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TITOLO DELLA TESI DI DOTTORATO

Role of Protein Tyrosine Phosphatase Receptor, Type Gamma (PTPRG) in the regulation of endothelial permeability.

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ABSTRACT

The vascular endothelium is a selective barrier that regulates, through paracellular and/or transcellular routes, the transport of macro-molecules and cells across the vessels. Modifications of endothelial cell barrier permeability are involved in many physio-pathological events, such as acute inflammatory response and cancer. Several signalling molecules regulate endothelial permeability, regulating actin cytoskeleton dynamics and/or junctional complex disassembly. Although the role of protein kinases in the regulation of endothelial permeability during physio-pathological conditions is well established, the involvement of protein phosphatases in endothelial cells function still remains poorly defined. In a recent study, our laboratory showed that the Protein Tyrosine Phosphatase Receptor, type gamma (PTPRG), which belongs to the protein tyrosine phosphatases receptor-like family, has a negative role on the regulation of recruitment of human primary monocytes. Indeed, activation of PTPRG tyr-phosphatase activity by means of two Trojan fusion proteins, namely TAT-ICD, that encompasses the complete intracellular active enzymatic domain of PTPRG, and P1-WD (P1-wedge domain), which activates the endogenous PTPRG activity, inhibits signalling pathways controlling integrin activation by chemoattractants.

In this study, by taking advantage of TAT-ICD and P1-WD, we investigated the role of PTPRG in the regulation of endothelial permeability. We provided evidence showing that PTPRG activation induces a time- and dose-dependent increase of permeability of endothelial cell monolayers. To corroborate these data, we evaluated the function of ZO-1, a tight junction-associated protein. The data indicated a dose-dependent reduction of ZO-1 expression upon PTPRG activation. Since ZO-1 protein expression critically regulates the stability of tight junctions, these data support the role of PTPRG in the regulation of endothelial permeability. We speculated that PTPRG activity may modulate, directly or indirectly, the phosphorylation state of signalling events controlling the expression and function of junctional proteins involved in the control of endothelial permeability.

INTRODUCTION

The vascular endothelium is a highly dynamic and heterogeneous organ, having metabolic, secretory and immunologic functions, in addition to barrier physical function. In adults, the endothelium consists of approximately $1x10^{13}$ cells, accounting for a surface area estimated in the range of 1 to 7 m^2. **[1; 2]**

The main cells constituting the vessel barrier are the endothelial cells (ECs). ECs are a heterogeneous cell population, widely differing in morphology and function along the vascular tree, according to organ-specific requirements. Their structural and functional integrity are important in the maintenance of the vessel wall and circulatory function.

The endothelium plays a central role in blood-tissue exchange of plasma fluid, proteins and cells. To maintain circulatory homeostasis and the physiological function of different organs, endothelium acts as a semi-permeable selective membrane that controls the passage of ions, macro-molecules and cells across the vessel wall. As a result, vessel barrier dysfunction and endothelial hyperpermeability represent crucial events in the development of a several diseases, such as ischemia-reperfusion injury (IRI), adult respiratory distress syndrome (ARDS) and tumour metastasis. **[3]**

There are two different mechanisms that regulate the endothelium permeability: transcellular pathway (or transcytosis), which involves the formation of vesicles such as caveolae and vesiculo-vacuolar organelle (VVO), and paracellular pathway, which involves the dynamic opening of intercellular junctions (***Figure 1***). **[4]**

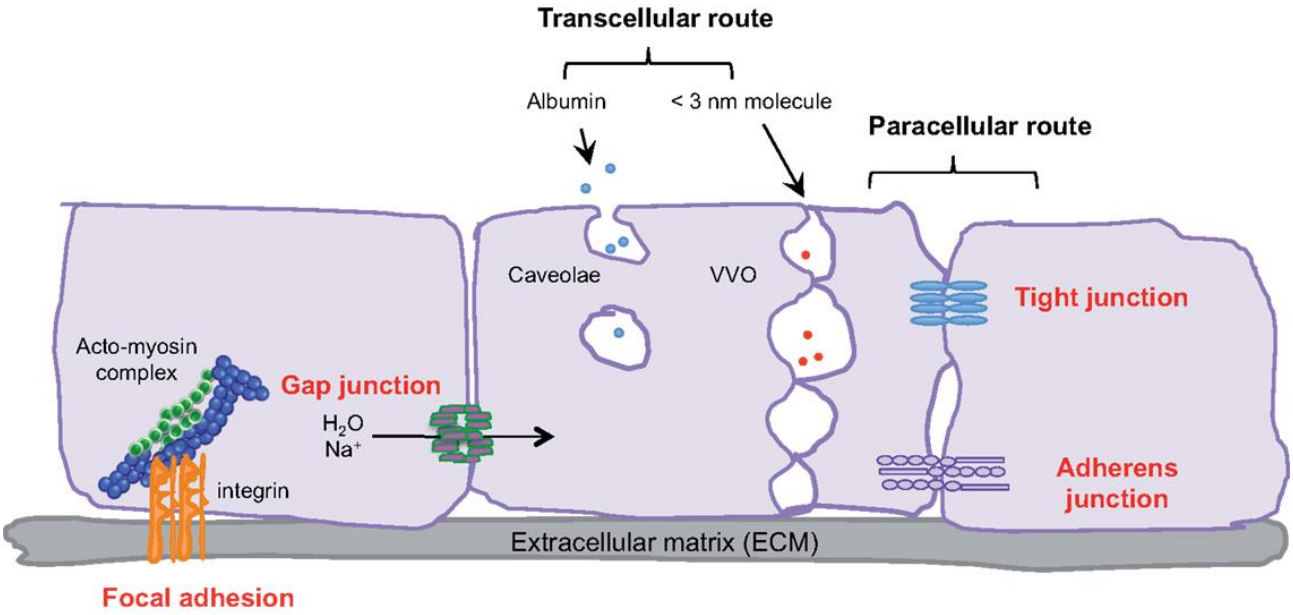


Figure 1. The passage of cells and macromolecules through the endothelial barrier can occur through transcellular or paracellular pathways.

The transcellular transport allows the passage of macromolecules larger than 3 nm through the cells cytoplasm via vesicles. The primary transcellular pathway in endothelial cells is represented by caveolae that uses an energy-dependent mechanism. Caveolin-1 (Cav-1) is the main structural and signalling protein of caveolae, transporting macromolecules from luminal to abluminal space and vice versa, mediating a receptor-dependent or –independent route. **[5]**

The paracellular transport is regulated by endothelial intercellular junctions (or inter-endothelial junctions, IEJs), including complex junctional structures, namely gap junctions (GJs), adherence junctions (AJs) and tight junctions (TJs), playing pivotal roles in cell–cell communication, tissue integrity and barrier function, respectively (***Figure 2***). **[6]**

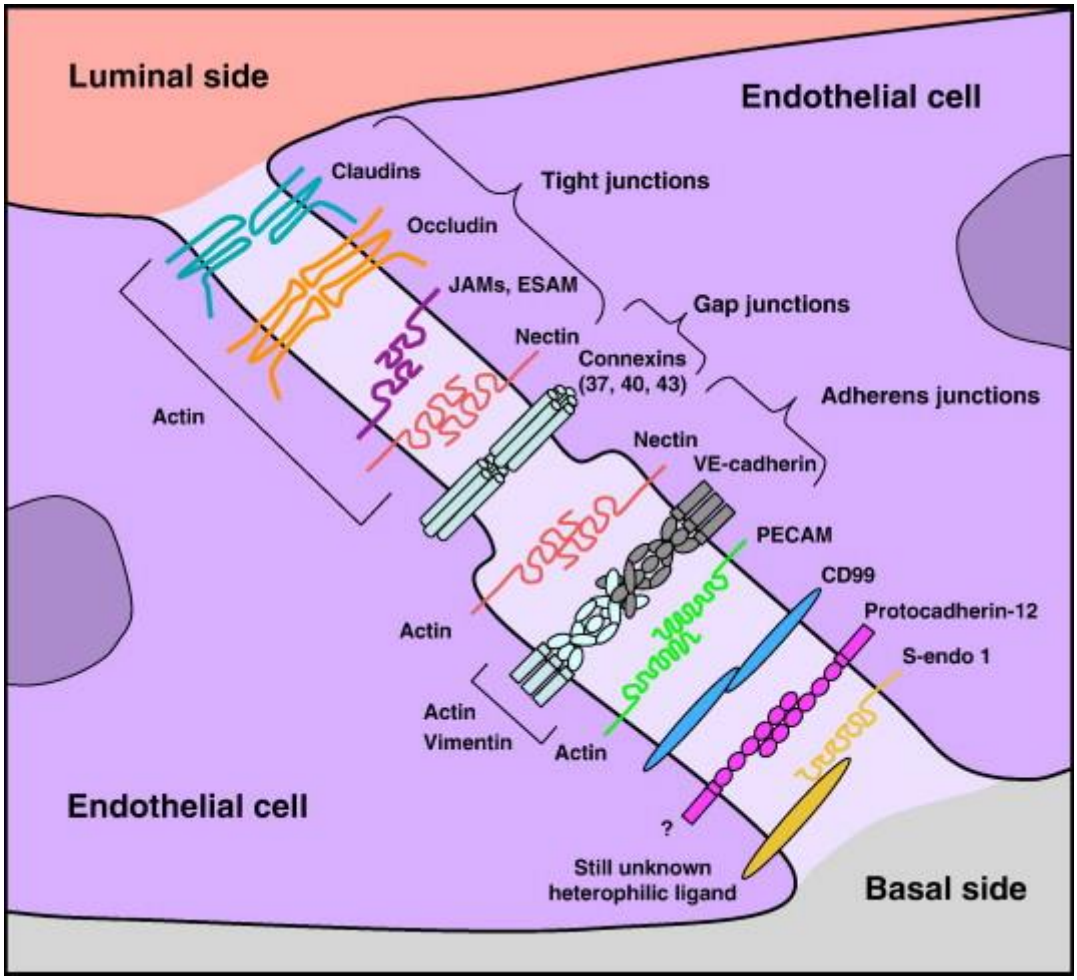


Figure 2. Structure of IEJs. TJs are the most apical structure and are constitute by claudins, occludins and JAMs. VE-cadherin is the main structural protein of AJs. The flux of solutes and ions between adjacent cells is regulated by the connexon which is the basal structure of GJs.

