Vessel Remodeling

Peri/Epicellular Protein Disulfide Isomerase Sustains Vascular Lumen Caliber Through an Anticonstrictive Remodeling Effect


See Editorial Commentary, pp 488-489

Abstract—Whole-vessel remodeling critically determines lumen caliber in vascular (patho)physiology, and it is reportedly redox-dependent. We hypothesized that the cell-surface pool of the endoplasmic reticulum redox chaperone protein disulfide isomerase-A1 (peri/epicellular=pecPDI), which is known to support thrombosis, also regulates disease-associated vascular architecture. In human coronary atheromas, PDI expression inversely correlated with constrictive remodeling and plaque stability. In a rabbit iliac artery overdistension model, there was unusually high PDI upregulation (∼25-fold versus basal, 14 days postinjury), involving both intracellular and pecPDI. PecPDI neutralization with distinct anti-PDI antibodies did not enhance endoplasmic reticulum stress or apoptosis. In vivo pecPDI neutralization with PDI antibody-containing perivascular gel from days 12 to 14 post injury promoted 25% decrease in the maximally dilated arteriographic vascular caliber. There was corresponding whole-vessel circumference loss using optical coherence tomography without change in neointima, which indicates constrictive remodeling. This was accompanied by decreased hydrogen peroxide generation. Constrictive remodeling was corroborated by marked changes in collagen organization, that is, switching from circumferential to radial fiber orientation and to a more rigid fiber type. The cytoskeleton architecture was also disrupted; there was a loss of stress fiber coherent organization and a switch from thin to medium thickness actin fibers, all leading to impaired viscoelastic ductility. Total and PDI-associated expressions of β1-integrin, and levels of reduced cell-surface β1-integrin, were diminished after PDI antibody treatment, implicating β1-integrin as a likely pecPDI target during vessel repair. Indeed, focal adhesion kinase phosphorylation, a downstream β1-integrin effector, was decreased by PDI antibody. Thus, the upregulated pecPDI pool tunes matrix/cytoskeleton reshaping to counteract inward remodeling in vascular pathophysiology. (Hypertension. 2016;67:613-622. DOI: 10.1161/HYPERTENSIONAHA.115.06177.)

Key Data Supplement

Key Words: cytoskeleton ■ endoplasmic reticulum ■ hydrogen peroxide ■ protein disulfide isomerase ■ vascular remodeling

Vascular remodeling involves changes for the whole-vessel circumference and crucially determines lumen caliber in physio(patho)logical situations, such as shear-stress responses, restenosis postangioplasty,1−3 or native atherosclerosis.1,4 Moreover, cellular mechanisms governing conduit vessel remodeling are likely shared by small-vessel remodeling, for example, in hypertension.5 Despite such relevance, mechanisms of vessel remodeling are not well known. The identification of endothelium6 and nitric oxide 7 mediator roles of redox processes, as indeed supported by many subsequent studies.8,9 We showed previously that superoxide dismutase underactivity favors constrictive remodeling after injury (AI), whereas exogenous replenishment of SOD3(ecSOD) rescued bioactive nitric oxide from inducible NO synthase and normalized vessel caliber by counteracting constrictive remodeling rather than neointimal growth.5 The randomized clinical trial Multivitamins and Probucol Study showed that the antioxidant probucol significantly prevented restenosis post balloon angioplasty, essentially by preventing constrictive remodeling rather than neointima formation.9 Such redox-responsiveness is in line with the importance of redox pathways in cytoskeletal dynamics10 and extracellular matrix organization,11 which are both crucially involved in vessel remodeling. However, molecular determinants of such redox signaling pathways remain unclear.

Received July 21, 2015; first decision August 3, 2015; revision accepted November 16, 2015.
From the Vascular Biology Laboratory (L.Y.T., H.A.A., G.K.H., T.L.S.A., A.I.R., A.S.C., F.R.M.L.), Interventional Cardiology Unit (C.K.T., P.A.L.-N.), and Pathology Laboratory (P.S.G.), Heart Institute (InCor), University of São Paulo School of Medicine, São Paulo, Brazil.
The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.115.06177/-/DC1.
Correspondence to Francisco R.M. Laurindo, Vascular Biology Laboratory, Heart Institute (InCor), University of São Paulo School of Medicine, Av Eneas C. Aguiar, 44, Annex 2, 9th Floor, São Paulo, CEP 05403-000, Brazil. E-mail francisco.laurindo@incor.usp.br
© 2016 American Heart Association, Inc.
Hypertension is available at http://hyper.ahajournals.org
DOI: 10.1161/HYPERTENSIONAHA.115.06177

© 2016 American Heart Association, Inc.
Several evidences suggest that the endoplasmic reticulum (ER) chaperone protein disulfide isomerase (PDI) is involved in redox homeostasis and signaling. PDI, a thioredoxin superfamily protein, is a 20-member family of multifunctional redox chaperones. The canonical role of PDI family members is to assist in disulfide bond formation/isomerization in nascent ER proteins. We showed, through loss- and gain-of-function experiments, that PDI associates with and functionally regulates growth factor–induced NADPH oxidase activation in vascular smooth muscle cells. Moreover, PDI is required for Nox1-dependent vascular smooth muscle cell migration, associated RhoGTPase activation, and cytoskeletal organization. Convergence between PDI and NADPH oxidases also occurs in macrophages and neutrophils. Such effects of PDI are part of growing evidences that have implicated the ER in redox pathophysiology. In particular, signaling of the unfolded protein response because of ER stress involves upstream and downstream oxidant or reductive processes, which can mediate both proadaptive or proapoptotic responses. Overt disruption of redox homeostasis, however, leads to cell death during the unfolded protein response. In addition to the ER pool, there is substantial evidence for an extracellular PDI pool that is actually composed by cell surface–attached PDI (epicellular) plus secreted PDI (pericellular). As pericellular PDI are often indistinguishable, we coined the term peri/epicellular (pec) PDI to designate the extracellular pool. PecPDI mediates many thiol redox-dependent processes, including virus internalization, galectin binding, metalloproteinase regulation, integrin signaling, and platelet aggregation. Importantly, pecPDI sustains injury-associated thrombosis, and inhibitors of PDI family proteins are among the most innovative antithrombotic strategies. Moreover, pecPDI exerts predominant thiol reductase activity and mediates transnitrosation reactions that govern nitric oxide bioavailability.

