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Protein disulfide isomerase externalization in endothelial cells follows classical and unconventional routes

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ABSTRACT

Extracellular protein disulfide isomerase (PDIA1) pool mediates thrombosis and vascular remodeling, however its externalization mechanisms remain unclear. We performed systematic pharmacological screening of secretory pathways affecting extracellular PDIA1 in endothelial cells (EC). We identified cell-surface (csPDIA1) and secreted non-particulated PDIA1 pools in EC. Such Golgi bypass also occurred for secreted PDIA1 in EC at baseline or after PMA, thrombin or ATP stimulation. Inhibitors of Type I, II and III unconventional routes, secretory lysosomes and recycling endosomes, including syntaxin-12 deletion, did not impair EC PDIA1 externalization. This suggests predominantly Golgi-independent unconventional secretory route(s), which were GRASP55-independent. Also, these data reinforce a vesicular-type traffic for PDIA1. We further showed that PDIA1 traffic is ATP-independent, while actin or tubulin cytoskeletal disruption markedly increased EC PDIA1 secretion. Clathrin inhibition enhanced extracellular soluble PDIA1, suggesting dynamic cycling. Externalized PDIA1 represents $< 2\%$ of intracellular PDIA1. PDIA1 was robustly secreted by physiological levels of arterial laminar shear in EC and supported alpha 5 integrin thiol oxidation. Such results help clarify signaling and homeostatic mechanisms involved in multiple (patho)physiological extracellular PDIA1 functions.

1. Introduction

The (patho)physiology of vascular signaling is, among all organ systems, one of those most directly redox-regulated via intracellular as well as extracellular pathways. The latter includes processes involving thiol proteins, among which the protein disulfide isomerase family has received increasing attention [1–3]. The PDI family includes more than 20 members, e.g., PDIA1, Erp57, Erp5 and Erp72, with PDIA1 as the prototype [1]. PDIA1, like most PDI family members, is a dithiol-disulfide oxidoreductase chaperone from the endoplasmic reticulum (ER). Such ER location is given by a N-terminal signal sequence [4], in addition to a C-terminal motif (KDEL for PDIA1) responsible for ER retrieval from Golgi. The main function of PDIA1 is oxidative protein folding in the ER lumen [5], while PDIA1 further supports proteostasis by contributing to ER-associated protein degradation (ERAD) pathway [6]. Moreover, PDIA1 associates with and regulates agonist-triggered oxidant generation via Nox family NADPH oxidases [7] and exerts increasingly evident signaling roles in the redox regulation of cell-surface and extracellular matrix proteins [3,8]. PDIA1 is externalized by: pancreatic cells [9], hepatocytes [10,11], tumor cells [12], T cells [13] and particularly platelets [14,15], vascular smooth muscle cells (VSMC) [16] and endothelial cells (EC)[17,18]. Many redox-modulated targets have been ascribed to such peri/epicellular PDIA1 (=*pecPDIA1*) pool, including: a) disulfide bond reduction of glycoprotein gp120 from HIV virus [19,20], tumor endothelial marker-5 [21], $\alpha_v\beta_3$ integrins in EC [22], tissue factor [23] and αMβ2 in neutrophils [24]; b) thiol-disulfide isomerization of ADAM17 [25] and $\alpha_{IIb}\beta_3$ in platelets [26]. Functional implications of such thiol switches include vascular thrombosis [17,27], platelet activation [15,28], cell adhesion [29], viral infection [12,19,20,30] and vascular remodeling [16]. In particular, the prothrombotic roles of pecPDIA1 [23,31] and other thiol

Abbreviations: PDI, protein disulfide isomerase; csPDI, Cell surface PDI; *pecPDIA1,*, peri/epicellular PDIA1; ER, endoplasmic reticulum; BFA, Brefeldin-A; Mon, Monensin; CytD, Cytochalasin-D; Noc, Nocodazole; SC, Sodium chlorate; Neo, Neomycin; Baf, bafilomycin; NEM, N-ethylmaleimide; Methy, methylamine; Glyb, glyburide; DNP, 2,4-dinitrophenol; Nyst, nystatin; GRASP, Golgi-associated peripheral membrane protein

