B*are representative fields of Adβ-gal-infected BAEC cells stained for VE-cadherin, SOD3 and DAPI; some nuclear background of the green-labeled secondary antibody can be observed.*d) A,B*representative fields of the staining of AdSOD-3-infected BAEC cells for the indicated markers. For c) and d) BAEC cells were analyzed after 3 days of infection(n=6 images/condition)

Fractionation of BAEC and 1G11 cells proves the nuclear localization of SOD-3

As mentioned above, an interesting hypothesis supported by different groups is that SOD3 is not only secreted to the extracellular space but also is located in the nucleus. Furthermore unpublished studies on 1G11 cells in our laboratory suggest that SOD3 is located in the nucleus using the method of subcellular fractionation. Using this approach we obtained four different fractions from the Ad-SOD3-infected BAEC cells. In the first fraction (F1) are detected the proteins that are located mostly in the cytosol; in the second fraction(F2) are the proteins located in the membrane; the third fraction (F3)contains proteins located in the nuclei, and the fourth fraction (F4)contains the insoluble cytoskeleton, including structural proteins.

At the first experiments we used 1G11 endothelial cells overexpressing SOD3 or GFP as control. The results of the western blot analysis with the different subcellular fractions are shown in the Figure 6. SOD3 was detected in the F3 (black arrows at 30 kDa), co-partitioning with the nuclear marker HNRP (Heterogeneous nuclear ribonucleoprotein), and in the Fraction 4, which refers to the cytoskeleton. It is noticeable that there was a small contamination of the nuclear marker in the fraction 4, which might be explained for the presence of cytoskeletal proteins in the nucleus. However, no SOD3 was detected in the cytosol (F1), identified by the marker GADPH (Glyceraldehyde 3-phosphate dehydrogenase).

Next, we performed a similar approach using Adβ-GAL- or AdSOD3-infected BAEC cells. The results of the western blot analysis of the fractions are shown in the Figure 7. In these cells, we found minor amounts of SOD3 in the membrane fraction (F2) and the cytoskeleton (F4), but the majority of the SOD3 in the nucleus (F3). The nuclear maker used here, histone H4, was located in F3 and F4; this is quite logical because the histones interact in the nucleus with proteins of the cytoskeleton. When we analyzed VE-cadherin in this fractionation, we found a strong signal in the fraction 2, which refers to the membrane

proteins, where VE-cadherin is expected to be located, and in F4 because VE-cadherin interacts tightly with several proteins of the cytoskeleton. Curiously, VE-cadherin bands appeared also in the nuclear fraction. This phenomenon may explained because of a possible localization of VE-cadherin in the nucleus during the division of the cells. Some contamination of cytosolic proteins was detected in the F2 of Adβ-GAL-infected cells.

. In summary, both experiments that operated with different endothelial cells have shown that a fraction of SOD3 partitioned in the nucleus, which is in accordance to previous findings of other laboratories [5]

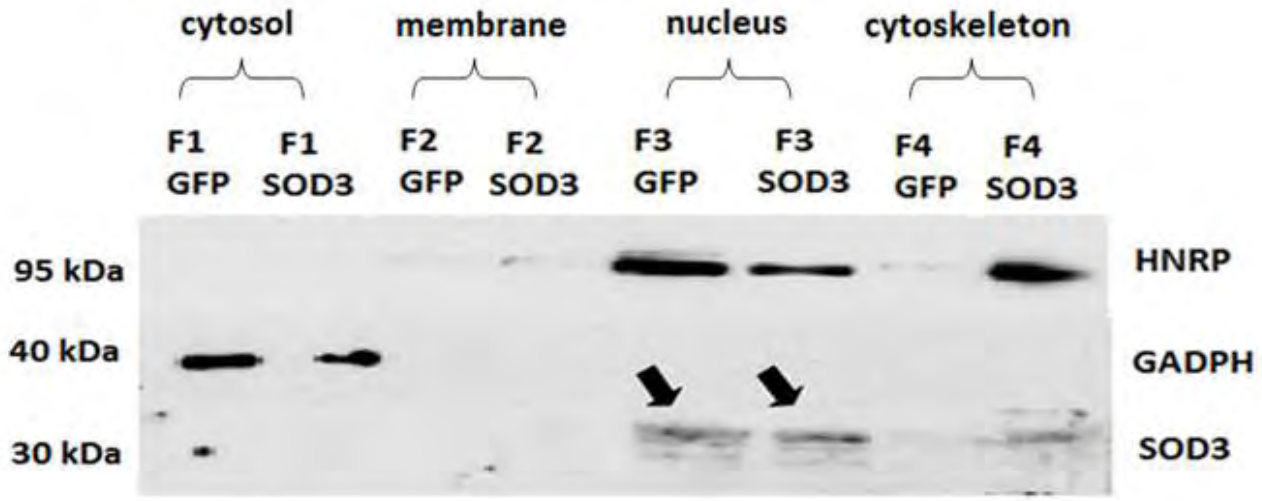


Figure 6. The results of the western blot analysis of the fractionation of the endothelial cell line BAEC. The western blot analysis of the fractionation of 1G11 cells where could be observed the bands for the control of fractionation and the detection of SOD3 in the nucleus (black arrow) and to other fractions.(n=3)

DISCUSSION

In this report, we investigate the role of extracellular superoxide dismutase on the endothelium. One of the most important structural molecules of the endothelium is VE-cadherin. Previous studies of our laboratory focused on a potential correlation of SOD3 with VE-cadherin. In this study we focused on 3 different topics. First, we tried to analyze the RNA levels of endothelial cells that overexpress SOD3 in order to observe its correlation with VE-cadherin levels. As a second thought, we tried to figure out if there is any correlation at the proteins levels of VE-cadherin in the cells that overexpress SOD3. And finally we tried to investigate the subcellular localization of SOD3 with an particular interest for its partitioning to the nucleus.

