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ORIGINAL PAPER

Cell-surface HSP70 associates with thrombomodulin in endothelial cells

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Abstract

Heat shock protein-70 (HSP70) is crucial for proteostasis and displays cell-protective effects. Meanwhile, enhanced levels of cell-surface (cs) and secreted HSP70 paradoxically associate with pathologic cardiovascular conditions. However, mechanisms regulating csHSP70 pool are unknown. We hypothesized that total and csHSP70 expressions are modulated by hemodynamic forces, major contributors to endothelial pathophysiology. We also investigated whether thrombomodulin, a crucial thromboresistance cell-surface protein, is a csHSP70 target. We used proteomic/western analysis, confocal microscopy, and cs-biotinylation to analyze the pattern and specific characteristics of intracellular and csHSP70. HSP70 interaction with thrombomodulin was investigated by confocal colocalization, *en face* immunofluorescence, proximity assay, and immunoprecipitation. Thrombomodulin activity was assessed by measured protein C activation two-step assay. Our results show that csHSP70 pool in endothelial cells (EC) exhibits a peculiar cluster-like pattern and undergoes enhanced expression by physiological arterial-level laminar shear stress. Conversely, total and csHSP70 expressions were diminished under low shear stress, a known proatherogenic hemodynamic pattern. Furthermore, total HSP70 levels were decreased in aortic arch (associated with proatherogenic turbulent flow) compared with thoracic aorta (associated with atheroprotective laminar flow). Importantly, csHSP70 co-localized with thrombomodulin in cultured EC and aorta endothelium; proximity ligation assays and immunoprecipitation confirmed their physical interaction in EC. Remarkably, immunoneutralization of csHSP70 enhanced thrombomodulin activity in EC and aorta ex vivo. Overall, proatherogenic hemodynamic forces promote reduced total HSP70 expression, which might implicate in disturbed proteostasis; meanwhile, the associated decrease in cs-HSP70 pool associates with thromboresistance signaling. Cell-surface HSP70 (csHSP70) expression regulation and csHSP70 targets in vascular cells are unknown. We showed that HSP70 levels are shear stress-modulated and decreased under proatherogenic conditions. Remarkably, csHSP70 binds thrombomodulin and inhibits its activity in endothelial cells. This mechanism can potentially explain some deleterious effects previously associated with high extracellular HSP70 levels, as csHSP70 potentially could restrict thromboresistance and support thrombosis/inflammation in stress situations.

Keywords Endothelial cells · Atherosclerosis · HSP70 · Thrombomodulin · Cell stress · Shear stress

Abbreviations

HSP70	70 kDa heat shock protein family
HSC70	Constitutive HSP70
HSP701A/1B	Stress-inducible HSP70
Cs	Cell surface
TM	Thrombomodulin
PLA	Proximity ligation assay
PCa	Protein C activated
VEGF	Vascular endothelial growth factor
LSS	Low laminar shear stress
EC	Endothelial cells
PDI, PDIA1	Protein disulfide isomerase A1

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Introduction

The 70 kDa heat shock protein family (HSP70) proteins have well-known intracellular roles as molecular chaperones and ancient components of cellular proteostasis networks

(Powers and Balch 2013; Borges and Ramos 2005). HSP70 is constitutively expressed (HSC70) and also induced by stress (HSP70 1A/1B) (Powers and Balch 2013). While HSP70 and other HSPs (27, 60, 90, or 110 kDa) are passively released by dying or damaged cells, they are also actively secreted by vascular, neuronal, epithelial, and tumor cells and also immune cells such as monocytes, macrophages, and B cells (Calderwood et al. 2007). Along the same line, the discovery that HSP70 and HSP90 including their ER paralogs such as GRP78 and GRP94, as well as PDI family ER redox chaperones, are actively externalized by cells had crucial implications to several diseases including cardiovascular conditions (Henderson and Pockley 2012; Soares Moretti and Martins Laurindo 2016).

Extracellular HSP70 exerts tumor cell survival effects during stress (Radons 2016) and can be both anti- and proinflammatory (Calderwood et al. 2007), via Siglec-5 or Siglec-14 lectin binding, respectively (Fong et al. 2015) or by exerting a cytokine-like role via CD14 (Asea et al. 2000). Interestingly, intranasal exogenous HSP70 administration protects against amyloid β42-induced neurodegeneration (Fernandez-Funez et al. 2016) and increases life span and cognitive functions in developing mice (Bobkova et al. 2015). Soluble HSP70 was also found in serum from healthy individuals (Pockley et al. 1998) and increases during cancer, acute lung injury, diabetes, chronic inflammation, infection, trauma, and also with aging. Recently, HSP70 was shown to interact with CLEC14a (C-type lectin domain family 14 member), a protein involved in tumor angiogenesis (Jang et al. 2017). HSP70 is an important anti-tumoral target, with several inhibitors described (Shrestha et al. 2016).