Gap Junctions (GJs) are maintained by connexins; particularly, endothelial cells express three connexins isoforms: Cx43, Cx40 and Cx37. The connexins are organized in connexons which act as channels for the passage of small molecular weight substances between adjoining cells.

The contacts between neighbour cells are established through two different junctional complexes: adherence junctions (AJs) and tight junctions (TJs), distributed along the intercellular cleft. In contrast to epithelial cells, in endothelial cells the junctional architecture is less defined and adherence junctions are intermixed with tight junctions. **[7]** The actin cytoskeleton is bound to each junctional complex and controls their integrity through actin remodelling. The dynamic ability of

endothelial barrier to regulate paracellular permeability derives in part from the junctional complexes and signalling interplay between AJs, TJs and cytoskeleton.

Adherence junctions are regulated by vascular endothelial (VE)–cadherin, consisting of five cadherin-like repeats that associate by means of Ca^{2+}-dependent homophilic interaction with VE-cadherin on the adjacent cells. At the cytoplasmic side, VE-cadherin interacts with p120-catenin (p120) through the juxtamembrane domain, whereas the C-terminal domain binds with β-catenin and γ-catenin (or plakoglobin). Both β-catenin and γ-catenin link α-catenin, which anchors the cadherin-catenin complex to actin filaments. Additionally, α-catenin can also bind α-actinin and vinculin which stabilize AJs association to actin cytoskeleton. **[8]** Although adhesion between VE-cadherin and catenins is required for maintaining AJs integrity, p120 binding to VE-cadherin is central to AJs stability. Depletion of p120 or β-catenin reduces basal fence function and impairs the restoration of normal barrier function. Several studies have demonstrated that p120 and catenins are required for maintaining VE-cadherin on the plasma membrane: deletion of p120 and catenins determines internalization of VE-cadherin and, subsequently, endothelial barrier disruption. **[5]** The scaffold function of p120 is important to regulate the interactions between cadherins, kinases, phosphatases, and Rho GTPases, which in turn control phosphorylation state and stability of cadherin mutual interaction, as well as with catenins. Importantly, Xiao et al. demonstrated that p120 may regulate surface expression of VE-cadherin. Indeed, the deletion of p120 using small interfering RNA (sRNA) in ECs causes a decrease in VE-cadherin expression, whereas overexpression of p120 increases cellular level of VE-cadherin. Thus, p120 can modulate endothelial permeability by multiple mechanism, primarily by influencing VE-cadherin expression and its interaction with multiple signalling molecules. **[6; 8; 9; 10]**

Another important component of adherence junctions is the complex nectin-afadin-ponsin. The nectin family includes four members (nectin-1, nectin-2, nectin-3 and nectin-4) that share the same general structure defined by three extra cellular immunoglobulin domains, a single

transmembrane helix and one intracellular domain. The cytoplasmic tail binds to the PDZ-containing protein afadin, which, in turn, connect nectin to the actin cytoskeleton. Ponsin binds afadin, vinculin and α-catenin, which is the connection between cadherins and nectin-afadin complex. Nectin may also associate with ZO-1 and afadin along with other TJs proteins, such as JAMs. Probably, nectin-afadin complex plays a role in the organization of different junctional structures, depending on cell type localization and organism stage development.

Tight junctions are the other important component of IEJs that mediate intercellular adhesion. They represent the one-fifth of the cell junctions in the endothelium and their complexity differs between endothelial cells of distinct vascular districts. TJs are maintained by the homophilic interaction of the cell-cell adhesion molecules occludin, claudins and junctional adhesion molecules (JAMs). **[11]** Both claudins and occludin contain four transmembrane domains generating homotypic bonds between adjacent endothelial cells. In contrast, JAMs are single pass transmembrane polypeptides, including a short cytoplasmic tail and a single extracellular domain. On cytoplasmic side, tight junctions are associated with a large array of scaffolding and signalling molecules that stabilize the junctional structure and regulate their formation and differentiation. The C-terminus of claudins and occludin connects with the N-terminus of ZO proteins, members of membrane-associated guanylate kinases (MAGUKs), which interact with F-actin through their C-terminus, stabilizing TJs complex. ZO-1 is the first discovered intracellular component and the most extensively studied; ZO-2 and ZO-3 are two other isoforms of ZO proteins family and both ZO-2 and ZO-3 share sequence homology and domain organization with ZO-1. **[5; 11; 12]** Transmembrane proteins of TJs interact also with other cytoplasmic proteins, such as PAR3 and PAR6, cingulin and JACOP/paracingulin, as well as myosin II and GEF-H1, a junction-associated activator of Rho GTPases. These scaffolding proteins recruit several types of signalling proteins to the TJs, including various protein kinases (such as different isoforms of protein kinase C, PKC), protein and lipid phosphatases, small monomeric GTP-binding proteins (such as Rho, Rab, and Ras small GTPases)

and their regulators, as well as heterotrimeric GTPases. This important network of scaffolding and signalling proteins is involved in the organization and stabilization of tight junctional complex, as well as in the regulation of interactions with actin cytoskeleton, which plays a major role in the regulation of endothelial barrier properties. Some junctional proteins interact not only with F-actin but also with other important actin-associated proteins and proteins that regulate actin dynamics, suggesting that they represent functional scaffolds that link signalling pathways to actin dynamics. For instance, cingulin, in addition to F-actin, also binds non-muscle myosin II as well as Rho signalling components that regulate myosin activity. **[13; 14]**

Beside intercellular junctions, the integrity of endothelial barrier depends also on another critical element, the focal adhesions (FAs), which regulate the anchorage of endothelial cells to underling basal lamina. These quite complex structures consist of transmembrane integrins and intracellular adapter proteins like paxillin, vinculin, talin and zyxin, connecting the whole structure to the actin cytoskeleton. Focal adhesions transmit both outside-in and inside-out signals, coordinating proteins of various signalling pathways such as focal adhesion protein kinase (FAK), members of Src family, tyrosine phosphatases and PKC. Several of these pathways are involved in maintenance of endothelial barrier integrity, including, for instance, FAK which provides both positive and negative regulation of endothelium integrity. FAK phosphorylation and activation were shown to play a central role in thrombin- and TGFβ-induced barrier disruption. Nevertheless, endothelial barrier strengthening concomitant with the FAK activation was also shown. Likely, the effects of FAK activation may depend on the experimental context, such as nature of stimuli and activation state and type of ECs. **[15; 16; 17]**

Overall, endothelial permeability is the result of inter-endothelial junctions and cytoskeleton dynamics regulation. Alterations of these regulatory mechanisms compromise vessel wall integrity, primarily by formation of gaps between endothelial cells, determining an increase in endothelial permeability to plasma proteins.

Various physiological and/or pathological processes can lead to modifications of endothelium integrity and, consequently, of vascular permeability. One of the most relevant is the inflammatory response, during which endothelial permeability increases to facilitate the flux of inflammatory mediators and the migration of immune cells in the inflamed tissue (***Figure 3***). **[15; 18]**

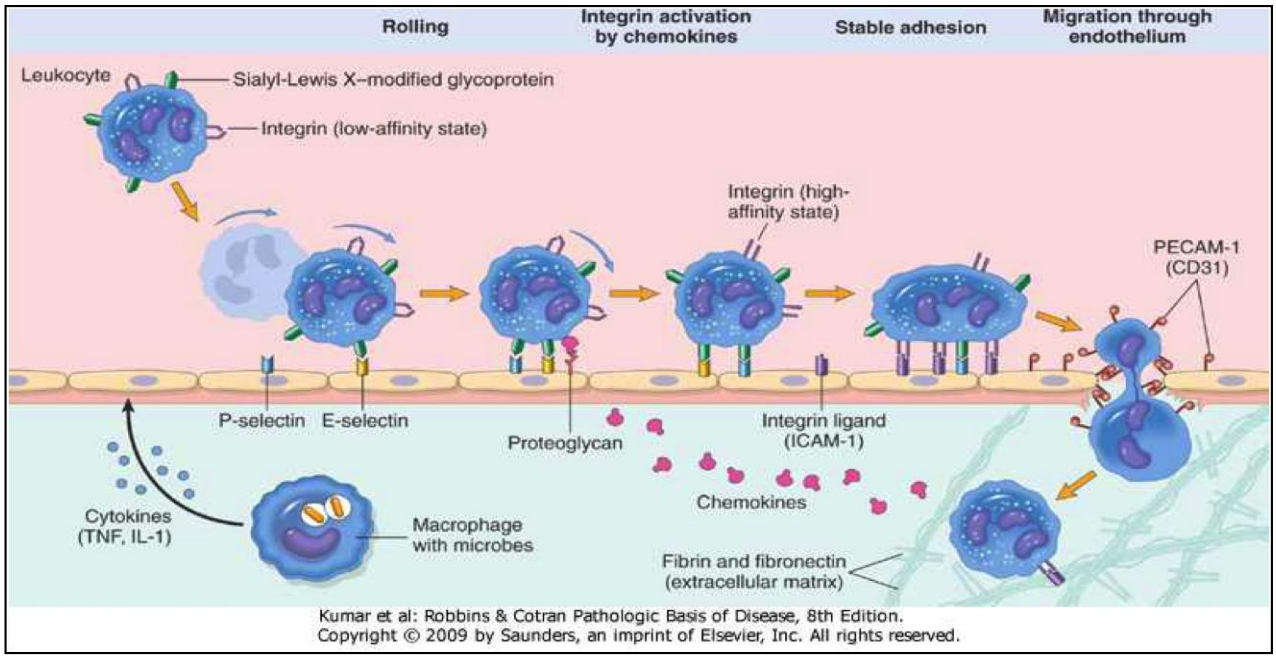


Figure 3. During inflammation, and more in general during the immune response, the first step is the adhesion of immune cells to endothelium, following by transmigration of leukocytes. Transmigration of immune cells from the vasculature into the tissues is a rapid process which does not damage the integrity of the endothelial barrier function. However, factor of leukocyte origin may modify endothelial permeability during inflammation.