Given the redox sensitivity of vessel remodeling, the involvement of PDI in redox signaling and homeostasis and, in particular, the characteristics of pecPDI discussed above, we hypothesized that the pecPDI pool regulates vascular remodeling. Here, we investigated such hypothesis using multiple approaches.

**Methods**

Detailed methods are available in the online-only Data Supplement. This study was approved by local Scientific Research/Ethics Committees.

In human coronary artery specimens from 20 consecutive necropsies in patients dying from acute myocardial infarction, left anterior descending and circumflex segments were classified as negative/constructive remodeling or outward/positive remodeling based on their internal elastic lamina area, defined against respective artery segments with lumen preservation, assumed as reference controls as reported.

Iliofemoral overdistension injury was performed by a coronary-type balloon catheter in normolipidemic male rabbits under fluoroscopy, with balloon: vessel ratio of 1.2, which is well within the range of analogous models and human interventions. At day 12 AI, the arterial segment was isolated, and the pecPDI pool was evaluated. The injury site was then examined for signs of pecPDI overexpression.

Cell-surface thiols were labeled by membrane-impermeable thiol-alkylator Alexa-Fluor maleimide (3 μmol/L per 20 minutes at 4°C). For immunospin trapping, freshly removed arteries were incubated with 40 mmol/L 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in the absence of serum (30 minutes at 37°C), followed by immunodetection of DMPO-protein adducts with specific antibody. Vascular hydrogen peroxide production was assessed using Amplex Red assay. Cell-surface biotinylation of extracellular-free thiols was performed after labeling with 200 μmol/L 3-(N-maleimidopropionyl) biocytin.

All data are means±SEM. Comparisons were performed by paired Student’s t test, 1-way ANOVA with Newman–Keuls post hoc or 2-way ANOVA with Bonferroni post hoc test using GraphPad Prism 6.0 (GraphPad Software Inc, La Jolla, CA). Significance level was 5%.

**Results**

**PDI Expression Inversely Correlates With Constrictive Remodeling in Human Coronary Plaques**

To address whether PDI correlates with remodeling of human coronary artery atheromas, PDI immunoreactivity was assessed in autopsy atheroma specimens from coronary heart disease patients (see Methods section of this article). There was enhanced PDI expression in media from plaques exhibiting outward remodeling (Figure 1A and 1B) and lower PDI expression in media/intima of plaques exhibiting constrictive remodeling (Figure 1D–F). These data correlate PDI expression to vessel remodeling in a disease-relevant model. To further investigate this process, we addressed an experimental injury model.

**Intracellular and PecPDI Are Markedly Overexpressed During Vascular Repair AI**

A previously validated model of balloon injury/repair-induced arterial remodeling was used. In this model, 75% overall caliber loss occurs by day 14 AI, with 50% loss occurring between days 7 and 14 AI, mainly through constrictive remodeling. Although PDI mRNA levels peaked at 7 days AI and decreased thereafter (Figure 2A), PDI protein expression further enhanced over time, reaching ca. 25-fold increase 14 days AI (Figure 2B) across all vessel layers (Figure 2C). PDI overexpression also occurred after rat carotid injury (Figure S1). ER chaperones Grp78, Grp94, and calreticulin were increased robustly, indicating ER stress (Figure 2D; Figure S3). However, compared with PDI, their increase was 10-fold lower and peaked at 7 days AI, decreasing/stabilizing thereafter. PDI overexpression during vascular repair seems unrelated solely to ER stress.

To investigate pecPDI pool expression in injured arteries, immunofluorescence experiments were performed without permeabilization, showing strong pecPDI increase at 14 days AI (Figure 2E). This PDI staining pattern was clearly diverse from that observed after permeabilization, as well as from that of cortactin (control for potential cortical protein artifacts; Figure S2). Experiments with extracellular thioprotein biotinylation depicted increased pecPDI in arteries 14 days AI (Figure 2F). Although mechanisms underlying PDI externalization remain unclear, the classical Golgi-dependent route is unlikely because incubation of artery segments with brefeldin-A did not inhibit and actually accentuated pecPDI

**Constrictive Remodeling in Human Coronary Plaques**

To address whether PDI correlates with remodeling of human coronary artery atheromas, PDI immunoreactivity was assessed in autopsy atheroma specimens from coronary heart disease patients (see Methods section of this article). There was enhanced PDI expression in media from plaques exhibiting outward remodeling (Figure 1A and 1B) and lower PDI expression in media/intima of plaques exhibiting constrictive remodeling (Figure 1D–F). These data correlate PDI expression to vessel remodeling in a disease-relevant model. To further investigate this process, we addressed an experimental injury model.