Furthermore, extracellular HSP70 plays increasingly evident roles in cardiovascular (patho)physiology. Its plasma levels are enhanced in hypertension of pregnancy (De Maio et al. 2012) and directly associate with the prognosis of heart failure (Jenei et al. 2013) as well as with increased risk (Zhang et al. 2010) and severity of acute coronary syndromes (Zhang et al. 2011), although association with lower risk and severity of coronary artery disease was also reported (Zhu et al. 2003). Recent investigations showed that ischemia-induced microvessel formation in vivo (Shiota et al. 2010) is prevented by HSP70 inhibition. Similarly, exogenous HSP70-1A enhances endothelial cell (EC) migration and tube formation in vitro at levels comparable to vascular endothelial growth factor (VEGF), while VEGF-induced EC migration was prevented by pharmacological or siRNA-mediated HSP70 inhibition (Kim et al. 2016). In both cases, it is likely that the observed HSP70 effects reflect to some extent its intracellular function (Shiota et al. 2010; Kim et al. 2016). It was previously shown that induction of HSP70 through HSF1 promotes enhanced thrombomodulin expression in endothelial cells (Uchiyama et al. 2007).

Thrombomodulin is an integral membrane protein that binds thrombin on the cell surface and changes its function from procoagulant to anti-coagulant by potentiating protein C activation (Martin et al. 2013). In vivo HSF1 activation promotes anti-thrombotic effects associated with induction of HSP70 and upregulation of thrombomodulin, while genetic deletion of inducible HSP70 enhances thrombosis (Allende et al. 2016). However, most such studies focused on total HSP70 pool and some were performed with exogenous HSP70 supplementation, rendering it difficult to extrapolate such data to the endogenous extracellular HSP70 pool. Overall, despite such a likely importance in several human disease states (De Maio et al. 2012; Calderwood et al. 2007; Xu 2002; Henderson and Pockley 2012; Krause et al. 2015; Jang et al. 2017), extracellular HSP70 functions, particularly in vascular cells, are yet poorly known (Pockley 2002; Xu 2002; Henderson and Pockley 2012) and are indeed controversial (Henderson and Pockley 2012; Krause et al. 2015; Pockley 2002).

We hypothesize that total and csHSP70 expressions are modulated by hemodynamic forces, major contributors to endothelial pathophysiology. In addition, following results from preliminary experiments, we also propose to investigate whether thrombomodulin, a crucial thromboresistance cell-surface protein, is a csHSP70 target. We show that endogenous HSC70/HSP70 (collectively named csHSP70) binds to the EC surface and is decreased under condition associated with cardiovascular disease such as low laminar shear stress (LSS, 4 dynes/cm^2). In the same condition, intracellular HSP70 levels display an impaired upregulation, as compared to that observed under physiologic high shear. Importantly, csHSP70 interacts with thrombomodulin and HSP70 immunoneutralization, in contrast to the results from atheroprotective shear-dependent expression, which enhances protein C activation, suggesting a negative regulation of thrombomodulin function. These data may help understand some apparent discrepancies regarding the roles of HSP70 in disease and suggest a possible physiological mechanism to counteract unrestricted thromboresistance during stress situations in which HSP70 externalization is enhanced.

Materials and methods

Cell culture Primary human umbilical vein endothelial cells (HUVECs) were acquired from Thermo Scientific (C0155C). Cells were cultivated in endothelial cell growth basal medium (EBM) (Lonza) supplemented with EGM (Lonza CC-3162) until passage five and 16 h starved before shear stress experiment. Then, cells were submitted to 11 or 4 dynes/cm^2 laminar shear stress for 24 h under serum starvation in EBM using a cone-and-plate system (Tanaka et al. 2016; Araujo et al. 2016). Control static cells were incubated in 1% fetal bovine

serum (FBS) for 24 h to maintain cell integrity. In some experiments indicated in the legend, a selection-immortalized HUVEC (Moraes et al. 2014) was maintained in RPMI containing 10% FBS, HEPES 25 mM, 100 mg/mL streptomycin, and 25 mg/mL penicillin.

Cell-surface biotinylation and Western analysis HUVECs were seeded at 4×10^5cells in petri dishes (60 mm) with 3 mL medium 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) was performed as described (Willems et al. 2010). Briefly, HUVEC was incubated with EZ-Link sulfo-NHS (*N*-hydroxysuccinimido)-biotin in phosphate-buffered saline (PBS) for 1 h at 4 °C. The reaction was interrupted with cell incubation in Tris–HCl 50 mM (pH 7.5) for 10 min following by cell lyses in 50 mM Tris–HCl (pH 7.4), 1% Triton X-100, and 150 mM NaCl for 1 h containing protease inhibitors. Then, the lysates were incubated with Streptavidin MagneSphere Paramagnetic Particles (Z5481) for 16 h at 4 °C. After that, beads were PBS washed and incubated with Laemmli buffer 30 min before 5 min boiled following by SDS-PAGE. Western blots were performed as described (Araujo et al. 2016) using anti-HSP70 (3A3, ab5439), anti-GRP94 (52031), anti-GRP78 (ab21685), and anti-thrombomodulin (ab94373) from Abcam, anti-PDIA1 RL90 (MA3-019) from Thermo Scientific, anti-HSP701A/1B from Millipore (AB9920), and β actin from Sigma.

Confocal immunofluorescence HUVEC was seeded (3×10^4/well) onto glass coverslips in 24-well plates for 24 h. After 24-h growth, 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% bovine serum albumin (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-HSP70 (1:200, ab5439) and anti-thrombomodulin (1:100, ab94373) from Abcam.