Leukocytes release many molecules that regulate the vascular permeability during immune response. Agonists such as histamine, thrombin or growth factors may increase endothelial permeability through an effect on cell contractility or junctional molecular organization. **[19]** Actin cytoskeleton remodelling is mediated by phosphorylation of myosin light chain (MLC) that promotes actin-myosin cross-bridging and leads to cellular contraction. This remodelling can be controlled by various kinases, such as p21-activated kinase (PAK), protein kinase A (PKA), PKC, Src and Ca^{2+}/CaM-dependent protein kinase II (CaMKII), activating myosin light chain kinase (MLCK). **[20;**

21; 22] Factors increasing endothelial permeability, released by inflammatory cells, also mediate inter-endothelial junction disorganisation through activation of many signalling pathways that involve protein kinases, such as Src, ROCK, MAPK and MLCK, and protein phosphatases, such as VE-PTP, SHP2, RPTP-μ and PTP1B. For instance, histamine-triggered signalling causes phosphorylation and disruption of components of the AJs and TJs. However, thrombin stimulates the dissociation of SHP2 from VE-cadherin-catenin complex, causing an increase of complex phosphorylation state and, consequently, of endothelial permeability. **[3; 23]**

Notably, and importantly, the increase in endothelial permeability is an event secondary to the adhesion of leukocytes to the surface of the vessels wall; thus, leukocyte recruitment itself may modulate the permeability of the endothelial barrier.

The general model describing leukocyte recruitment implies a multi-step cascade of adhesive interactions and activating signals, which is classically divided in six main different phases: capture (or tethering), rolling, activation, firm adhesion, crawling and transmigration through the endothelium into the tissue (***Figure 3 and 4***). **[18; 24]**

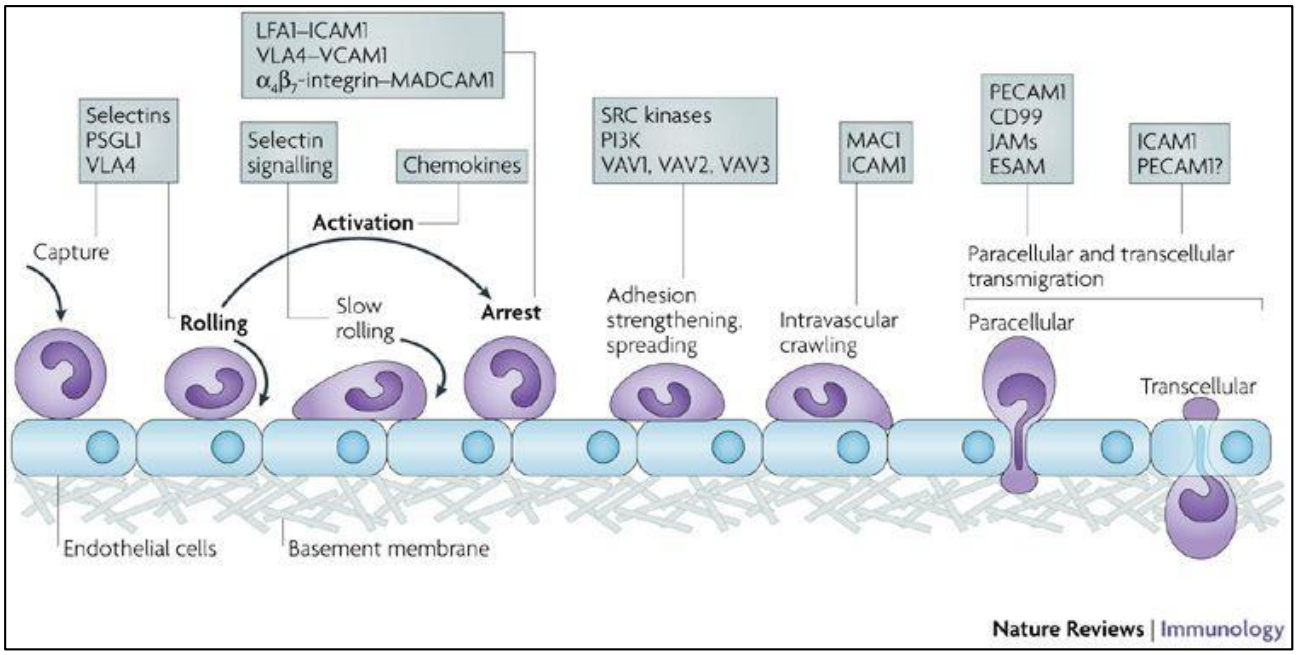


Figure 4. Leukocytes adhesion cascade.

Whereas tethering and rolling phases are mediated by selectins, rapid stable adhesion on endothelium depends on interaction between integrins, such as VLA-4 and LFA-1, expressed by leukocytes, and their cognate receptors, VCAM-1 and ICAM-1 respectively, expressed by endothelial cells. Rapid engagement of rolling cells by chemoattractants, such as chemokines, leads to activation of a complex intracellular signalling cascade, including protein and lipid kinases and phosphatases, in turn controlling rapid integrin affinity activation. For instance, protein tyrosine kinases (PTKs) of the JAK family are upstream transducers linking the chemokine receptor CXCR4 to the hierarchical activation of Rho and Rap small GTPases, thus controlling integrin affinity upregulation and arrest of T-lymphocytes. **[25]** Furthermore, recent study from our laboratory showed that the Protein Tyrosine Phosphatase Receptor, Type Gamma (PTPRG) inhibits JAK2 tyr-phosphorylation by chemoattractants, resulting in down-regulation of LFA-1 affinity triggering and mediated adhesion. **[26]** Thus, leukocyte trafficking is under tight control of phosphorylating and de-phosphorylating events which regulate signalling mechanism, controlling integrin activation and mediated adhesion.

Overall, protein phosphorylation and de-phosphorylation events play a central role in the regulation of both leukocyte recruitment and endothelial hyperpermeability, the two initial phases of immune cells migration during inflammation. However, while the role of protein kinases has been extensively studied, the role of protein phosphatases remains quite elusive, likely due to lack of techniques allowing specific up-modulation of protein tyrosine-phosphatases activity in *in vitro* and *in vivo* models.

The protein tyrosine-phosphatases (PTPs) superfamily is classified into four groups: the classical receptor PTPs (RPTPs), the classical non-receptor PTPs (nrPTPs), the dual specificity PTPs (dsPTPs) and the low-molecular-weight PTPs (***Figure 5***). The RPTPs include seven different classes characterized by a variable extracellular domain and an intracellular region commonly shared with other components of the superfamily. **[27; 28]** The intracellular region is typically composed of two

domains (ICD): the membrane proximal D1 domain, which possesses catalytic activity, and the distal D2, which is possibly involved in substrate specificity, stability and protein-protein interaction of the phosphatase.

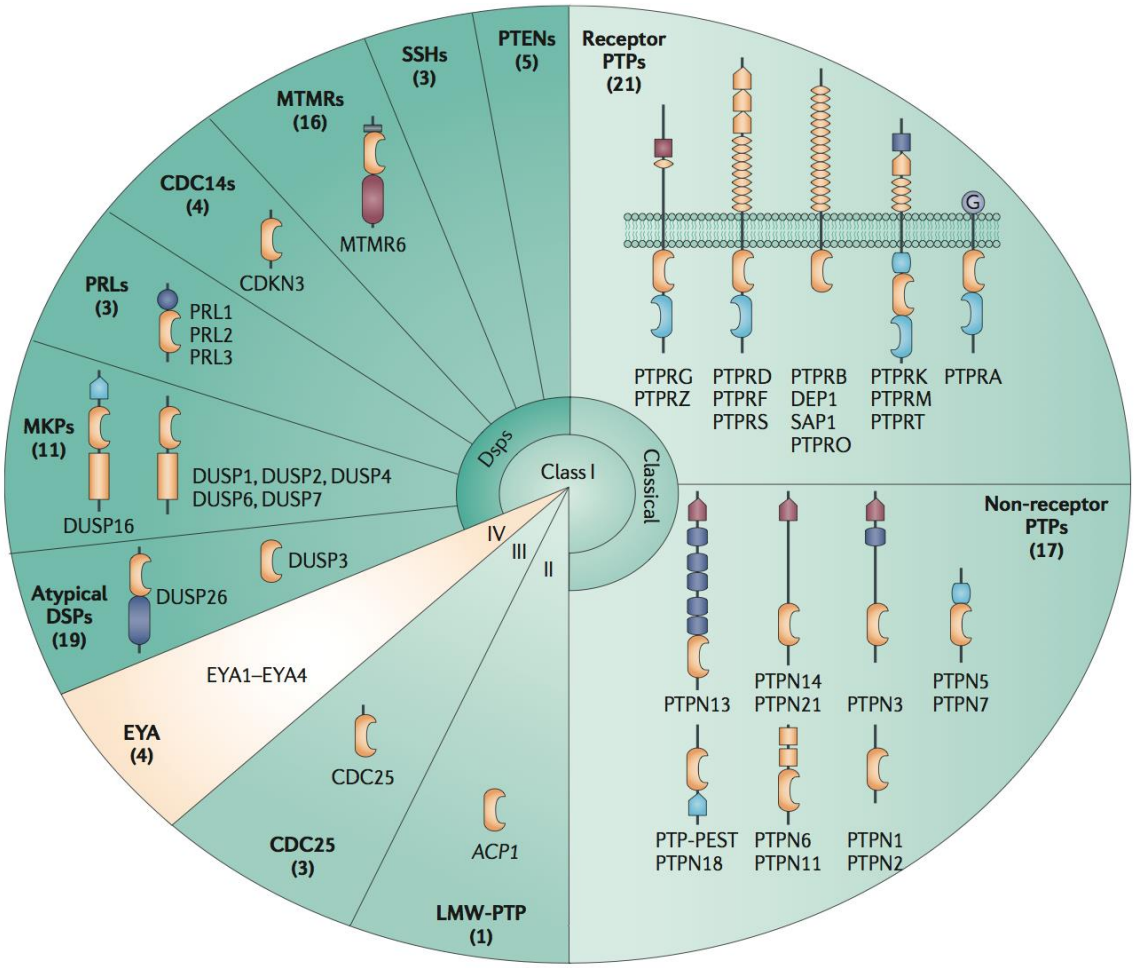


Figure 5. The PTP family of protein tyrosine phosphatases.