**Intracellular and PecPDI Are Markedly Overexpressed During Vascular Repair AI**

A previously validated model of balloon injury/repair-induced arterial remodeling was used. In this model, 75% overall caliber loss occurs by day 14 AI, with 50% loss occurring between days 7 and 14 AI, mainly through constrictive remodeling. Although PDI mRNA levels peaked at 7 days AI and decreased thereafter (Figure 2A), PDI protein expression further enhanced over time, reaching ca. 25-fold increase 14 days AI (Figure 2B) across all vessel layers (Figure 2C). PDI overexpression also occurred after rat carotid injury (Figure S1). ER chaperones Grp78, Grp94, and calreticulin were increased robustly, indicating ER stress (Figure 2D; Figure S3). However, compared with PDI, their increase was 10-fold lower and peaked at 7 days AI, decreasing/stabilizing thereafter. PDI overexpression during vascular repair seems unrelated solely to ER stress.

To investigate pecPDI pool expression in injured arteries, immunofluorescence experiments were performed without permeabilization, showing strong pecPDI increase at 14 days AI (Figure 2E). This PDI staining pattern was clearly diverse from that observed after permeabilization, as well as from that of cortactin (control for potential cortical protein artifacts; Figure S2). Experiments with extracellular thioprotein biotinylation depicted increased pecPDI in arteries 14 days AI (Figure 2F). Although mechanisms underlying PDI externalization remain unclear, the classical Golgi-dependent route is unlikely because incubation of artery segments with brefeldin-A did not inhibit and actually accentuated pecPDI
(Figure S4A). Moreover, PDI externalization was enhanced by tunicamycin-induced ER stress, as detected by extracellular biotinylation (Figure S4B) or immunofluorescence (Figure S4C). The latter also showed pecPDI colocalization with α2-integrin, a known PDI-regulated cell surface/transmembrane protein. Thus, ER stress may contribute to PDI externalization during vessel repair.

Neutralization of PecPDI Does Not Enhance ER Stress

To address vascular pecPDI effects, we performed loss-of-function experiments using well-validated neutralizing antibodies: either the RL90 (Thermo-Pierce) or 34(BD) clones. Because intracellular PDI supports protein folding and protects against ER stress, we addressed whether pecPDI affects ER stress. Neutralization of pecPDI in cultured rings from control or injured arteries (for model validation see Figure S6) did not enhance ER stress–related KDEL-chaperones, nor did it alter differentiation or proliferation markers (Figure 3A–3C). In contrast, downregulation of total PDI pool via siRNA in cultured rings from injured vessels decreased calponin expression, increased proliferating cell nuclear antigen expression, and upregulated KDEL-chaperones and apoptosis, whereas uninjured vessel rings were unaltered (Figure S5).

We also investigated pecPDI-dependent effects in redox processes known to alter vascular remodeling and biomechanics. The increased hydrogen peroxide production because of vessel injury/repair was attenuated by pecPDI neutralization (Figure 3D), whereas in vivo pecPDI neutralization abrogated immunospin-trapping signals for DMPO-protein radical adducts, which is consistent with lower reactive oxygen species (ROS) production (Figure 3E). Overall, pecPDI silencing yields specific effects that do not simply mirror the total PDI pool.

PecPDI Counteracts Constrictive Remodeling AI

To investigate the role of pecPDI in vascular remodeling in vivo, we performed perivascular incubation of PDIAb using pluronic gel at day 12 AI and evaluated the arterial lumen after 48 hours (14 days AI). For validation, we used immunofluorescence with secondary antibody only to document homogeneous PDIAb uptake in arteries 48 hours after gel application (Figure 4A). Importantly, pecPDI immunoneutralization with the RL90 (Figure 4B) or the BD34 (Figure S7) antibody induced an average 25% decrease in lumen caliber at arteriography. In this model, caliber loss results predominantly from constrictive remodeling, but neointima growth could also participate. Thus, we performed optical coherence tomography (Figure 4D), which not only confirmed PDIAb-induced lumen loss but also importantly showed that such effect was because of smaller total vessel circumference rather than decreased wall thickness (Figure 4E). Histological analysis confirmed that PDIAb did not change neointimal size (Figure 4C). Vasomotoricity was unlikely to affect vascular caliber assuming that nitroglycerin was administered before arteriography or tomography. Thus, our findings indicate a novel anticonstrictive remodeling effect of pecPDI. Importantly, PDIAb in control intact arteries affected none of those variables (Figure S8).
Disrupted Matrix/Cytoskeletal Architecture Accounts for PDI-ab-Induced Inward Remodeling

Collagen matrix\(^2\) and cytoskeletal restructuring\(^1,6,7\) are cornerstones of vascular remodeling. We investigated pecPDI effects in arterial collagen under polarized light. In PDI-ab-exposed arteries 14 days AI, there was a shift toward thick strongly birefringent red fibers (resembling type I collagen) and lower density of thin weakly birefringent green fibers (resembling type III collagen), suggesting a stiffer collagen matrix (Figure 5A). In parallel, PDI-ab promoted remarkable collagen architecture changes; particularly, collagen bundle orientation was switched from normal circumferentially aligned fibers toward radially oriented fiber patches (yellow arrows, Figure 5A).