***En face* immunofluorescence** Animal studies were performed in male C57BL/6 6-week old following approval from to Ethics Committee of the Heart Institute and School of Medicine from University of São Paulo, Brazil. After euthanasia with CO_2 inhalation, the abdominal aorta was carefully removed and cut along the longitudinal axis. Aorta segment were fixed in paraformaldehyde 4% (20 min, 25 °C), blocked with BSA 4% (30 min, 25 °C) and antibodies, as follows: primary rabbit-anti-thrombomodulin (1:50, ab94373) and mouse-anti-HSP70 (1:200, ab5439) in BSA 1% were incubated overnight at 4 °C. Fluorescent secondary antibodies (Invitrogen) with DAPI (Invitrogen) were incubated for 1 h at 25 °C and analyzed in confocal microscopy (Zeiss LSM 510 META). For layer specific measurements, z-stacks (1-μm interval) were scanned since the intimal face until the media.

SILAC Cells were cultivated according to Mann and collaborators (Ong and Mann 2006; Mann 2006). Briefly, cells were grown in DMEM/F12 SILAC (stable isotope labeling by amino acids in cell culture) Media (Thermo Scientific, A2494301) supplemented with EGM, 2% dialyzed FBS, arginine $^{13}C_6$, $15N_4$ (Sigma #608033), lysine $^{13}C_6{}^{15}N_2$ (Sigma#608041) for heavy media, or regular arginine and lysine for light media. Cells were replicated until passage five, when > 90% of proteins were labeled with heavy amino acids. After labeling, cells were submitted to starving in heavy or light media for 16 h without EGM and FBS. Then, using a cone-and-plate system, cells were submitted to 11 or 4 dynes/cm^2 SS for 24 h in starving heavy or light media (see above). Proteins were extracted with 8 M urea, 150 mM NaCl, 50 mM Tris, 1 mM EDTA add protease cocktail inhibitor (Sigma, P8340), and phosphatase cocktail inhibitor (Sigma P2859). Protein was quantified by BCA method (Thermo Scientific, 23225), according to manufacturer recommendations, and 100 μg protein from each condition was pooled (11 dynes/cm^2 heavy with 4 dynes/cm^2 light or 11 dynes/cm^2 light with 4 dynes/cm^2 heavy), followed by proteomic analysis.

Proteomic analysis Proteomic analysis was performed in Mass Spectrometry Research Center in Vanderbilt University. Briefly, proteins were reduced with 10 mM DTT, alkylated with 300 mM iodoacetamide, and digested with trypsin 0.1 μg/μL. Peptides were desalted using zip-tip and submitted to LC-MS with 11 steps of MudPIT fractionation, followed by QExactive analysis. For peptide and protein identification, data were analyzed using the Maxquant software package, version 1.3.0.5 (Cox and Mann 2008; Cox et al. 2011). MS/MS spectra were searched again a human subset database created from the UniprotKB protein database. Precursor mass tolerance was set to 20 ppm for the first search, and for the main search, a 10-ppm precursor mass tolerance was used. The maximum precursor charge state was set to 7. Variable modifications included carbamidomethylation of cysteines (+ 57.0214) and oxidation of methionines (+ 15.9949). Enzyme specificity was set to Trypsin/P, and a maximum of 2 missed cleavages were allowed. The target-decoy false discovery rate (FDR) for peptide and protein identification was set to 1% for peptides and proteins. A multiplicity of 2 was used, and Arg10 and Lys8 heavy labels were selected. For SILAC protein ratios, all reported protein groups were identified with two or more distinct peptides and quantified with two or more ratio counts. Protein groups identified as reverse hits were removed from the datasets.

Proximity ligation assay by DUOLINK Proximity ligation assays (PLA) provide robust evidence for protein–protein interaction, revealed by analysis of secondary antibody-coupled mutually complementary DNA strands via in situ PCR (Söderberg et al. 2006). After primary antibodies, anti-HSP70 (ab5439) and anti-thrombomodulin (ab94373) slices were washed in wash buffer A (provided into the Sigma kit) and the PLA Duolink following the product recommendation regarding probes, ligation, and amplification reactions. In control cells, primary antibodies were omitted. After the PLA amplification reaction, cells were PBS washed and incubated with Phalloidin 633 and DAPI in PBS with 1% BSA at 37 °C for 60 min. Slices were then mounted with PBS/Glycerol 1:1 and kept in the dark until image analysis.

Immunoprecipitation For co-immunoprecipitation, HUVECs from seven confluent 150 mm plates were pooled. Plates were PBS-washed and incubated for 20 min on ice in lysis buffer (20 mM Tris–HCl pH 7.8, 250 mM sucrose, 1 mM $MgCl_2$ and 1 mM $CaCl_2$) contained protease (1 mM PMSF, 1 μg/mL leupeptin and aprotinin) and phosphatase inhibitors (50 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate). Cell suspensions were transferred to a 15-mL nitrogen cavitation bomb by 30 min in 400 psi of N_2 pressure following by centrifugation for 1000×*g* (10 min, 4 °C). Lysates were incubated overnight at 4 °C under agitation with 10 μg of anti-thrombomodulin antibody (rabbit, ab109189), followed by incubation with 100 μl protein G-coated magnetic (GE) beads for 4 h at 4 °C. Beads were washed in sucrose resuspended in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1% CHAPS) with protease and phosphatase inhibitors for 1 h at 25 °C. HSP70 was detected by western blot using anti-HSP70 antibody (ab5439). Fluorescent immunoblotting was scanned with the Odyssey near-infrared imaging system and repeated in three independent experiments.