The activity of RPTPs is controlled by different mechanisms, including, as most likely critical mechanism, the reversible transition from a homodimeric inactive form to a monomeric active form. Indeed, the PTP domains are organized in symmetrical dimers, where an inhibitory helix-turn-helix wedge motif from one monomer may block the enzymatic active site of the partner monomer. This reciprocal occlusion of the active sites leads to an inhibition of the catalytic activity of the RPTPs (***Figure 6***). **[29; 30; 31]** However, at present, although the dimer-to-monomer mechanism of RPTPs

activity regulation is accepted, the activation of monomerization under physiological conditions and the nature of RPTPs ligands capable of triggering phosphatase activity are poorly described, or mainly unknown.

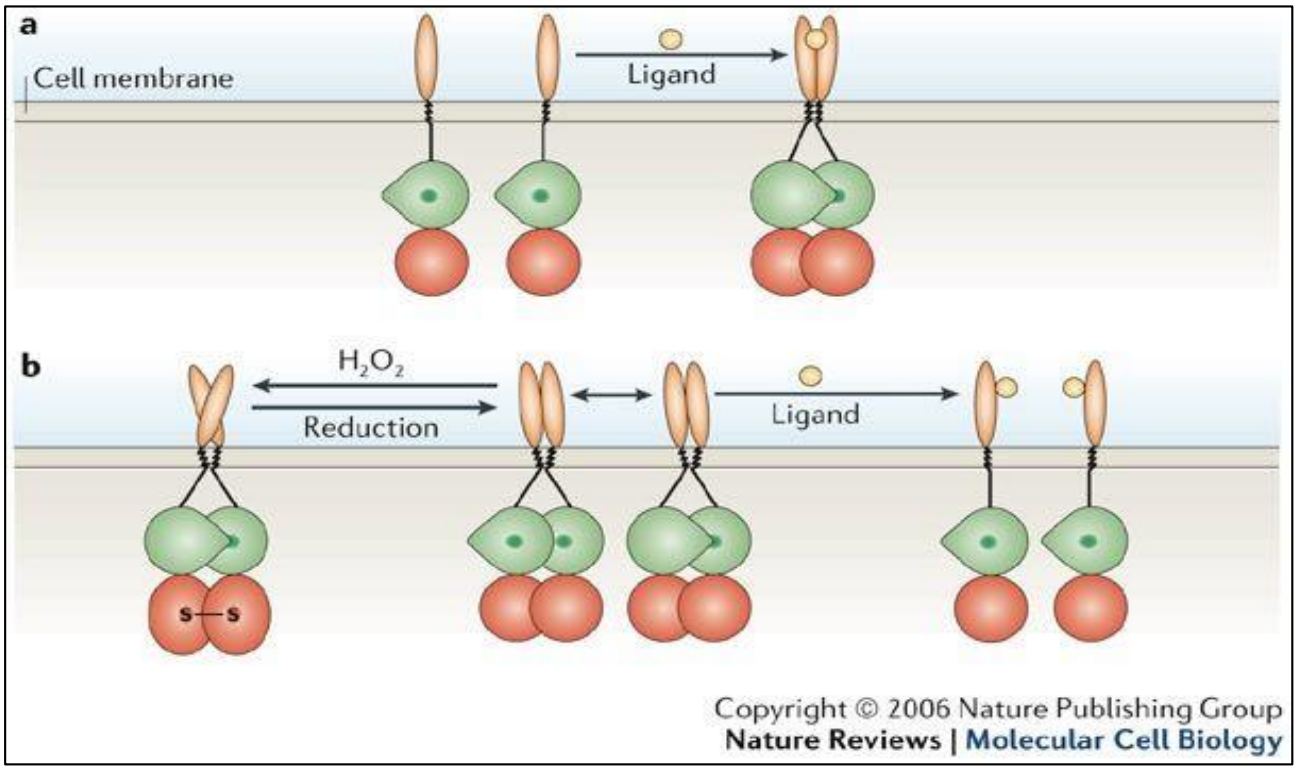


Figure 6. Regulation of receptor PTP function by dimerization.

Recently our laboratory developed a novel, patented, approach based on Cell Penetrating Peptides (CPPs, trojan peptides) to investigate, in primary human cells, the functional role of signalling protein tyrosine de-phosphorylation triggered by Protein Tyrosine Phosphatase Receptor, type gamma (PTPRG) activation. This approach is based on the unmasking of PTPRG phosphatase activity by means of two Trojan fusion proteins: TAT-ICD, which encompasses the complete intracellular, constitutively active, enzymatic domain of PTPRG, and P1-WD (P1-wedge domain), which induces, by steric hindrance, endogenous PTPRG activity by triggering the separation of the

two PTPRG monomers (***Figure 7***) (See also our YouTube movie at https://www.youtube.com/watch?v=wf8VF6SQaHo). **[26]**

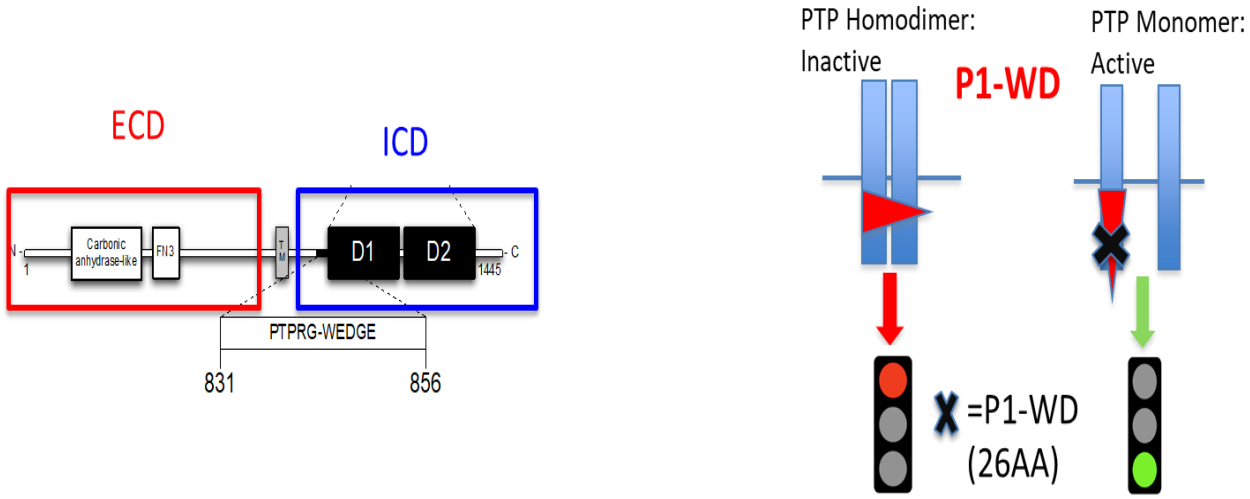


Figure 7. Diagram of Cell Penetrating Peptides strategy.

Protein tyrosine phosphatase receptor, type gamma (PTPRG) is widely expresses in many tissues, including vessel endothelium. **[32]**

In this study, we investigated the role of PTPRG in the regulation of endothelial permeability. We found that PTPRG plays an important role in the regulation of vessel barrier. In particular, up-regulation of PTPRG activity by CPPs triggers a consistent increase of endothelial permeability. We, then, hypothesized that PTPRG-mediated permeability increase may depend on junctional complexes disorganization and consequently gaps formation between neighbouring cells. Our hypothesis was corroborated by the data showing a dose-dependent decrease of ZO-1 expression in endothelial cells treated with P1-WD. The data, although preliminary, highlight for the first time the regulatory role of PTPRG on endothelial cells function. These findings may open interesting opportunities of pharmacological modulation of endothelial permeability in inflammatory and neoplastic contexts.

AIM OF THE STUDY

Endothelium hyperpermeability and leukocytes transmigration are mediated by a variety of signalling pathways, involving protein phosphorylation and de-phosphorylation. In this context, the role of protein tyrosine-phosphatases remains poorly investigated.

Recent study from our laboratory showed that Protein Tyrosine Phosphatase Receptor, type gamma (PTPRG) is a negative regulator of signalling mechanisms controlling leukocyte integrin activation and mediated adhesion. Particularly, activation of PTPRG triggers tyrosine de-phosphorylation and inhibition of JAK2 PTK; consequently, rapid LFA-1 affinity triggering and mediated adhesion by chemoattractants in monocytes is prevented.

Vezzalini et al. showed that PTPRG is widely expressed by many normal and neoplastic human tissues, including high endothelial venules in lymph nodes. **[32]** Importantly, JAK PTKs have been shown to regulate endothelial permeability. **[33; 34]** Thus, PTPRG does emerge as a logical target of investigation in the endothelial context.

In this study we took advantage of our previously CPPs technology, fully validated in primary human cells, to study the role of PTPRG activity in the regulation of endothelial permeability.

MATERIALS AND METHODS

Cell cultures

bEnd3, an immortalized mouse brain endothelial cell line, were purchased from American Type Culture Collection (ATCC® CRL2299™). bEnd3 cells were grown in DMEM (Lonza, 12-604F) with 4.5 g/L glucose, sodium pyruvate, L- glutamine, phenol red, 10% FBS and 1% penicillin/streptomycin (Lonza, 17-602E). Cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2. The cells were trypsinized and seeded at specific density, based on the type of experiment. Monocytes were purified from whole blood of healthy donors, provided by the Transfusion Medicine Department of Borgo Roma, headquarters of the Azienda Ospedaliera Universitaria Integrata di Verona.