PecPDI inhibition also promoted cytoskeletal disruption that was expressed by more irregular stress fiber orientation; also, there was 30% decrease in the coherence coefficient of medial actin fibers, indicating their lower cell-to-cell alignment. Neointima actin coherence was decreased versus media but unaffected by PDI-ab (Figure 5B). Hessian matrix–based analysis (Methods section in the online-only Data Supplement) post injury showed increased stress fiber number and thickness after PDI-ab versus IgG exposure (Figure 5D; Figure S10). Overall, this suggests impaired stress fiber anchoring distribution or turnover rather than formation per se. Elastic fiber amount/morphology was unaffected by PDI-ab (not shown). Collectively, these results support pecPDI roles in sustaining outward remodeling via matrix/cytoskeleton structural reorganization.

Changes in Viscoelastic Properties of Injured Arteries After PecPDI Inhibition

To investigate vascular architecture–associated viscoelastic properties, we assessed isometric tension response to externally applied stretching. PDI-ab, both in organ culture (Figure S11) and in vivo (Figure 5E), accentuated loss-of-tension poststretch (viscoelastic stress relaxation) in injured, but not in intact arteries. In progressive tension protocols, despite having no effect on rupture tension, PDI-ab exposure decreased distance to rupture (Figure 5F). These data reinforce that pecPDI counteracts exacerbation of vessel wall stiffness.
PecPDI Inhibition Disables β1-Integrin and Focal Adhesion Kinase Signaling in Injured Arteries

Integrins, which support cytoskeleton and matrix remodeling, are redox/pecPDI-regulated in platelets and endothelial cells. Thiol reductants generally mediate integrin activation. We addressed whether β1-integrin is a pecPDI target in our model. In injured arteries, pecPDI neutralization decreased extracellular thiol levels (Figure 6A). PDIAb decreased both total and immunoprecipitated β1-integrin (Figure 6B). Importantly, levels of reduced β1-integrin were less detectable by cell-surface biotinylation after PDIAb (Figure 6C). In addition, FAK-Tyr397 phosphorylation, a known integrin-dependent target, was attenuated, both in culture and in vivo (Figure 6D; S12). Intriguingly, pecPDI inhibition also downregulated the mRNA expression of both focal adhesion kinase (FAK) and β1-integrin (Figure S13). PecPDI neutralization did not alter the mRNA expression of collagen (I and III) and proteins involved in collagen synthesis, such as transforming growth factor-β and connective tissue growth factor (Figure S13). Together, this suggests that pecPDI effects on collagen (Figure 5A) predominantly relate to maturation rather than synthesis.

Discussion

We show that PDI is markedly overexpressed during vascular injury repair and pecPDI pool coordinates the extracellular matrix and cytoskeletal architecture to preserve lumen caliber and counteract inward remodeling. PecPDI neutralization does not exacerbate ER stress. Constrictive remodeling on pecPDI neutralization is associated with decreased oxidant levels and marked disorganization of stress fibers and collagen, which translates into lower vessel ductility. β1-integrin and FAK are likely targets of pecPDI effects. In parallel, PDI expression inversely correlates with inward remodeling and stability in human plaques.

The unusually high PDI overexpression during vascular repair, which is seemingly coordinated across distinct cell types, is surprising and reminiscent of a fundamental tissue repair process. Mechanisms orchestrating this response deserve further investigation and may relate, at least in part, to the severe ER stress during vessel repair because sustained ER stress is often associated with increased PDI expression in several diseases, including atherosclerosis. Our data (Figure 2) as well as recent studies from our group (T. Araujo et al, unpublished data, 2015) suggest that pecPDI levels directly correlate with total PDI expression. This is consistent with the correlations between total PDI and vessel remodeling observed in human specimens (Figure 1). Although our study focused on PDIA1, we cannot rule out possible cross-talk with PDI family members in vessel lumen modulation. Indeed, such cross-talk is expected based on the complementary specificities of distinct PDIs. Moreover, Erp57 (PDIA3) is reportedly involved in thrombosis and a known nonspecific target of the RL90 antibody. In our study, distinct neutralizing anti-PDIA1 antibodies that are targeted against the whole protein (RL90) or to a specific sequence of the final α and initial β domains (BD34) induced similar vessel lumen loss. In addition, Erp57 expression was negligible compared with pecPDI (data not shown). Together, these considerations speak against any significant role of Erp57 in our model.

Previously reported vasculoprotective effects of PDI focused mainly on prosurvival intracellular PDI effects in ER dysfunction associated with oxidized lipids and hypoxia. Our data add to other specific effects of pecPDI pool, which include immune reactions, pathogen response, and thrombosis. Subcellular routes of PDI externalization remain elusive. Endothelial PDI externalization is an early response-to-injury signal, supporting integrin-mediated platelet accumulation, fibrin formation, and neutrophil recruitment. Platelet pecPDI, in turn, enhances integrin-mediated adhesion and supports
thrombosis in mice. In our model, platelet and leukocyte accumulation are no more evident at day 14 AI, although they play significant roles at earlier stages. Thus, pecPDI may have specific roles at distinct stages of vascular repair.