Protein C activation assay Protein C activation (APC), used to assess thrombomodulin activity, was measured as described with modifications (Ammollo et al. 2011). Primary HUVECs seeded in 24-well plates (6×10^4/well) for 24 h were incubated in 0.25 mL of medium 200 without serum with either anti-HSP70 (3A3, 1:100), anti-HSP70 (9920, 1:150), or anti-PDI (RL90, 2 μg/mL) for 25 min at 37 °C following by 0.1 μM protein C (Enzyme Research Laboratories) incubation for 5 min. To activate protein C via cell surface–thrombomodulin, we added thrombin 1 U/mL for 10 min at 37 °C. The medium (50 μL) was removed, and the reaction was stopped with hirudin (20 μg/mL) for 5 min. The kinetics of protein C activation in 96-well plate was determined using 50 μL 2 mM chromogenic substrate (S-2366, Chromogenix) in Opti-MEM using a SpectraMAX reader at 405 nm. Since HSP70 antibodies 9920 and 3A3 were developed in whole rabbit serum and ascites fluid, respectively, their exact quantification was not possible and experiments were based on antibody dilutions as indicated.

Statistical analysis Data are mean ± SEM. Densitometric analysis of all immunoblots was performed using Odyssey Li-Cor Software. Each experiment was repeated at least three independent times. Data were analyzed by paired *t* test to compare two groups or one-way ANOVA plus Student–Newman–Keuls multiple-range test, to compare three groups. A p value < 0.05 was considered significant. All analyses were performed using GraphPad Prism v.5.

Results

cs HSP70 pool in human ECs

We first confirmed the occurrence of a basal csHSP70 pool in EC (Kim et al. 2016) through cell-surface biotinylation assays followed by western analysis of HSP70 (Fig. 1).

Modulation of HSP70 expression in EC under shear stress

In blood vessels, the major physiological hemodynamic force acting in endothelium is shear stress (SS) (Nigro et al. 2011). Physiological arterial levels of laminar SS are generally considered atheroprotective, whereas disturbed or low-level laminar shear patterns are well accepted to correlate with proatherogenic conditions and vascular dysfunction (Yurdagul et al. 2016). Thus, we investigated intra- and extracellular HSP70 levels in

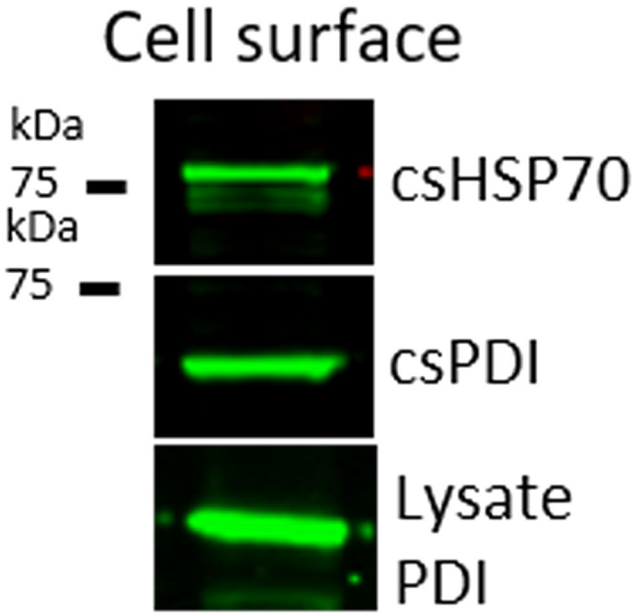

Fig. 1 A pool of HSP70 is externalized by endothelial cells. HUVECs were incubated in PBS for 5 min following by cell-surface biotinylation assay for analysis of anti-HSP70 or anti-PDI antibodies

EC submitted to either physiological (11 dynes/cm^2) or low laminar SS (4 dynes/cm^2, LSS). EC proteomic analysis was performed as part of a larger investigation addressing differential regulation under LSS vs. physiological SS (Venturini et al., unpublished observations). Such analysis revealed that an important anti-thrombotic protein, thrombomodulin, was robustly decreased under LSS (Fig. 2a) as expected (Sperry et al. 2003). In contrast, the levels of GRP78/Bip and GRP94, which are respectively the ER-located HSP70 and HSP90 paralogs, were unaffected, similarly to PDIA1 (Fig. 2a, b). Investigation of these proteins was performed in other experiments to provide technical and conceptual cross-comparisons, since some PDIs and GRP78 functions at the cell surface have been well investigated (Takemoto et al. 1992; Wiest et al. 1997). Remarkably, the total cytosolic inducible HSP70 expression diminished under LSS compared with physiological SS (11 dynes/cm^2) (Fig. 2a). These data were confirmed through western analysis with specific antibodies (Fig. 2b).