The first experiments that operated were the RNA analysis of BAEC cells that overexpress SOD3 in comparison to control cells (uninfected, Adβ-GAL). We tried three different periods of infection, for 3 days, 5days and at 10 days. SOD-3 overexpression enhanced VE-cadherin mRNA levels. Although the results obtained from the analysis of qPCR were quite similar, there was a tendency to increased VE-cadherin mRNA at longer infection

times. Nevertheless, we could not establish a linear correlation between SOD3 and VE-cadherin levels, suggesting that SOD3 is only a part of the machinery involved in VE-cadherin regulation. Maybe the RNA levels of VE-cadherin in the endothelial cells increase until one specific amount. It is possible that endothelial cells only needed a limited amount of VE-cadherin. Thus, maybe the overproduction of RNA of VE-cadherin has a ''threshold'' and after that, the cells stop the production of RNA. Overproduction of SOD3 has no limit, however, because is secreted instead of located in the nucleus.

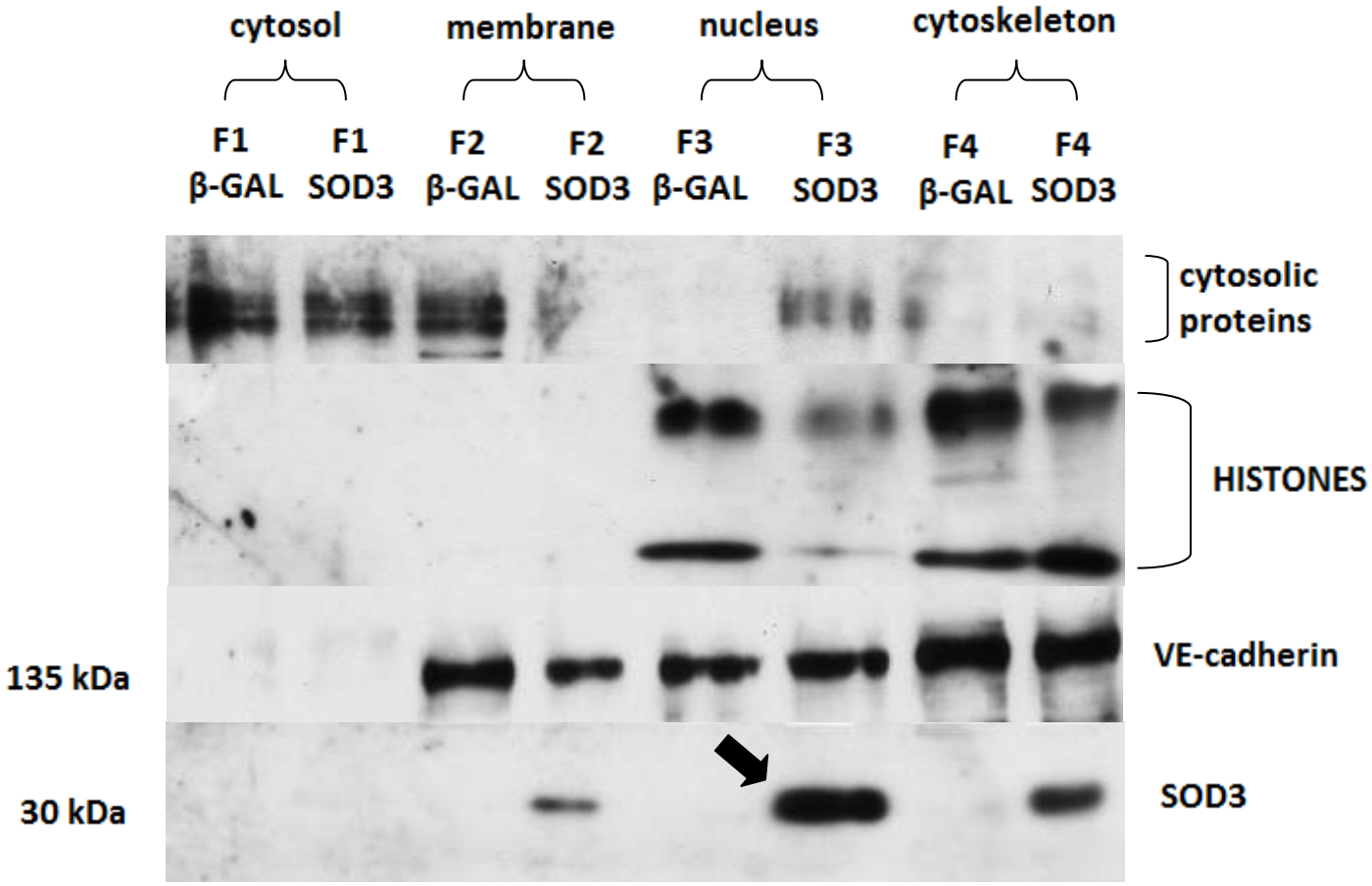


Figure 7. The results of the western blot analysis of the fractionation of the endothelial cell line BAEC. The western blot analysis of the fractionation of BAEC cells where could be observed the bands for the control of fractionation, the detection of VE-cadherin and the detection of SOD3 in the nucleus (black arrow) and to other fractions.(n=3)

Moreover, we operated experiments in order to observe whether the protein levels of VE-cadherin are upregulated by the overexpression of SOD3 in BAEC cells. To address this point we had two different approaches. The first, approach was the extraction of proteins of the cells and western blot analysis. The second approach was the staining of cells in order to visualize VE-cadherin and SOD3 in BAEC cells. The analysis of the protein levels was only studied after 3 days of infection. The results that we obtained from the western blot analysis support that the overexpression of SOD3 for three days was not enough to enhance the

protein levels of VE-cadherin. However, confocal microscopy showed a slight increase in the brightness of VE-cadherin in the membrane of SOD3-expressing cells. It is noticeable that the clear SOD3-induced enhancement of VE-cadherin mRNA is not translated directly in increased VE-cadherin protein levels, suggesting that VE-cadherin might be regulated at the post-transcriptional level. We need to use more quantitative techniques to ascertain this point.

In conclusion, we can say that the overexpression of SOD3 is responsible for an increase of the RNA levels of VE-cadherin; around 4- to 5-fold higher than Adβ-gal-infected cells. But the protein levels of VE-cadherin change slightly with the techniques used, suggesting additional regulatory mechanisms.

The final topic that we focused on was the subcellular localization of SOD3. Other laboratories in various of works had investigated this subject [5, 6]. On the one hand, most of the studies support that SOD3 potentially located in the nucleus. [5]. On the other hand, other studies reported that SOD3 is not located in the nucleus [6]. This dispute boost us to investigate the subcellular localization of SOD3 in BAEC and 1G11 endothelial cells. To address this problem we used a protocol of fractionation of cells in four different fractions as it was described extend above. Both cell lines gave us the same results that indicate the localization of SOD3 in the nucleus. Indeed, when the fractionation of cells was successful we observed that SOD3 is located in fraction 3, which refers to the nucleolic proteins.