Trojan fusion proteins

Control TAT and Penetratin 1 (P1) peptides and the fusion protein P1-PTPRG wedge domain (P1-WD) were synthesized by GeneScript.

PTPRG TAT-ICD fusion protein was produced in our laboratory, using the protocol previously described in Mirenda et al., 2015. **[26]**

P1-WD peptide (RQIKIWFQNRRMKWKKGKQFVKHIGELYSNNQHGFSEDFEEVQ) includes the complete P1 sequence (16 aa; RQIKIWFQNRRMKWKK), an inserted glycine to allow flexibility of the fusion peptide and the computationally identified PTPRG wedge domain sequence encompassing aa 831 to 856 of human PTPRG (26 aa; KQFVKHIGELYSNNQHGFSEDFEEVQ) (***Figure 8***).

The cDNA of PTPRG TAT-ICD, coding for the complete intracellular domain of PTPRG (ICD, aa 797 to 1445) (***Figure 8***), was cloned into pRSET-TATa vector between BamHI and EcoRI sites. Expression of recombinant TAT-ICD fusion protein was controlled to allow purification under non-denaturing conditions. Overnight culture of the *E. coli* strain BL21 pLysS harboring recombinant

plasmid expressing (His)6-TAT-ICD was diluted in fresh LB medium containing ampicillin 50 µg/ml and chloramphenicol 35 µg/ml and grown at 37°C to an OD600 of 0.6. Protein expression was induced overnight at 16°C with 0.1 mM isopropylthio-ßgalactoside (IPTG). Harvested cells were suspended in 1/50 culture volume of lysis buffer containing 20 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 7.3 and protease inhibitors without EDTA. Samples were sonicated 5 times for 30 sec at 4°C and centrifuged for 15 min in a Sorval SS34 rotor at 12500 rpm at 4°C. After centrifugation, the supernatants were 0.45 µm porous filtered and were collected to the HisTrap™ HP 1 ml charged with nickel ions. The clarified lysate was applied to the column at 1 ml/min flow rate. After washing with binding buffer, TAT-ICD was eluted with binding buffer containing 500 mM imidazole, with a linear gradient from 10 mM to 500 mM imidazole in 90 min at 1 ml/min flow rate. Eluted proteins were concentrated on Amicon® Ultra-15 Centrifugal Filter Devices (Millipore). Concentrated stock samples of purified native TAT-fusion proteins were stored at -20°C.

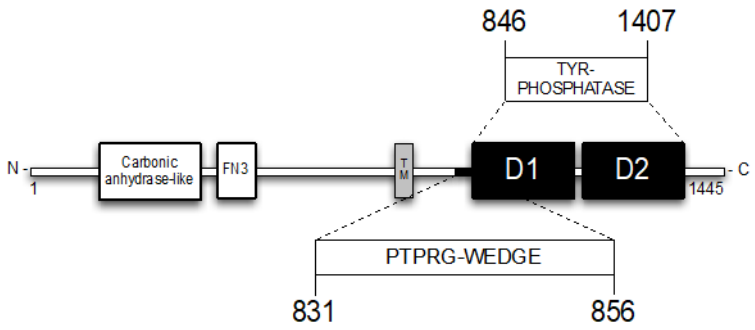


Figure 8. Diagram of PTPRG protein domains.

For the monolayer treatment, TAT-ICD (2 µM) and P1-WD (10, 25 e 50 µM) were diluted in DMEM+1% Pen/Strep, the medium used during the starvation (see *Permeability assay*).

Permeability assay

bEnd3 cells were seeded in fluoroblock transwell insert (Corning® FluoroBlok™ Cell Culture Inserts 351151) with a selective size membrane of 3 µm, that permits the diffusion of medium components, at a density of $7{,}5x10^5$ cells/ml. Cells grew in sterile incubator until reaching the confluence. Before the experimenters, cells were maintained in starvation (serum deprivation, DMEM + 1% Pen/Strep) for three days to promote the translocation of tight junction proteins to plasma membrane, as reported in literature. **[35]** Starved cells were, then, treated separately with the two Trojan fusion proteins, TAT-ICD and P1-WD, for two hours in presence of BSA-FITC (66 KDa, Sigma A9771) or DEXTRAN-FITC (150 KDa, Sigma 46944), added to the medium at a concentration of 1 mg/ml, to evaluate the permeability increase upon treatment.

Variations in permeability were evaluated by quantifying BSA-FITC and DEXTRAN-FITC absorbance (488nm) which crossed the transwell membrane, using spectrophotometer (VICTOR X5 Multilabel Plate Reader).

The data were visualized using a stacked bar graph. In this type of graph each bar represents the whole and segments in the bar represent different categories of that whole. Different colours are used to illustrate the different categories in the bar.

Immunofluorescence staining

bEnd3 cells were seeded on coverslip (VWR 631-0713) at a density of $1{,}6x10^5$ cells/ml. After 24 hours, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Then, the cells were washed in PBS1x and were incubated for one hour at room temperature with blocking solution (PBS1x + 2% of NGS, normal goat serum). At the end of incubation, primary antibody solution was added to the cells: anti-PTPRG antibody (LSBio, LS-C162314) was used at the dilution of 1:100 and was incubated overnight at 4°C. Following, the proper secondary antibody, goat anti-rabbit Alexa Fluor 488-conjugated (Invitrogen A32731), was added at the dilution of 1:700 and was incubated for

1 hour at room temperature. Nuclei were stained with DAPI (Sigma D9542). After mounting with an anti- fading solution (Sigma, DABCO 10981), slides were observed using Zeiss AxioImager.Z1 with objective lens 40x. The images were analysed with Software Zen 2.6 from Zeiss.

Western Blot

bEnd.3 cells and monocytes were lysed in ice-cold 1% Nonidet P-40 buffer containing complete protease inhibitor mixture (Roche 11836170001). Total cell lysates were quantified by Bradford assay (Bio-rad). Equal amounts of total lysates were resuspended in sample buffer and denatured at 98°C for 5 min. Lysate were subjected to 7.5% SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane. The membranes were blocked with BSA 5% in PBS1x containing 0.1% Tween for 1 hour at room temperature. Then, the solution of mouse anti-PTPRG (P4), rabbit anti-ZO-1 1:1000 (ThermoFisher 40-2200) and rabbit anti-actin 1:3000 (Sigma, A2066) were incubated overnight at 4°C. After washes in PBS1x + 0.1% Tween, the appropriate secondary antibodies were incubated for 1 hour. Immunoreactive bands were visualized by ECL detection (EMD Millipore) and intensities of band signals were quantified by densiometric analysis by using ImageQuant Las4000. The anti-PTPRG antibody P4 (***Figure 9*****C**) was kindly provided by Prof. Claudio Sorio (Department of Medicine, University of Verona).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was carried out by calculating mean and SD between different experiments. All statistical analyses were performed using the GraphPad Prism version 5 software.

RESULTS

To investigate the regulatory role of PTPRG activation on endothelial permeability, we first wished to verify whether PTPRG was expressed on bEnd.3 cells. This analysis was essential to justify the usage of P1-WD Trojan fusion peptide, that activates endogenous PTPRG by triggering receptor monomerization, as previously shown. **[25]**

Immunofluorescence staining showed expression of PTPRG on bEnd.3 plasma membrane, with a diffuse surface distribution (***Figure 9*B**). PTPRG expression in bEnd.3 cells was, then, confirmed by western blot analysis (***Figure 9*C**), showing that bEnd.3 cells express the canonical PTPRG, as evidenced by the molecular weight, compared to control human monocytes.