A classical response of EC under SS is enhanced nitric oxide production via endothelial nitric oxide synthase, associated with vessel dilation (Vanhoutte et al. 2016). In line with this, the levels of nitrite in the EC conditioned medium were significantly higher under physiological SS as compared with LSS (Fig. 3a). We further quantified csHSP70 levels in these experiments. Importantly, csHSP70 expression under LSS was decreased compared with physiological SS (Fig. 3b). Moreover, the significant upregulation (vs. static condition) of HSP70 expression observed with physiological SS and LSS (Fig. 3c), further with increased intracellular HSP70 expression in EC exposed to SS compared to LSS. In this assay, we further validated the proteomic results using a different antibody which recognizes only inducible form HSP701A/1B, in contrast with the antibody in Fig. 2b, which recognizes two members: HSC70 and inducible HSP70. Therefore, levels of csHSP70 and intracellular HSP70 are decreased in LSS (Fig. 3), an effect not observed for PDI.

Thrombomodulin and csHSP70: evidences for interaction and functional modulation

Expression of thrombomodulin was reportedly increased by overall HSP70 induction in aortic tissue (Allende et al. 2016) or EC (Uchiyama et al. 2007); however, the specific role of csHSP70 in thrombomodulin activity is unknown. We first investigated whether thrombomodulin and HSP70 co-localize at the surface of non-permeabilized EC. Confocal fluorescence microscopy revealed a partial colocalization of both proteins (Fig. 4a), indicating that they share the same region at the cell surface. While one cannot rule out a small degree of permeabilization during the fixing step, the staining patterns clearly differ between the non-permeabilized and permeabilized cells. Indeed, co-localization is evident in the non-permeabilized condition (Fig. 4a). We then assessed if cell-surface thrombomodulin and HSP70 can interact physically, using proximity ligation assay technology and co-immunoprecipitation. These experiments detected a positive interaction signal in non-permeabilized EC (Fig. 4b). Furthermore, we showed that immunoprecipitation of thrombomodulin brings a fraction of HSP70 (Fig. 4c).

To investigate functional consequences of csHSP70-thrombomodulin interaction, we assessed if extracellular HSP70 modulates thrombomodulin activity through measurement of protein C activation (APC activity) in the presence of specific validated inhibitory antibody against HSP70 (Gutiérrez et al. 2010). We used two distinct antibodies which recognize either the nucleotide binding domain (9920,

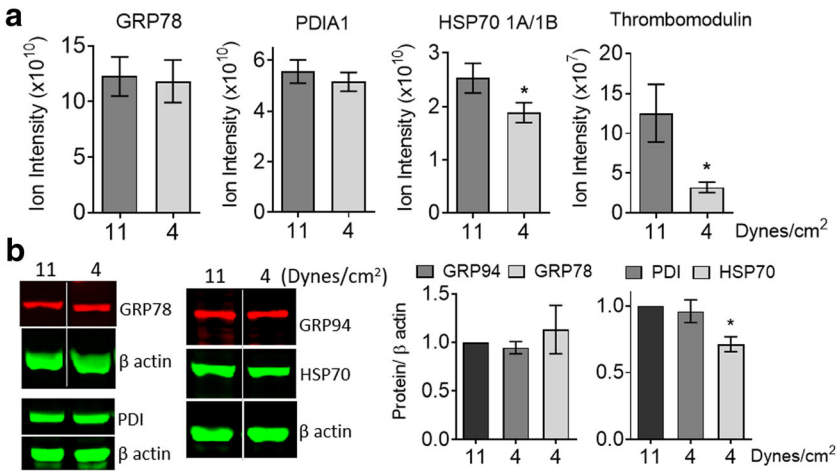

Fig. 2 Intracellular HSP70 levels decrease after low laminar shear stress. **a** Primary HUVECs were exposed to physiological arterial-level laminar shear stress (11 dynes/cm^2) or low laminar shear stress (4 dynes/cm^2) for 24 h, followed by proteomic/mass spectrometry analysis, as described in detail in "Materials and methods." **b** Western analysis from **a** with anti-HSP70 (A3A), anti-PDI (RL90), anti-GRP78, or anti-GRP94. Data are representative of three separate experiments with mean ± SEM, *t* test with $*p < 0.05$ compared to 11 dynes/cm^2. The white bars represent cut western blot membranes from the same gel