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isomerases have been a focus of great interest [3,32] and led to clinical trial(s) with pharmacological inhibitors [32]. Despite such importance, PDIA1 externalization mechanisms remain enigmatic [32,33]. Previous reports indicated that pecPDIA1 can be detected without evident cell rupture [9,30,34] and maintains the C-terminal KDEL-sequence [11,34], while mechanisms allowing PDIA1 escape from KDEL receptor are unknown. Cell-surface externalization of PDIA1 through classical secretory pathway was described in ECs infected by dengue virus, but not constitutively [35], while PDIA1 secretion through this pathway has been reported in pancreatic [36] and thyroid FRTL5 cells [37]. Furthermore, it is unclear to what extent PDIA1 externalization in vascular cells occurs following injury/inflammation or also under physiological conditions. In this context, a comprehensive systematic analysis of cellular secretion routes and diverse extracellular PDIA1 pools is lacking. Understanding such pathways in EC is required to help elucidate a mechanistic basis for pecPDIA1 functions under conditions associated with vascular pathophysiology and thrombosis. Systematic use of pharmacological inhibitors can provide a useful, often-used screening platform to functionally assess a general secretory pathway profile [38], particularly the Golgi dependence through compounds such as brefeldin-A (BFA), which are well known to inhibit ADP-ribosylation factor (ARF1) GTPase, leading to structural Golgi collapse [39]. Here, we performed such a screening in EC, addressing essentially all classes of currently described secretion pathways [40,41] to explore their regulatory roles in PDIA1 externalization under different stimuli. Our results provide evidence that pecPDIA1 comprises a cell-surface, as well as a soluble secreted pool. In EC, secreted PDI predominantly follows Golgi-independent secretory route(s), which showed to be GRASP55 and syntaxin-12 independent, while both classical and Golgi-bypass routes contribute to the cell-surface pool. We ruled out unconventional type I, II and III pathways to support PDIA1 externalization. Interestingly, secreted PDI undergoes endocytosis mediated by clathrin. Finally, we provide evidence for robust PDIA1 secretion under physiologically relevant shear stress in EC and that PDIA1 oxidizes alpha5 integrin thiols.

2. Methods

Antibodies were as follows: PDIA1 (SPA891) from Enzo; GM130 (35/GM130) and anti-PDI BD34, from BD-Transduction Laboratories; Erp72, thrombomodulin, GORASP2 (GRASP55), GRP78, from Abcam; PDIA1 (RL90), from Thermo-Scientific; Syntaxin 12, from Santa Cruz; p44/42 MAP kinase (ERK1/2, 4696) and p44/42 MAPK (ERK1/2, 4377) (Thr202/Tyr204), from cell Signaling. Biotin Peroxidase (BN-34), β-actin, Hoeschst, Bafilomycin, Brefeldin A, Monensin Sodium, Nocodazole, Filipin III, Glybenclamide (Glyburide), Neomycin trisulfate salt hydrate, Sodium Chlorate, 2,4-Dinitrophenol were from Sigma. Cytochalasin-D was from Calbiochem. Cell culture material was from Gibco.

2.1. Cell culture

A selection-immortalized human umbilical vein endothelial cell line (HUVEC) [42] was maintained in RPMI containing 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, 25 mg/mL penicillin. To retrieve PDIA1 from conditioned culture medium, confluent adhering cells were subcultured using trypsin/EDTA and seeded at 2.5×10^6 cells in plastic petri dishes (100 mm) with a final volume of 8 mL culture medium (containing FBS) per dish. After culturing for 22–24 h, distinct treatments were performed in 5 mL serum-free medium, followed by medium centrifugation in Centricon (10/30 kDa cut-off, 40x medium concentration), and the concentrated medium was stored at 80 °C for < 30 days until use. Primary HUVEC were obtained from Invitrogen (C-003-5C), and maintained in Medium 200 supplemented with Low Serum Growth Supplement (LSGS) according to the supplier instructions. HUVEC were used between passages 3 and 7. In the Shear Stress (SS) experiments, cells were plated on collagen type IV-coated plates (20 μg/mL) in medium with 0.5% SFB (instead of 2% SFB used for cell growth). HUVEC were exposed to SS of 15 Dyn/cm^2 for 10 min in a cone-plate device [43] in serum-free Medium 200.