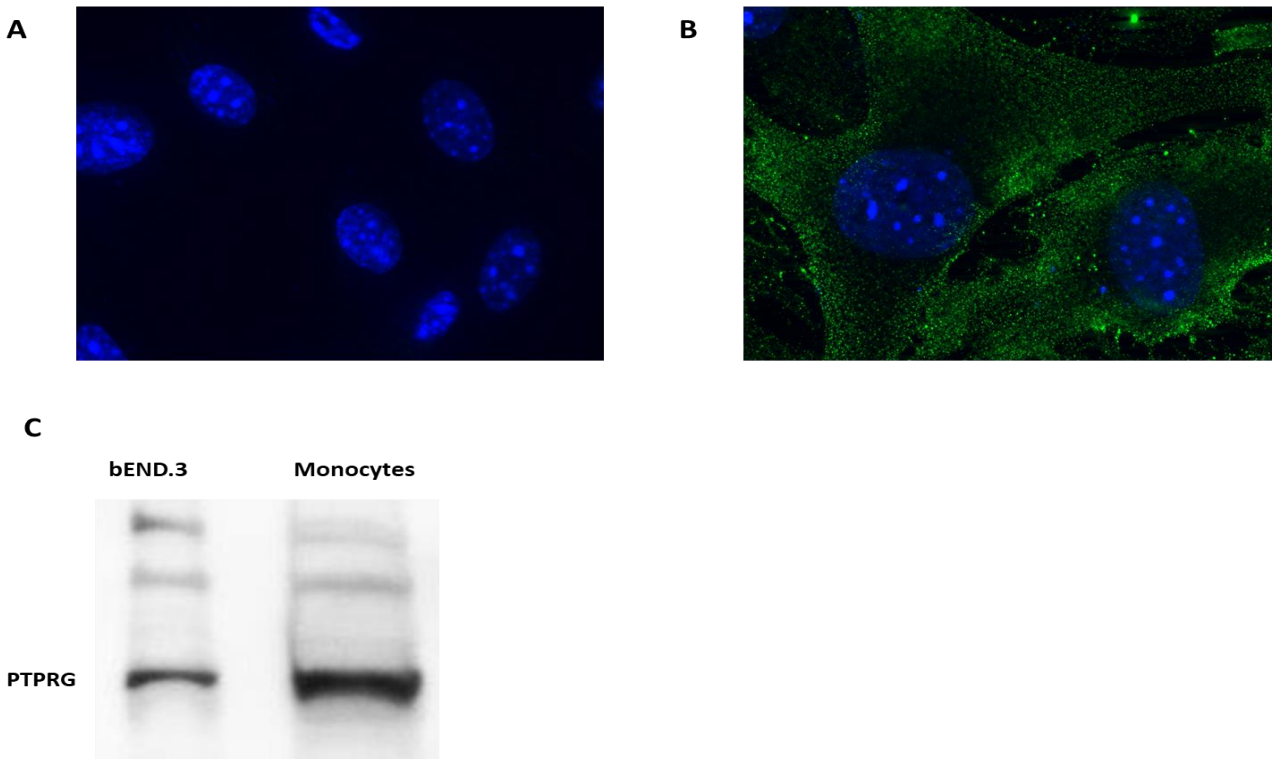
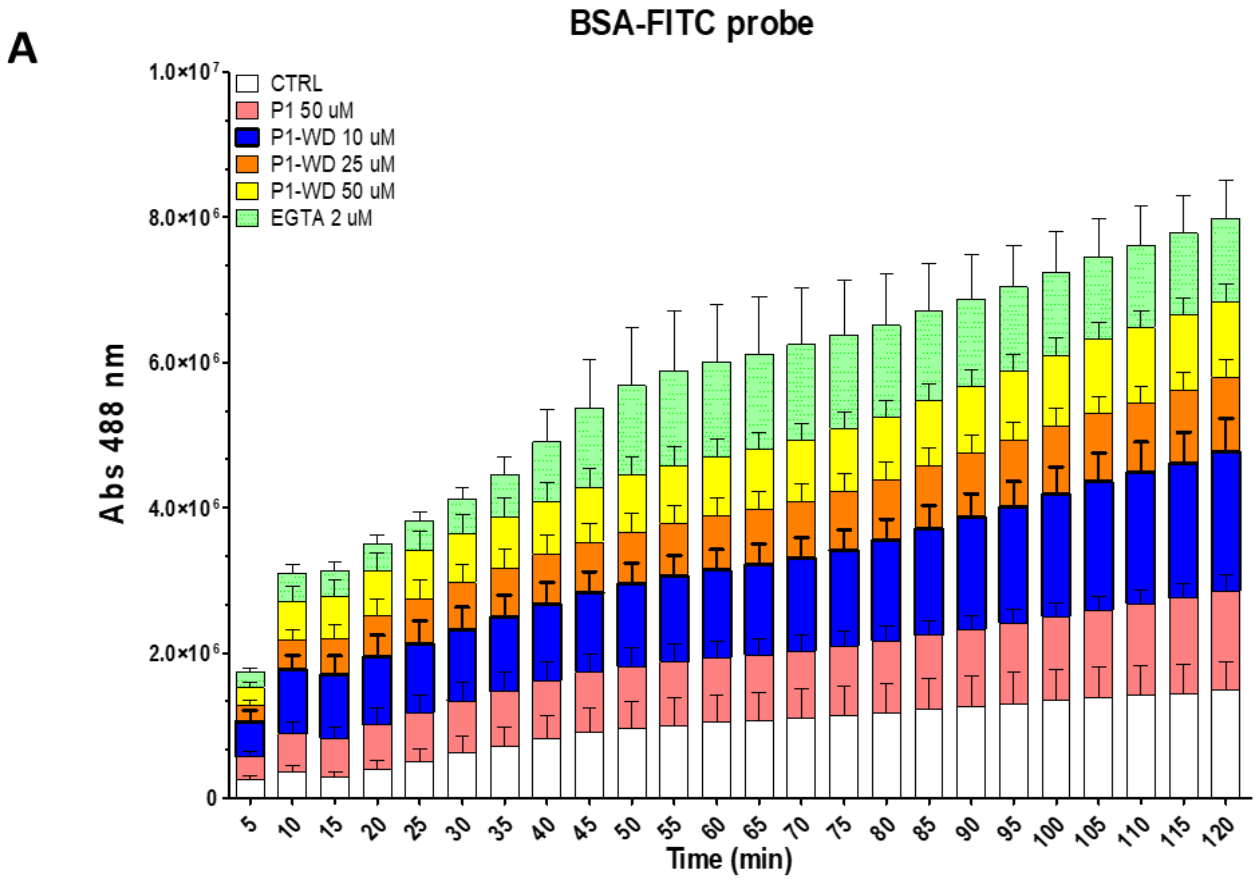


Figure 9. Evaluation of PTPRG expression in bEnd.3 cell line, using immunofluorescence staining and western blot. The bEnd.3 monolayer was stained with rabbit anti-PTPRG primary antibody and a goat anti-rabbit AF488 secondary antibody. Nuclei were stained with DAPI. The comparison between (**A**) control and (**B**) PTPRG stained cells showed that the phosphatase was expressed on bEnd.3 surface. (**C**) Western blot analysis confirmed PTPRG expression. Monocytes are shown as positive control.

Having confirmed the expression of PTPRG in our endothelial cell model, we proceeded to evaluate the functional effect of PTPRG activation on bEnd.3 cells permeability, by first testing the effect of P1-WD, to activate endogenously expressed PTPRG.