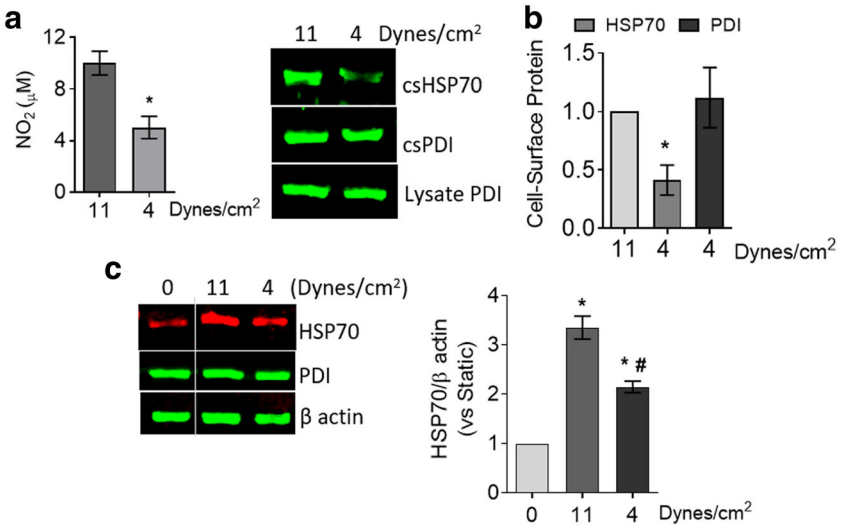

Fig. 3 Extracellular HSP70 levels are downregulated upon low laminar shear stress. **a** Primary HUVEC exposed to arterial-level physiological laminar shear stress (11 dynes/cm^2) or low laminar shear stress (4 dynes/cm^2) for 24 h, followed by nitrite measurement in conditioned medium using chemiluminescence-based *NO analyzer*. Data represent mean ± SEM from three separate experiments, *t* test with $*p < 0.05$. **b** The similar experiment in **a** followed by cell-surface biotinylation assay for analysis of anti-HSP70 (3A3) and anti-PDI antibodies. **c** Western blot analyses of intracellular pools using anti-HSP70-9920 which recognizes inducible HSP70 and anti-PDI. Primary HUVECs were incubated in 1%FBS at static condition (0 dynes/cm^2) as an essential requirement to sustain their viability after 24 h. Data represent mean ± SEM from three separate experiments, $*p < 0.05$

Millipore) or the substrate binding domain (3A3, Abcam). Neutralization of csHSP70 increased APC activity, suggesting that csHSP70 acts as inhibitor of thrombomodulin (Fig. 5). Of note, PDIA1 inhibition with the RL90 monoclonal antibody (Fig. 5) or BD34 monoclonal antibody (not shown) did not affect APC activity, as reported (Smith et al. 2015).

To further address such interaction, we performed similar co-localization experiments using an ex vivo protocol, since some in vivo interactions in whole vessels can differ from those observed in culture conditions. A similar co-localization between csHSP70 and thrombomodulin was observed in the EC monolayer from mice aorta (Fig. 6a) through *en face* multicolor immunofluorescence imaging. We also found that extracellular HSP70 inhibition increases aorta thrombomodulin activity (Fig. 6b) and that proatherogenic shear stress decreased HSP70 content in aortic arch compared with thoracic aorta (Fig. 6c) which is associated with in vivo physiological shear stress. The distinct pattern of inhibitory effects of 9920 vs. 3A3 antibodies on APC activity in Fig. 5 vs. Fig.6b assays may be due to the type of preparation, endothelial cells vs. whole tissue. Overall, our finding that diminished expression of HSP70 accompanied conditions associate with vascular pathology, such as low shear stress (LSS), fits into such a paradigm of HSP70-mediated vasculoprotection.

Discussion

We showed for the first time the systematic screening of intracellular HSP70 (Fig. 2) and csHSP70 (Fig. 3b) and HSP70 pool downregulation in EC (Figs. 2 and 3) and mouse aorta (Fig. 6c) exposed to proatherogenic *shear stress* (SS). In line with a previous report showing that after 6-h exposure to physiological SS the intracellular HSP70 levels rise from a static baseline (Wang et al. 2007), we showed that upregulation was also observed after 24 h of SS and is impaired by LSS (Fig. 3c). Accordingly, HSP70 mRNA has been reported to increase after arterial-level laminar shear stress compared with oscillatory shear stress (White et al. 2011).

In contrast with the extracellular pool of chaperones from the protein disulfide isomerase family, well recognized to support thrombosis initiation (Flaumenhaft and Furie 2016; Essex and Wu 2018), vascular functions of the extracellular HSP70 pool have been relatively unexplored. In fact, there are no described inhibitors specific to this pool (Shrestha et al. 2016). Meanwhile, potential csHSP70 receptors are still being unraveled; proposed receptors in antigen-presenting cells (LRP/CD91, CD40, TLR2, TLR4) (Henderson and Pockley 2012) were shown not to support HSP70 cell-surface binding (Thériault et al. 2005). On other hand, lectin receptors Siglec-5 and Siglec 14 (Fong et al. 2015), as well as the scavenger receptors LOX-1 (Thériault et al. 2005); SRA-I, FEEL-I, and SREC-I (Murshid et al. 2011); and more recently CLEC14a (Jang et al. 2017) were shown to bind csHSP70. It is unknown, however, to what extent the stability of such binding contributes to HSP70 retention at the cell surface.

We showed here that csHSP70 is distributed in a cluster-like pattern in EC (Fig. 4a) and that csHSP70 interacts with thrombomodulin in cultured EC (Fig. 4b, c). A reproducible co-localization between HSP70 was found in cultured EC and