Mechanisms underlying pecPDI effects on vessel caliber seemingly involve tonic extracellular matrix/cytoskeletal remodeling because PDIAb effects occurred after short-term exposure. Switching to a more rigid collagen type and to its radial fiber orientation (Figure 5A) probably translates into disrupted intrawall force distribution and energy dissipation, as suggested by findings reported with matrix metallopeptidase-13 knockdown. In parallel, marked cytoskeletal disorganization on pecPDI neutralization in vivo was evident from several results (particularly Figure 5D). Such disturbed matrix/cytoskeletal pattern correlates with decreased vessel ductility (Figure 5E and 5F), that is, the capacity to deform without rupture and enhanced stress relaxation. An important question is whether pecPDI affected cytoskeletal signaling as a specific, repair-associated signal or through an upstream effect as a general mechanoresponsive mediator. We provided evidence for the latter with the observation that stress fiber induction in vascular smooth muscle cells undergoing cyclic stretching (10%–12%) as well as in endothelial cells submitted to laminar shearr-stress was prevented by pecPDI neutralization (Figure 6E). This suggests that pecPDI may behave as a general regulator of mechanoadaptation across distinct cell types; this intriguing possibility deserves further investigation. In parallel, these results highlight one limitation of our study, which is the lack of discrimination of the specific vessel wall cellular targets of PDIAb.

Redox processes are candidate mediators of pecPDI effects in vessel remodeling, in line with emerging roles of PDI in Nox/redox signaling and known anticonstrictive remodeling effects of SOD3 during vascular repair. PecPDI neutralization reduced hydrogen peroxide, which is a mediator able to sustain vessel caliber either directly or via endothelial nitric oxide synthase. Hydrogen peroxide depletion enhances vascular stiffness. Vascular repair augmented extracellular protein thiols, together with enhanced oxidant generation, whereas pecPDI neutralization decreased extracellular thiols (Figure 6A) despite lowering oxidant production (Figure 3D and 3E). This apparent paradox means that ROS production might sustain PDI reductase activity or that PDI-dependent ROS generation and thiol reductase activity occur in distinct spatial/temporal compartments. Cell-surface ROS sources are yet unclear, although Noxes are natural candidates. Indeed, Nox inhibition prevents microparticle-released PDI reductase activity. Importantly, PDI/pecPDI downregulation may directly affect the cytoskeleton given the recently reported complexation between PDI and β-actin cysteine. Finally, increased ROS production converges with integrin activation.
The finding that β1-integrins and FAK phosphorylation are likely targets of pecPDI is relevant because integrins connect the extracellular matrix/extracellular environment to the cell cytoskeleton. Importantly, integrins are redox sensitive, and distinct integrin subunits can be (re)activated by reducing agents.27 Accordingly, as PDI has been reported to interact with F-actin (Alexa-Phalloidin635), quantified in equally divided areas along media and neointima through the coherence coefficient, calculated using orientation-J plugin from Image-J, with elastic fibers excluded from quantifications. C, Coherence coefficient in media/neointima; P<0.05 vs InjIgG; n=3. Scale bar, 20 μm. D, F-actin fiber pattern analysis using Hessian algorithms along transversal vessel wall lines (dotted lines in B), depicting fiber thickness distribution according to phalloidin-fluorescence intensity. Graphs are means of 6 transversal line measurements from 4 independent experiments. The 10th percentile values were 38±3, 6±1, and 20±2 for control, InjIgG, and InjPDIAb, respectively (P<0.05 for all comparisons). E, Isometric tension loss in pretensioned arterial rings recorded for 180 s during passive relaxation; P<0.05 vs CtrIgG and CtrPDIAb, n=4 to 5. F, Arterial rings were rapidly tensioned until rupture or failure to sustain tension, and distance to rupture was measured; **P<0.05 vs InjIgG, n≥5. PDIAb, PDI antibody.

Figure 5. Peri/epicellular protein disulfide isomerase (PecPDI) reshapes the matrix and cytoskeleton architecture in injured arteries. In vivo experiments as in Figure 4. A. Picrosirius-stained sections for collagen analyses on polarized light. Color graph depicts quantitations of red/orange (collagen I) or green/yellow (collagen III) fibers identified with filter adjustments. *P<0.05 vs InjIgG, n=3. B, F-actin alignment analysis in injured arteries stained for F-actin (Alexa-Phalloidin635), quantified in equally divided areas along media and neointima through the coherence coefficient, calculated using orientation-J plugin from Image-J, with elastic fibers excluded from quantifications.

Perspectives
We showed that pecPDI is overexpressed during vascular repair response and supports vascular caliber preservation through an anticonstrictive remodeling effect, which is associated with reshaping of actin stress fibers and collagen in a way to prevent enhanced vessel stiffness. β1-integrin is a likely pecPDI redox target in these effects. Because vascular remodeling pathways are relatively understudied, the identification of a novel molecular mechanism of vascular remodeling is relevant to clarify how processes involved in vascular...
pathology affect the lumen caliber. Such processes include vascular smooth muscle cell activation, matrix/cytoskeletal reorganization, and integrin signaling, and are also shared by arteriolar remodeling, for example, in hypertension, indicating further potential implications for our results. Because pecPDI has been crucially implicated in arterial thrombosis, pecPDI is a plausible connection between mural thrombosis and vascular remodeling, as indeed suggested by the observed correlation between PDI expression with human atheroma remodeling (Figure 1) and instability (Figure S14). Overall,
pecPDI effects further underscore reported evidence implicating redox processes as remodeling mediators.

Acknowledgments
We are grateful to Ana L. Garippo for technical support.

Sources of Funding
This study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP no. 09/51654-5, 10/06360-0, and 13/06241-0), Centro de Pesquisa, Inovação e Difusão FAPESP (CEPID Processos Redox em Biomedicina, no. 13/07937-8), Instituto Nacional de Ciência, Tecnologia e Inovação (INCTRedoxoma, CNPq), and Fundação Zerbini.

Disclosures
None.