2.2. GRASP55 silencing

HUVEC were transfected with GRASP55-specific small interference RNA sequence– SiRNA (CAGAGCUGGUUUGGAGCCUUUCUUU /AAAGAAAGG CUCCAAACCAGCUCUG) or a Scrambled sequence (CAGGUCUUGGGUC GAUUCCUAGUUU/ AAACUAGGAAUCGACCCAAGACCUG) using the AMAXA nucleofector system (Lonza) according to manufacturer's recommendations.

2.3. Classical secretory and unconventional pathway disruption

All treatments were performed in serum-free RPMI with each concentration indicated in the respective legend. Validated concentrations of each of these agents were used as guides for our selection, adjusting according to cell viability or other cell effects investigated in specific cases (see Results). References for these concentrations were: Brefeldin-A (BFA) [44], Nocodazole (Noc) [45], Cytochalasin-D (CytD) [46], Monensin (Mon), Sodium chlorate (SC), Neomycin (Neo) and N-ethylmaleimide (NEM) [38], bafilomycin (Baf) [38], methylamine (Methy), glyburide (Glyb), 2,4-dinitrophenol (DNP) [47].

2.4. Cell-surface biotinylation

HUVEC were seeded at 4×10^5cells in Petri dishes (60 mm), with 3 mL final volume/dish (containing FBS) for 24 h before treatments in serum-free medium, as indicated in each legend. The biotinylation experiment with EZ-Link sulfo-NHS(*N*-hydroxysuccinimido)-biotin (Thermo-Scientific) or MPB [3-(*N*-maleimido-propionyl) biocytin] (Molecular Probes) was performed as described [25]. All western blots were performed with RL90 clone anti-PDIA1, unless specified.

2.5. Cytotoxicity assay lactate dehydrogenase and annexin-V/propidium iodide

Lactate dehydrogenase assays were performed by *Cytotoxicity Detection Kit* (Roche), as described by the manufacturer. Annexin-V and propidium iodide were evaluated by flow cytometry after incubation for 15 min at 25 °C of PBS-washed, trypsin-harvested cells with annexin-V 1:20 (Invitrogen), followed by propidium iodide (0.1 mg/mL).

2.6. Confocal immunofluorescence

HUVEC were seeded (3×10^4/well) onto glass coverslips in 24-well plates for 24 h. After treatments, cells were fixed in 4% paraformaldehyde (20 min at 25 °C), rinsed in PBS, permeabilized or not in 0.1% Nonidet p40 (30 min at 37 °C) and blocked with 2% BSA for 30 min at 37 °C. Primary antibodies were incubated overnight at 4 °C and secondary antibodies for 2 h at 25 °C, diluted in PBS containing 1% BSA. Working dilutions were as follows: anti-PDIA1 (SPA891; 1:200), anti-Erp72 (1:200), anti-GM130 (1:300); Alexa Fluor 488 (1:200) and Alexa 546 (1:200). Nuclei were stained with DAPI (Invitrogen, 10 μg/mL) or Hoechst-33258 (1:200). Immunostained coverslips were mounted on microscope slides using glycerol/PBS (1:2 v/v). Cells were observed on an inverted laser confocal microscope (Zeiss LSM510-Meta) from our local “Rede Premium” Facility. Pinhole was adjusted according to objective and sample thickness. All images shown are representative from at least three independent experiments.

2.7. CRISPR assay

Specific gRNAs against STX12 were designed using CRISPR design tool (http://crispr.mit.edu/). A scrambled sequence was also designed