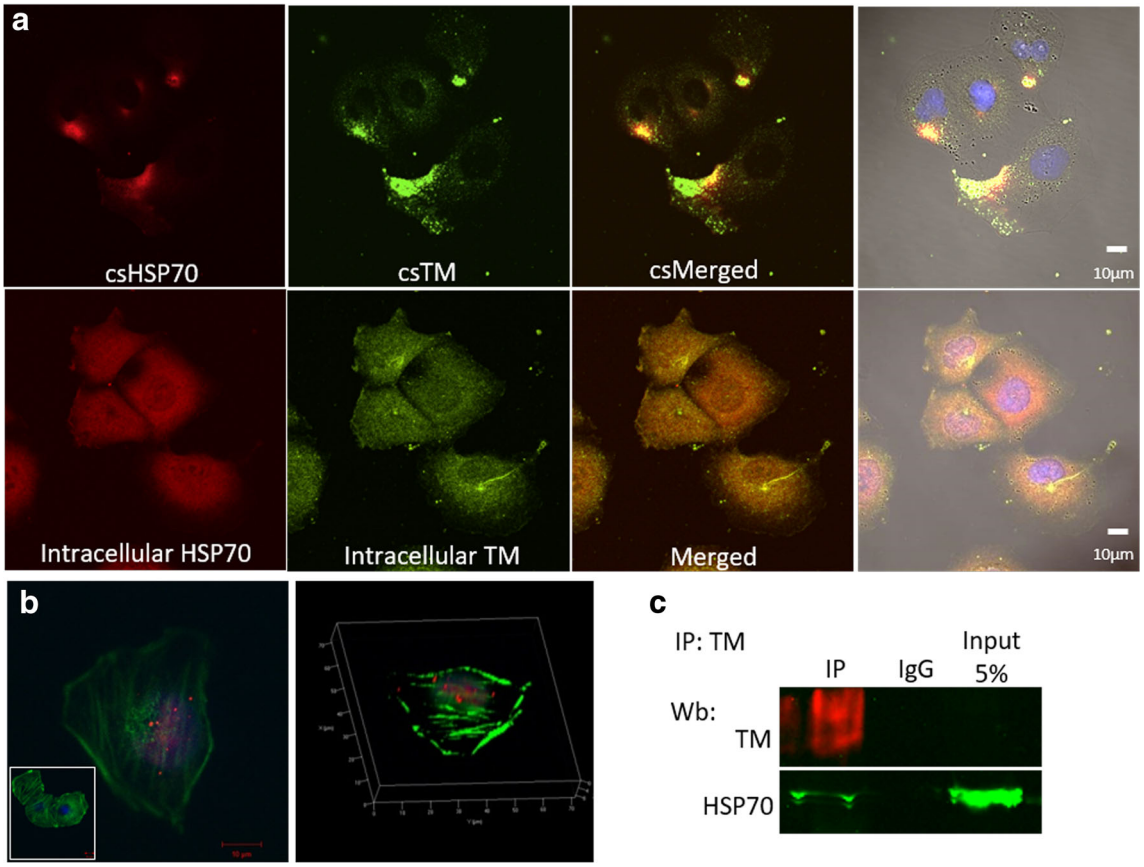

Fig. 4 HSP70 interacts with thrombomodulin at endothelial cell-surface. **a** Immunofluorescence staining of HSP70 and TM (thrombomodulin) in non-permeabilized or permeabilized HUVEC (intracellular) for 1 h in serum-free medium. Data are representative of three separate experiments. **b** Representative confocal image of proximity ligation assay analysis showing the interaction between HSP70 and TM in non-permeabilized HUVECs. Positive signal of protein interaction is represented as red dots; nuclei are stained with DAPI (blue) and F actin with phalloidin (green). The inset confocal image shows PLA negative controls in which primary antibodies were omitted. The right panel depicts 3-D reconstruction of confocal z-stack. **c** Immunoprecipitation assay in basal HUVEC, representative of three separate experiments

ex vivo in the abdominal mouse aorta (Figs. 4a and 6a) reinforcing its possible in vivo importance. The finding that csHSP70 neutralization significantly enhanced thrombomodulin activity, as measured by APC assay, was unexpected considering the upregulation of csHSP70 by an atheroprotective hemodynamic pattern (Fig. 5 and 6b). This is consistent with a possible specific effect of enhanced csHSP70 to limit EC thromboresistance and, conversely, with a protective effect in proatherogenic situations in which csHSP70 pool is underexpressed.

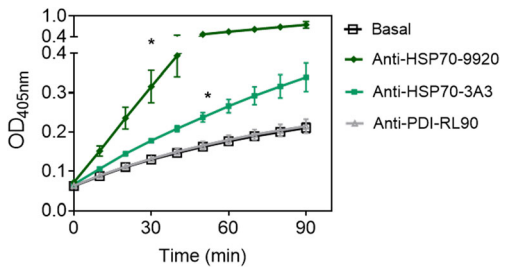

Fig. 5 csHSP70 restricts thrombomodulin activity in EC. Two-step protein C activation assay in primary HUVEC after incubation by 25 min with respective antibodies, anti-HSP70 (3A3, 1:100), anti-HSP70 (9920, 1:150), or anti-PDI (RL90, 2 µg/mL). Data represent mean ± SEM from three separate experiments, $*p < 0.05$, one-way ANOVA plus Student–Newman–Keuls

Thrombomodulin is a vasculoprotective integral membrane glycoprotein which is a crucial determinant of EC thromboresistance through anti-coagulant, anti-fibrinolytic, and anti-inflammatory effects (Martin et al. 2013). These actions support a robust anti-coagulant effect of thrombomodulin. An important structural feature of thrombomodulin is the presence of an amino terminal C-type lectin-like domain (CTLD) (domain 1) which connects to six epidermal growth factor (EGF)-like repeat domains and an extracellular serine–threonine-rich region (domains 2 and 3, linked to anti-coagulant effects). Of note, CTLDs are characteristic of LOX-1 and EGF-like domains of scavenger receptors, both previously shown to bind extracellular HSP70 (Murshid et al. 2011) possibly via hydrophobic motifs. CTLDs have, in fact, many hydrophobic regions accounting for potential HSP70 binding. We propose that HSP70 binding to CTDLs may lead to conformational changes affecting the EGF four to six domains, which are regions supporting protein C and thrombin binding. However, the possibility that HSP70 binds to domain 2/3 region of