References


What Is New?
- We identified that overexpressed peri/epicellular protein disulfide isomerase counteracts constrictive vascular remodeling during vessel repair after injury.
- Peri/epicellular protein disulfide isomerase effect is associated with redox β1-integrin regulation and involves cytoskeleton and matrix restructuring, which balances vessel stiffness.

What Is Relevant?
- Mechanisms of vascular remodeling are understudied, and the identification of a novel molecular mechanism able to orchestrate such events opens new perspectives.

Novelty and Significance

Summary
Peri/epicellular protein disulfide isomerase immunoneutralization during vascular injury repair induces lumen narrowing because of whole-vessel remodeling that involves reorganization of the extracellular matrix and actin cytoskeleton architecture. This effect involves peri/epicellular protein disulfide isomerase–mediated thiol reductase activity, reactive species generation, and it is associated with β1-integrin regulation. In human coronary plaques, PDI expression directly correlates with remodeling.
Peri/epicellular protein disulfide isomerase sustains vascular lumen caliber through an anti-constrictive remodeling effect

Leonardo Y. Tanaka
Haniel A. Araújo
Gustavo K. Hironaka
Thaís L. S. Araujo
Celso K. Takimura
Andres I. Rodriguez
Anelise S. Casagrande
Paulo S. Gutierrez
Pedro Alves Lemos-Neto
Francisco R. M. Laurindo

SUPPLEMENTAL MATERIAL

Supplemental material includes the following:

1) Expanded Materials and Methods Section
2) Table S1
3) Figures S1 to S14, with legends
EXPANDED MATERIALS AND METHODS

Reagents

All chemicals were from Sigma-Aldrich, unless specified. Alexa maleimide, Amplex red, Reverse Transcriptase kits, Sybr Green, Phalloidin and fluorescent-red TUNEL kit were from Invitrogen; OCT Tissue Tek embedding compound was from Fisher Scientific or Sakura; primary antibodies and dilutions used for immunobloting (IB), immohistochemistry (IHC) or immunofluorescence (IF) were from: Abcam (PCNA, 1:1000 IB; Integrin beta1, 1:1000 IB; Enzo (mouse-PDI, 1:1000 IB /IHC, 1:200 IF; rabbit-PDI, 1:200 IF; KDEL, 1:1000 IB); RD (total-FAK, 1:1000 IB); Santa Cruz (pospho-FAKtyr497 1:500 IB); Sigma (β-actin, 1:5000 IB; Calponin, 1:1000 IB) and Thermo-Pierce (PDI, 1:1000 IB/IF). Secondary anti-mouse and anti-rabbit antibodies conjugated with HRP-peroxidase for IB (Calbiochem); visible spectrum-fluorescein for IF (Invitrogen) and infra-red spectrum-fluorescein for IB (Licor) and Vectastain Elite ABC System for IHC (Vectastain). Culture medium DMEM, penicillin (100mg/mL), streptomycin (100 mg/ml) and trypsin were from Gibco BRL-Life Technologies; FBS was from Cultilab, collagenase type IV and elastase were from Worthington Biochemical Corporation. Non-immune mouse-IgG was from Santa Cruz or Thermo-Pierce. For surgical procedures: Ketamine hydrochloride (Ketamina®) and sodium pentobarbital (Tiopental®) were from Cristália;xylazine hydrochloride (Rompun®, Bayer).

Ethical approval

This study was approved by the Scientific Research and Ethics Committees, under the protocol number #1086/09, of the Heart Institute and School of Medicine, respectively, University of São Paulo, Brazil.

Human arterial specimens

We examined coronary artery specimens from the collected from 20 consecutive necropsies performed between 2008 and 2011 at the Pathology Laboratory, Heart Institute (InCor), University of São Paulo Medical School. All patients died from acute myocardial infarction. For histological analysis, sections were cut perpendicularly to the long axis of the artery in a serial mode, approximately every 10 mm, in the left anterior descending (LAD) and left circumflex (LCX) coronary arteries. For classification of the type of remodeling present in the more distal sections, the internal elastic lamina (IEL) area of each section was compared with the IEL area of the reference section and to the expected amount of “normal” arterial tapering. Positive (outward/expansive) remodeling was defined as an increase in IEL area in successive sections. Negative (inward/constrictive) remodeling was identified as a decrement in IEL area, relative to the adjacent proximal sections, that was greater than the anticipated change with normal vessel tapering, according to described. Imunohistochemistry analysis was performed with a mouse-monoclonal anti-PDI antibody (Enzo, 1:1000). For quantification of histological assay staining, captured photomicrographs were analyzed with an image analysis system (ImageProPlus 5.1, Media Cybernetics, Rockville, MD).

Rabbit iliac-femoral artery injury model

Normolipidemic male New Zealand White rabbits weighing 3-4 kg were anesthesized with ketamine (25 mg/kg)/xylazine (5 mg/kg) IM and kept anesthetized during surgical procedures with sodium pentobarbital (10-20 mg/kg in fractioned doses) IV. The right carotid artery was dissected and a 4F vascular sheath was inserted within the artery under direct view. Unfractionated heparin (250 IU/kg) was administered, and a hydrophilic wire guide was directed toward descending aorta under fluoroscopic guidance by means of digital angiography equipment (Phillips BV Pulsera). A Balloon angioplasty catheter (30 mm in length and 3.0 mm in inflated diameter) was introduced over the guide,
positioned at the beginning of femoral artery and inflated to 8 atm for 60 seconds (3 times with recovery of 30 seconds). Balloon-to-artery ratio was approximately 1.2. After both right and left arteries were injured, the carotid artery was ligated and benzatine-penicillin and gentamycin were given. At the 12th after injury, rabbits were again anesthetized and right and femoral arteries were dissected and exposed for perivascular delivery of 4 ml pluronic gel 25% (w/v) preloaded with 1 µg/mL of mouse-IgG (IgG, Santa Cruz or Thermo-Pierce) or anti-PDI (PDI Ab, clone RL.90, Thermo-Pierce or clone 34, BD). After 48 h, (day 14AI), rabbits were anesthetized as described above and left carotid artery was dissected for in vivo measurements: angiography and optical coherence tomography. Thereafter, rabbits were euthanized with sodium pentobarbital and iliac and femoral arteries were excised, cut into rings and either separated for immediate analysis or frozen in liquid nitrogen or OCT compound.