The experiments of the fractionation had been operated several times in both cell lines. The most attractive results are in the fractionation of BAEC cells that is shown in the Figures 7. Cells that overexpress SOD3 have a big band in the F3, which refers to the proteins of the nucleus. In this case we can observe also a weaker band in the membrane, which can be explained by either a possible contamination of the membrane and nuclear fractions, or by a possible association of SOD3 with a protein in the membrane. It is noticeable the appearance of SOD3 in the fraction of cytoskeleton in almost every experiment. Due to the control hybridizations, it could be a contamination between nuclear and cytoskeletal fraction; nonetheless, the hypothesis we favor is that SOD3 would associate to cytoskeletal proteins in the nucleus. This hypothesis is really attractive to investigate further in future studies. In conclusion, according to our recent results we can support the hypothesis of the nuclear localization of SOD3, which in turn will translate in an increase in VE-cadherin mRNA levels.

Conclusions and future perspectives

In this study we tried to investigate furthermore the role of SOD3 on the endothelium and the subcellular localization of SOD3. In order to investigate the role of SOD3 on the endothelium, we tried to find a correlation of SOD3 with the basic structural molecule of endothelial cells, VE-cadherin. Our results suggest that the overexpression of SOD3 upregulates 4- to 5-fold the RNA levels of VE-cadherin**.** Also, the upregulation of RNA levels is constant because the quantity of RNA is higher until one specific amount of RNA

(appeared in 5 days of infection). After this amount of RNA, VE-cadherin is independent of SOD3 expression. Furthermore, we could not detect a clear increase in the protein levels of VE-cadherin due to the SOD3 overexpression with the techniques that we used here. These results suggest that VE-cadherin might be regulated differentially at the transcriptional and post-transcriptional levels. Finally, based on our results on 2 different endothelial cell lines we can support that SOD3 partitions into the nucleus.

Our future studies have to focus more on the role of SOD3 on the endothelium. More experiments are needed with different endothelial cell lines and different conditions of infections. More specific, it is necessary to use HUVEC cells in order to clarify if the effect of SOD3 also occurs human cell lines. Furthermore, more experiments are needed with different conditions of infections in order to clarify the proper conditions of upregulation of VE-cadherin. Also, experiments with SOD3-deficient cells would also be helpful strengthen the association between SOD3 and VE-cadherin. Finally, the nuclear localization of SOD3 must be further examined using different fractionation systems. If this is confirmed a big question is to understand how SOD3 is located into the nucleus.

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