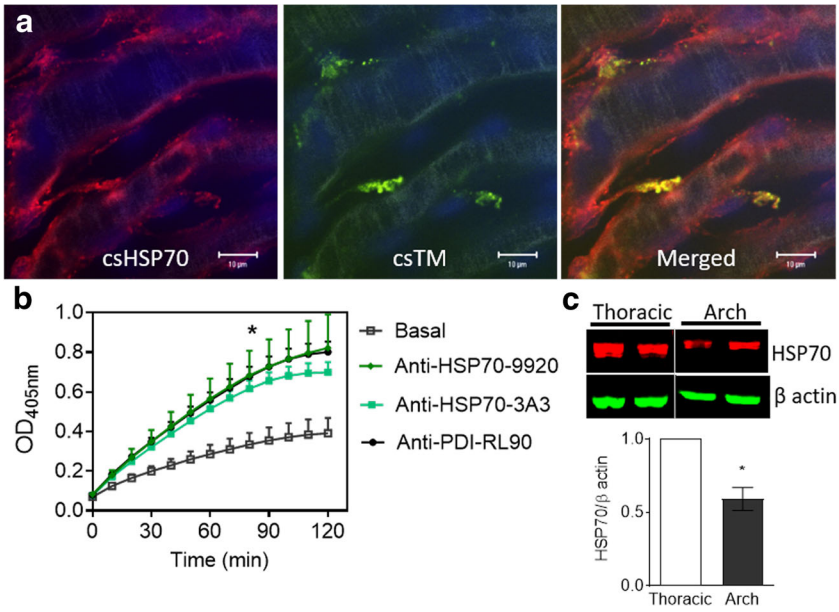

Fig. 6 csHSP70 restricts thrombomodulin activity in aorta. **a** *En face* staining of HSP70 (red) and thrombomodulin (TM, in green) and merged image (yellow) are seen in EC monolayer from mouse abdominal aorta. Nuclei (blue) and elastic fibers (clear threads) are depicted in all panels. Scale bar, 10 μm. Data are representative of four animals. **b** Two-step protein C activation assay in abdominal aorta. **c** Western blot analyses of intracellular pools using anti-HSP70-9920 in thoracic and arch aortas. Data represent mean ± SEM from three separate experiments, *t* test with $*p < 0.05$

thrombomodulin cannot be excluded, similar to other proteins (Mayer and Bukau 2005; Schlecht et al. 2011). Further studies are needed to reveal the mode of interaction between HSP70 and thrombomodulin. HSP70 is a versatile chaperone requiring several modulators including 53 HSP40 co-chaperones bearing J-domains responsible for stimulating ATPase activity and client protein delivery (Mayer 2018). Thus, we cannot rule out that partners such as HSP40 are required for HSP70 binding and regulation of thrombomodulin. In addition, another possibility is that HSP70 binds thrombomodulin within the secretory pathway and/or requires specific conformational thrombomodulin change.

Overall, our results highlight potentially important differences between the implications of intracellular and cell-surface pools of HSP70. In fact, one motivation of our study was the distinct reports of protective effects of total HSP70 pool, which are well supported by the nature of its intracellular effects (Mayer and Bukau 2005), vs. the apparently noxious roles of circulating HSP70 in several cardiovascular diseases. Our data suggest that csHSP70 may limit thromboresistance in stress situations associated with its increased pool. This mechanism can potentially explain some deleterious effects associated with high extracellular HSP70 levels in disease pathophysiology, including correlation with vascular calcification (Krepuska et al. 2011), pro-inflammatory actions in immune system (Henderson and Pockley 2012; Xu 2002), contributions to atherosclerosis development (Leng et al. 2013; Xu 2002) or to type2 diabetes (Krause et al. 2015), and biomarker of heart failure (Jenei et al. 2013). The recent proposal that the balance of intracellular and cell-surface effects of HSP70 likely determines the global phenotype (Krause et al. 2015) becomes specially fitting. Unraveling the precise mechanisms underlying HSP70-thrombomodulin interaction may reveal unsuspected pathways which potentially implicate selective extracellular HSP70 inhibition as a novel therapeutic modality in thromboinflammatory disease.

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Author contributions T.L.S.A. conceived the project, designed and performed most experiments, analyzed data, and wrote the manuscript; G.V. and A.C.P. designed, performed, and discussed shear stress-associated proteomic analysis; L.Y.T. performed *en face* immunofluorescence experiments; A.I.S.M. performed DUOLINK and immunoprecipitation experiments; and F.R.M.L. conceived the project, analysis results, discussed experiments, and wrote the manuscript.

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Compliance with ethical standards

Animal studies were performed in male C57BL/6 6-week old following approval from to Ethics Committee of the Heart Institute and School of Medicine from University of São Paulo, Brazil.

Conflict of interest The authors declare that they have no conflict of interest.

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