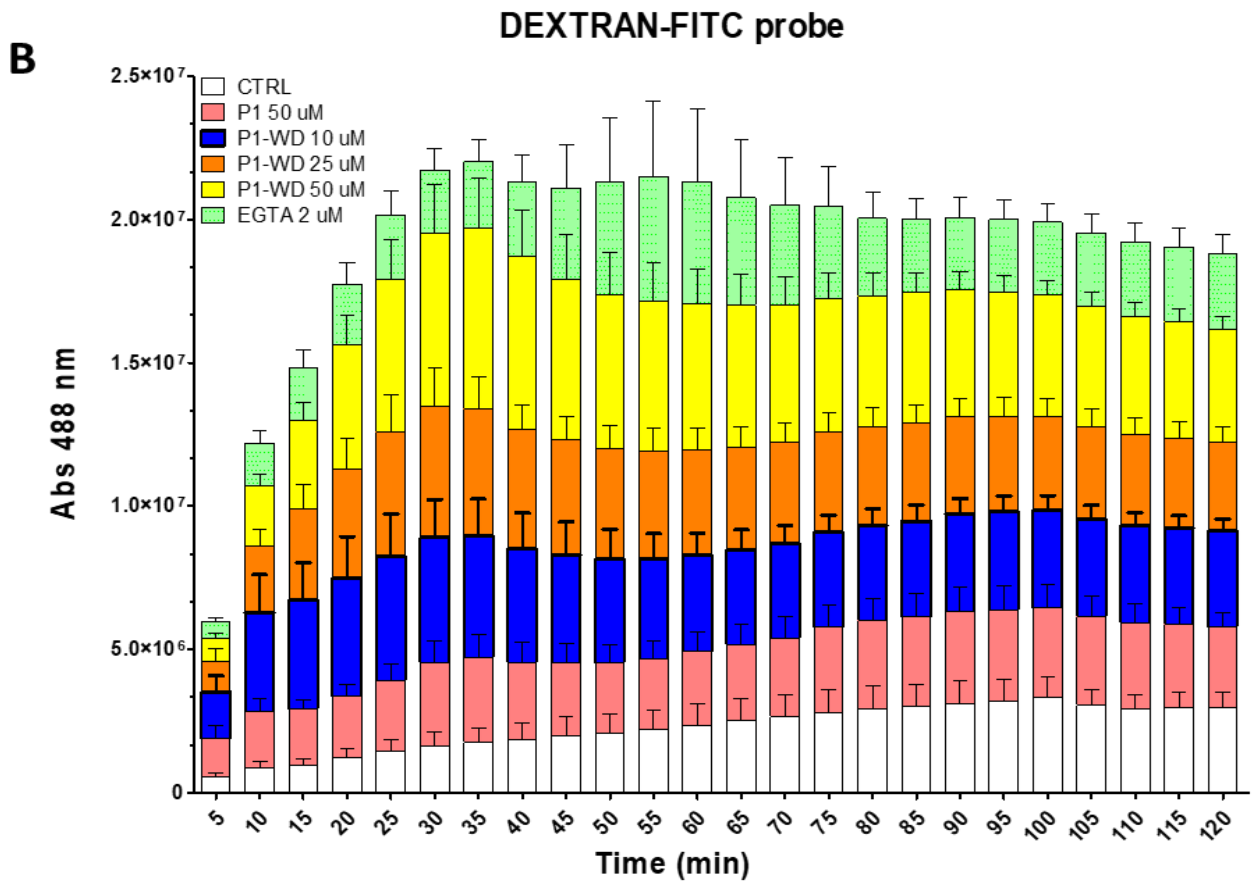


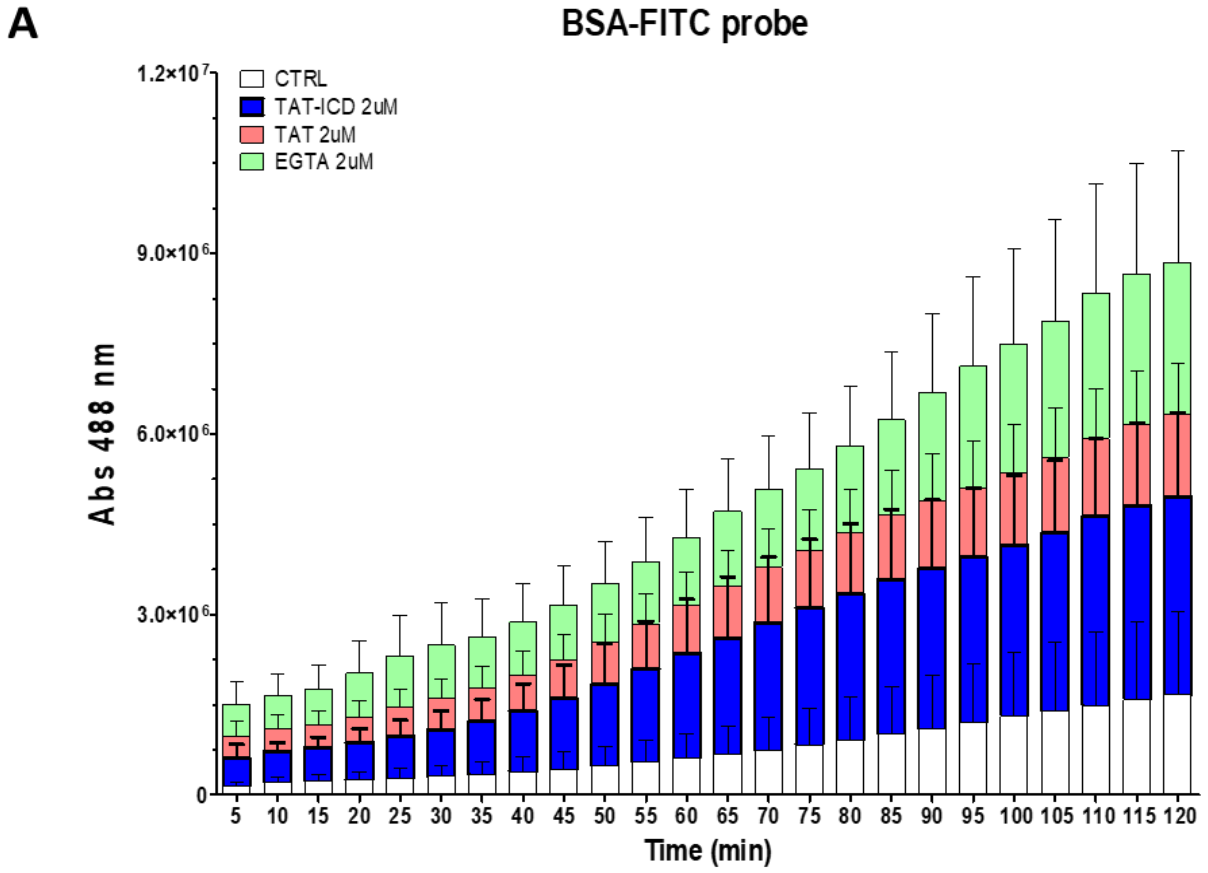
Figure 10. For the experiment with P1-WD, 6 conditions were considered: 1) not-treated cells (negative control), 2) cells treated with P1 50 µM (control carrier peptide), 3-4-5) cells treated with three different concentrations of P1-WD (10-25-50 µM), 6) cells treated with EGTA 2 µM (positive control). The permeability changes were evaluated using two probes with different molecular weight: (**A**) BSA-FITC, MW 66 KDa, and (**B**) DEXTRAN-FITC, MW 150 KDa. Absorbance was read at 488 nm with Victor X5 multiplate reader.

We observed a dose- and time-dependent increase in the permeability of bEnd.3 cell monolayers. Interestingly, the effect of P1-WD treatment was more evident when we used DEXTRAN-FITC as probe, with respect to BSA-FITC. Indeed, after twenty-five minutes of treatment with the lowest concentration of P1-WD (10 µM), we measured an absorbance of $4{,}3x10^6$ (arbitrary units), twice as much the absorbance of positive control (EGTA treatment $2{,}2x10^6$). The absorbance of negative control (untreated cells) was much lower ($1{,}4x10^6$). Treatment with the isolated control carrier (P1, corresponding to the Trojan sequence) at the highest concentration (50 µM) showed an

absorbance of $2,5x10^6$, thus quite lower than the cells even treated with the lowest concentration of P1-WD (***Figure 10*B**). Notably, this was particularly true at the earlier time points and with the lowest concentrations of P1-WD, suggesting the possible emergence of non-specific effects at the highest concentrations at late time points.

The data obtained using BSA-FITC as probe showed a similar trend in cells treated with the lower dose of P1-WD, whereas in the other conditions the permeability increase was lower than in the experiment with DEXTRAN-FITC. Moreover, the absorbance of cell monolayer treated with P1 peptide was comparable with the absorbance of not treated cell monolayer, confirming that control carrier minimally influences monolayer permeability. (***Figure 10*A**) Overall, these data suggest the involvement of PTPRG in signalling mechanisms controlling endothelial cells permeability regulation.

To confirm these findings, we proceeded with the alternative approach we previously developed to evaluate the functional effect of PTPRG activation and based on TAT-mediated intracellular delivery of the entire enzymatic domain of PTPRG, showing constitutive tyr-phosphatase activity. Treatment of bEnd.3 monolayer with TAT-ICD confirmed the regulatory role of PTPRG on bEnd.3 cell permeability.


A
BSA-FITC probe
CTRL
TAT-ICD 2uM
TAT 2uM
EGTA 2uM
Abs 488 nm
Time (min)

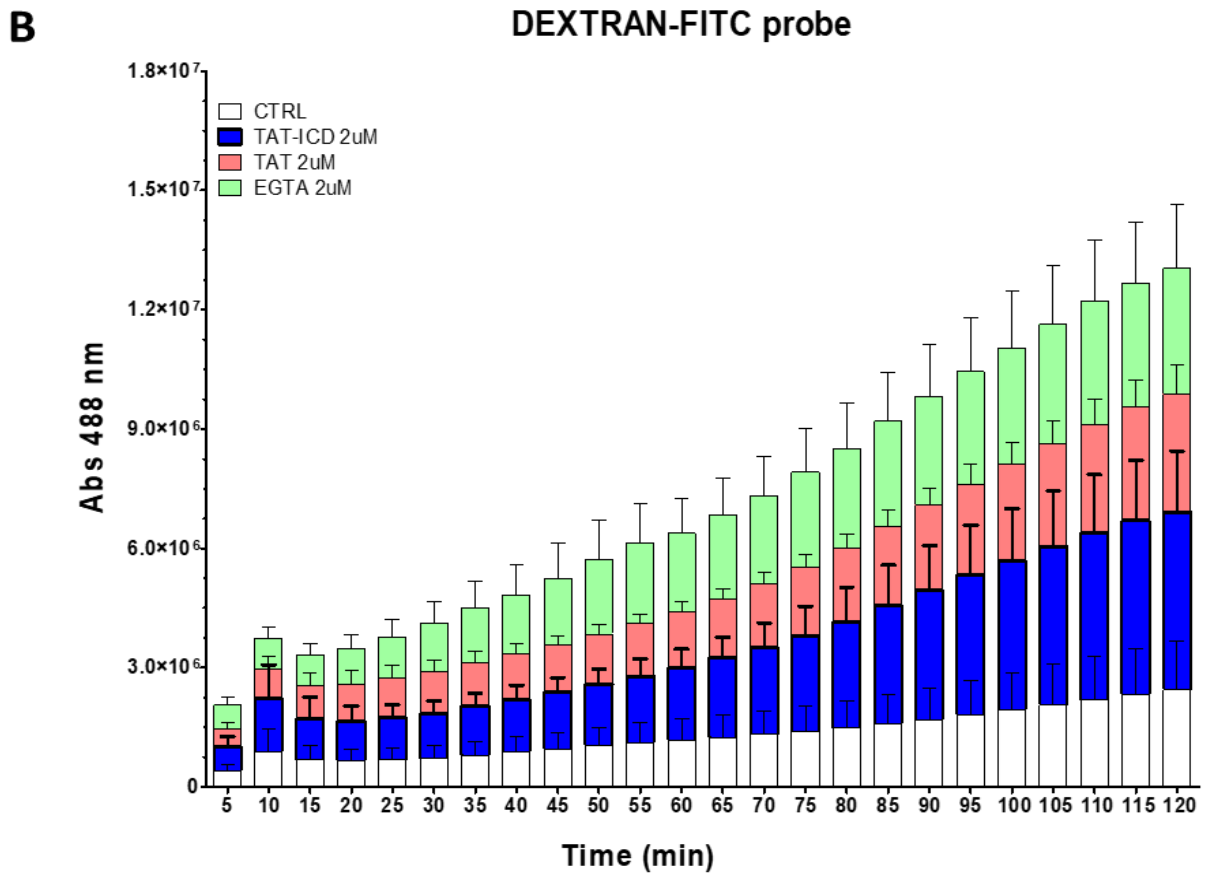

B
DEXTRAN-FITC probe
CTRL
TAT-ICD 2uM
TAT 2uM
EGTA 2uM
Abs 488 nm
Time (min)

Figure 11. For the experiment with TAT-ICD, 4 condition were considered: 1) not-treated cells (negative control), 2) cells treated with TAT-ICD 2 µM, 3) cells treated with TAT 2 µM (control carrier peptide) and 4) cells treated with EGTA 2 µM (positive control). The permeability changes were evaluated using two probes with different molecular weight: (**A**) BSA-FITC, MW 66 KDa, and (**B**) DEXTRAN-FITC, MW 150 KDa. Absorbance was read at 488 nm with Victor X5 multiplate reader.

Particularly, when we used BSA-FITC as probe (***Figure 11*****A**), the permeability increase could be rapidly detected. Indeed, after 1fifteen minutes of cell monolayer treatment with TAT-ICD the absorbance was of $0{,}6x10^6$, comparable with absorbance of positive control (EGTA treatment $0{,}5x10^6$), while the absorbance of negative control cell monolayer (untreated cells) was much lower ($0{,}2x10^6$). Moreover, at late time points, the permeability continued to increase; the TAT-ICD treated cell monolayer showed an absorbance of $1x10^6$, much higher than that of the negative control ($0{,}3x10^6$). Interestingly, cell monolayer treated only with the TAT peptide showed a trend similar to negative control. These data indicated that the TAT peptide alone did not trigger any effects on endothelial cells.