For organ culture and other experiments, a similar model was used, except that iliac and proximal femoral segments were injured through retrograde introduction of the balloon angioplasty catheter from a distal femoral artery branch. Balloon overdistention was performed as above. After selected times (4, 7 or 14 days AI), rabbits were euthanized with sodium pentobarbital and iliac and femoral arteries were excised, immediately frozen in liquid nitrogen and stored or processed for tissue culture experiments (in this case, always 14 days AI).

**Histology and immunohistochemistry (IHC)**

Arterial segments were fixed in buffered formalin (4%) for 24 h, included in paraffin and cut into 3µm-thick slices. For structural analysis, the segments were removed after perfusion-fixation at physiological pressures. Hematoxilin-eosin (HE), Picrosirius red and Verhoeff stainings were performed to analyze intima-media ratio, collagen and elastin, respectively. Collagen was evaluated using circularly polarized light coupled to Leica microscope. Polarizing filter was first positioned to identify red/thick fibers and turn to green/thin fibers. All parameters were captured and measured using Image J software (NIH). Analyses of open empty space area and elastin were performed with Leica microscope and Qwin software (Leica).

For IHC, sections were trypsin-treated for antigen retrieval, blocked with BSA 5%, incubated overnight with primary mouse anti-PDI (Enzo, 1:1000) and them with secondary (1:2000, Zymed, Invitrogen). Immunostaining was detected with 3,3'-diaminobenzidine (DAB). Images were recorded in Leica Optical Microscope and quantified using Image J software. All of parameters were analyzed by a blinded observer.

**Immunofluorescence (IF)**

Tissue IF was performed in cryosections (10 µm) from OCT-included arteries. Briefly, slices were put in silanized slides, fixed in cooled acetone (-20°C for 20 min); permeabilized or not with Triton 0.1% (10 min at RT); blocked with bovine albumin serum (4%BSA for 2 hours at RT); incubated overnight with primary antibodies and thereafter with fluorescein-conjugated secondary antibodies (1:200 v/v, Invitrogen) and nuclei marker DAPI (20 µg/mL, Invitrogen). Slides were mounted in PBS/glycerol (1:1 v/v). In some experiments aimed at testing specificity of peri/epicellular antibody labeling, IFs were performed before OCT inclusion. In this case, cryosectioning was performed under light protection and acetone incubation was omitted.

**Biotinylation protocols for reduced pecPDI or cell-surface proteins**

Freshly removed iliac arteries at baseline control or 14 days AI (5mm/~30mg) were washed with saline buffer, followed by incubation in serum free medium (45 min). Free thiol were labeled with 200 µmol/L membrane-impermeable 3-(N-maleimidopropionyl) biocytin; (MPB) for 1 hour at 4°C. Unreacted MPB was quenched twice with 400 µmol/L GSH (15 min at 4°C) and remaining
sulfhydryl groups were quenched with 800 µmol/L iodoacetamide for 10 minutes at room temperature. Biotin-labelled tissue was lysed (50 mmol/L Tris/HCl, pH 7.4, containing 1% (v/v) Triton X-100, 150 mmol/L NaCl) by at least 2 hours in the presence of protease inhibitors (1:100 aprotinin, 1:100 leupeptin, 1:50 PMSF). The samples were sonicated and incubated with streptavidin magnetic beads (2µg/mL; Promega) overnight at 4°C. Beads were washed three times with saline buffer to remove unspecific bead bound-proteins and boiled in Laemmli buffer for 5 min. All bead bound-proteins were subjected to SDS/PAGE and western blotting using different antibodies against PDI or antibody against biotin- HRP conjugates, to detect total surface thiol-proteins.

Tissue culture and siRNA experiments

After excision, iliac and femoral arteries were cleansed from connective tissue and washed several times in sterile phosphate-buffered saline (PBS, pH 7.4) supplemented with antibiotics (penicillin 100 U/mL and streptomycin 100 µmol/L) and cut in rings (2-3 mm). For siRNA experiments (primer sequences at Table S1), arterial rings were maintained in full Dulbecco's modified Eagle medium (DMEM) with antibiotics and fetal bovine serum (FBS, 10%) for 12 h, starved (DMEM without serum) for additional 12 h and changed to transfection medium (without serum and antibiotics) with mix of siRNAs against PDI (PDIsiRNA205: 33.3 nmol/L; PDIsiRNA449: 33.3 nmol/L; and PDIsiRNA1217: 33.3 nmol/L) or Scrambled sequences (siScr449: 50 nmol/L; and siScr1217: 50 nmol/L) during 14 h with lipofectamine 10 µg/mL. Next, full DMEM was replaced and maintained for 48h for mRNA expression or 72h for protein expression. Inhibition of pecPDI was performed by 48-h incubation in full DMEM with 1µg/mL of anti-PDI (clone RL90, Thermo-Pierce) or non-immune mouse anti-IgG (Santa Cruz or Thermo-Pierce).