The data obtained using DEXTRAN-FITC as probe were similar, although with quite different kinetics. Indeed, TAT-ICD treatment led to higher permeability increase at later time points, with respect to BSA-FITC assay (***Figure 11*****B**). Treated cell monolayer showed an increase in permeability ($1{,}6x10^6$), thus higher than negative control cell monolayer ($1{,}1x10^6$) after fifty-five minutes. A possible interpretation on these data is that the bigger dimension of DEXTRAN-FITC molecule required the formation of larger gaps between cells. Notably, as consequence of the reported experimental variability of this experimental setting, absolute values of absorbance were quite lower in these set of experiments, with the respect to the previous ones. Nevertheless, the data remained consistent and fully confirmed the regulatory role of PTPRG on endothelial cell permeability.

Altogether, these data show, for the first time, that activation of the tyr-phosphatase activity of PTPRG controls the permeability of the endothelium. To better understand the role of PTPRG on

endothelial permeability, we focused the analysis on the TJs molecule ZO-1, as a prototype molecule controlling TJs structure, by looking at its expression level in bEnd.3 cells after PTPRG activation. To more closely simulate the functional effect of the endogenous activation of PTPRG by a natural ligand, we studied the effect on ZO-1 expression upon PTPRG activation by P1-WD.

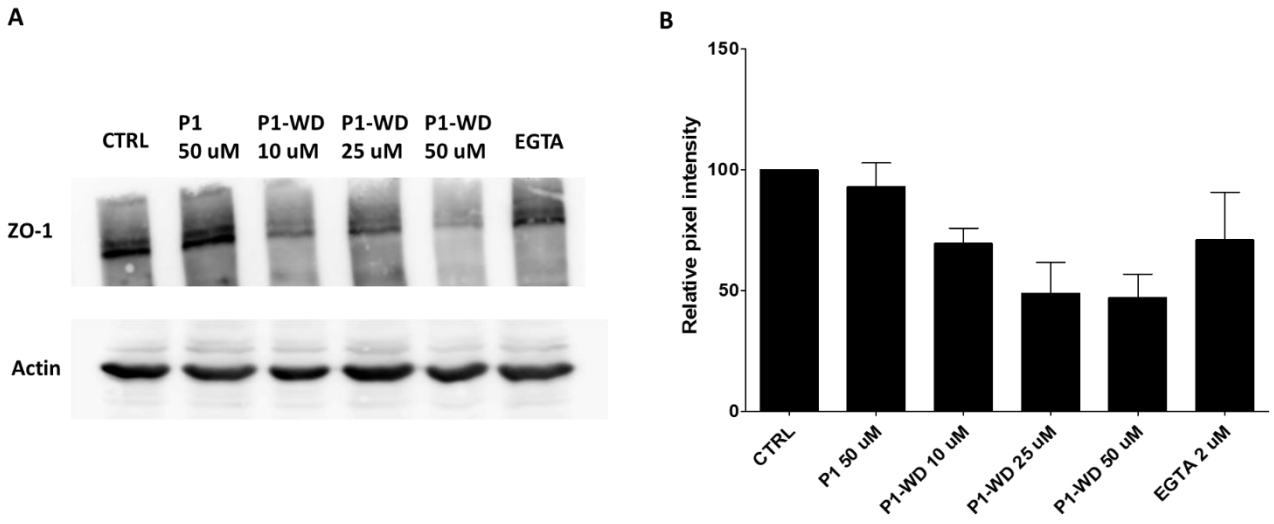


Figure 12. Effect of P1-WD treatment on ZO-1 expression was evaluated by western blot analysis. (**A**) bEnd.3 monolayers were treated with the same concentration using for permeability assay for 40 minutes. (**B**) Western blot immunoreactive band quantification.

Western blot analysis (***Figure 12*****A**) showed a dose-dependent reduction of ZO-1 in P1-WD-treated bEnd.3 cells, confirmed by quantification of immunoreactive bands (***Figure 12*****B**). Interestingly, the cells treated with P1 peptide showed a quantity of ZO-1 comparable with not treated cells, suggesting the specificity of P1-WD. Notably, preliminary data (not show) from immunofluorescence imaging, seemed to show a decrease of ZO-1 immunofluorescence signal intensity and a migration of the protein from the sub-membrane localization to the cytosol in cell monolayer treated with P1-WD, thus confirming the previous observation and further suggesting that ZO-1 is a target of PTPRG activity.

Overall, these data indicate that PTPRG may regulate endothelial cell permeability by modulating the expression and/or sub-cellular localization of junction proteins.

DISCUSSION

The barrier function of the blood vessels is tightly controlled by a network of highly interacting signalling and cytoskeleton proteins, leading to formation of complex signalosomes showing specific intracellular topography. These signalosomes regulate not only the organization and stability of inter-endothelial junctions, but also the cytoskeleton dynamics and its interaction with junctional complexes. **[3; 5]** While the role of kinases has been extensively studied, the regulatory function of phosphatases in the regulation of endothelial permeability remains still poorly defined. Quite likely, this depends to the lack of known RPTPs natural ligands and/or selective agonists, acting in cis or in trans. **[36]**

In this study, we examined the role of receptor protein tyrosine-phosphatases (RPTPs) in the regulation of endothelial permeability, specifically focusing the analysis on Protein Tyrosine Phosphatase Receptor, type gamma (PTPRG). To investigate the functional effects of PTPRG activation, we took advantage of two Trojan fusion proteins, TAT-ICD and P1-WD, that were developed and described in previous studies by our laboratory **[26]**, and set up a conventional model of endothelium monolayer exploiting the murine endothelial cerebral cell line bEnd.3. **[35]**

The results showed that treatment with both PTPRG-activating Trojan fusion peptides may trigger an increase in the monolayer permeability in a dose- and time-dependent manner. The data also showed diversity between BSA-FITC and DEXTRAN-FITC passage, thus suggesting that PTPRG activation may differently affect the size of endothelial gaps.

Notably, previous studies showed the capability of protein phosphatases in maintaining the integrity of vascular barrier. For instance, Grinnell et al. demonstrated that tyrosine-phosphatase SHP2 maintains the phosphorylation state of VE-cadherin, β-catenin and p120-catenin, preserving AJs integrity. **[37]** Moreover, treatment with vanadate, a potent inhibitor of PTPases, increases basal and thrombin-induced MLC (myosin light chain) phosphorylation in pulmonary ECs, which, in turn, provokes an increase of endothelial permeability. **[38]** In contrast, our data suggest that PTPRG

activation, by increasing endothelial permeability, may lead to vascular barrier disruption, thus highlighting a novel modality of regulation of endothelial barrier function by PTPs.

Furthermore, and importantly, the analysis showed that the expression, and perhaps the localization (preliminary observation), of junctional protein ZO-1, a member of Zonula Occludens proteins, was altered by PTPRG activation. Indeed, ZO-1 underwent a dose-dependent expression decrease in cells treated with the PTPRG-activating P1-WD CPP.

Based on these preliminary data, we hypothesized that the increase of endothelial permeability triggered by PTPRG activation may be due to modulation of the phosphorylation state of specific signalling mechanisms responsible of preserving the expression and/or localization of TJs proteins, such as ZO-1.

A high level of interplay between AJs and TJs is important in maintaining endothelial permeability. Perturbation in AJs organization could be reflected on TJs stability. Notably, among 31 previously identified PTPRG targets **[26]**, JAK PTKs were shown to be target of de-phosphorylation and inhibition by PTPRG. **[26]** In this context**,** Jayshree M. et al. demonstrated that JAK3-mediated phosphorylation of β-catenin maintains epithelial barrier functions by AJs localization of phosphorylated β-catenin by means of interaction with α-catenin. The loss of JAK3-mediated phosphorylation sites in β-catenin abrogates its AJs localization and compromises epithelial barrier function. **[34]** Thus, it is logical to suppose that the same functional outcome can be mediated by inhibition of the kinase activity of JAK3 by PTPRG. The consequent AJs perturbation could cause a destabilization of TJs and a possible delocalization and degradation of TJs proteins, such as ZO-1.

Another important signalling axis that regulates the cell-cell junctions involves rho GTPases activity. Rho protein activation level could disrupt junction integrity. **[12]** Three members of Rho family (RhoA, Rac and Cdc42) were demonstrated to play a central role in the regulation of endothelial permeability. In particular, RhoA promotes, under stimulation by chemoattractants, cell contractility by inhibiting myosin-associated protein phosphatase and indirectly promoting MLC

phosphorylation and actin-myosin contraction. By contrast, Rac and Cdc42 have been generally accepted as barrier protectors. In fact, Cdc42 plays a critical role in adherence junction restoration and re-distribution during the recovery phase of inflammation, with a resulting decrease of endothelial permeability. **[3]**

Notably, JAK PTKs are upstream regulators of Rho GTPases activity; specifically, JAKs are a critical upstream regulator of chemokine-mediated Rho GTPasas activation, by controlling phosphorylation and activation of four distinct rho GEFs, all involved in the integrin activation cascade. **[25]** Thus, it is conceivable that JAK PTKs inhibition by PTPRG activity could provoke an impairment of the precise balance of Rho GTPases activation level, producing junctional complexes destabilization and increase of endothelial permeability.

Further efforts are required to more deeply understand the precise mechanisms whereby PTPRG regulates endothelial permeability and to identified the signalling targets of PTPRG, under physio- and pathological stimuli. In literature is largely demonstrated that the increase of endothelial permeability is a significant event in inflammatory conditions associated with trauma, ischemia-reperfusion injury, sepsis, adult respiratory distress syndrome, diabetes, thrombosis. **[3]** Moreover, and quite importantly, cancer cells establish a functional, often quite deleterious, interplay with blood vessels, by reducing endothelial permeability, thus preventing the access of drugs to the neoplastic mass. This is, for instance, one mechanism of therapy evasion by Glioblastoma, one of the deadliest human cancer. Unfortunately, the mechanisms involved in the regulation of endothelial permeability in GBM remain still very poorly defined. **[39; 40]** Thus, an important outcome of our project is the possibility of exploiting PTPRG in the perturbation of permeability of blood vessels during pathological conditions, such as in cancer.

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