Immunoprecipitation and Western analysis

Arterial segments (pulverized using mortar and pestle under liquid nitrogen) were incubated in lysis buffer (Tris 20 mM, pH 8, NaCl 137 mM, NP-40 1% and glycerol 10%), with protease inhibitors (aprotinin 10 µg/mL, leupeptin 10 µg/mL, PMSF 1 mM), briefly sonicated and maintained for 1h on ice for protein solubilization with gentle mix. Tissue debris was pelleted (centrifugation 12,000 rpm for 20 min at 4°C) and protein supernatant quantified with Bradford method. For immunoprecipitation, 100µg protein were incubated overnight (4°C) with mouse anti-PDI 2.5µg/ml (Enzo) with gentle mix. Magnetic beads with protein G were added and incubated for additional 4h. Protein/antibody complex was precipitated through magnetic separation, washed (3x) with lysis buffer containing protease inhibitors and incubated in non reducing Laemmli sample buffer. Samples were boiled (100 ºC, 10 min) and separated by electrophoresis in polyacrylamide gel (8-15%) in denaturing condition. Proteins were transferred to a nitrocellulose membrane using semi-dry or in specific cases wet conditions (total/phosphoFak and β1 integrin). Nonspecific binding was blocked with non-fat milk 5% (2h at RT); primary antibodies were incubated overnight (4°C); fluorescein (LI-COR) or peroxidase-conjugated (Invitrogen) secondary antibodies incubated for 1h. Antibody-protein complex was detected by fluorescent scan (Odysssey) or luminescent detection (GE). Protein loading was corrected by β-actin expression and quantified using Odyssey software (LI-COR) or Scion software (NIH). Except for β1 integrin, protein detections were performed under reducing conditions.

Quantitative polymerase chain reaction (Q-PCR)

Vascular rings were pulverized (as above) and mRNA extracted using RNA extraction kit (Invitrogen) according to manufacturer instructions. The mRNA concentration was measured using NanoDrop (Thermo-Scientific) and purity confirmed by absorbance ratio 260/280 > 1.8. Reverse transcription was performed using 1 µg mRNA and following manufacturer protocol (RT Invitrogen). PCR was assayed using cDNA, primers and SyberGreen in 96-well plates (Invitrogen). Selected
housekeeping gene was GAPDH. The mRNA expression was quantified using $2^{\Delta\Delta CT}$ method. The primers sequences were as follows: PDI (forward, CGGCTACCCCACGATCAA; reverse, CCTCCCTGCGAGCTGTGTAC) and GAPDH (forward, GGGCGTGAAACACGAGAA; reverse, GGTGCAGGATGCGTTGCT).

Amplex Red

Vascular hydrogen peroxide production was estimated using Amplex Red assay adapted from previous studies. Briefly, vascular rings (2-3 mm) were incubated with Amplex Red (100 µmol/L, Molecular Probes-Invitrogen) in Krebs-HEPES solution (pH 7.4) with 3 µg/mL of anti-PDI or IgG. Fluorescent oxidation product (resorufin) was detected during 60 min at 37ºC with 590 nm emission and 560 nm excitation in spectrofluorometer MI5 (Perkin Elmer). Additional experiments were performed including horseradish peroxidase (HRP 1 U/mL) into reaction mixture, but results were similar to those without HRP, which are reported in Fig. 3D. Controls with PEG-catalase were also assessed to validate the method.

Immuno-spintrapping

Freshly-collected arteries or tissue culture rings were incubated with DMPO (Sigma) 40mmol/L in starved DMEM (30 minutes at 37ºC). After PBS washes, segments were OCT-included and the IF detection of DMPO-protein adducts performed as described above, using a mouse-anti-DMPO protein adduct antibody, a kind gift from Dr. Ronald Mason (NIES, North Carolina). For method validation, controls with sodium hypochlorite or PEG-SOD were performed.

Angiography

Following intraarterial nitroglycerin infusion (200 µg), angiography (Phillips BV Pulsera) was performed using contrast/saline solution (1:1 v/v). Arteriograms were recorded before, during, immediately after and 14 days after injury. Caliber diameter analysis was performed at 5 interspaced sites along balloon-treated segments and calculated by a blinded observer, using image J software.

Optical coherence tomography

The optical coherence tomography catheter DragonflyTM (St. Jude Medical) was positioned at the balloon-injured location, as confirmed by previously recorded angiography. Automatic pullback scan (St Jude Medical) was performed during physiological saline solution (0.9% NaCl) intraarterial infusions. Nitroglycerin and papaverin were administered before scanning to minimize vasospasm. Lumen, wall and vessel area were measured in 5 fixed positions by a blinded observer using St. Jude software.

Vascular viscoelastic properties

Vascular isometric tension responsiveness was tested in different conditions to infer mechanical alterations. Short-term stabilization (5 min) was allowed in organ bath with saline solution containing 100 µmol/L papaverin at 0.5g resting tension. Vascular rings (~4 mm in length) were stretched by rapid manually-induced distention and passive relaxation was recorded during 3 min. Control and PDIAb-treated injured artery segments were tested in parallel every experiment. Tension and distance-to-rupture (corrected by dry weight) were calculated after progressive stretching until reaching incapacity to maintain tension or in some cases rupture.

Cell culture model

Primary vascular smooth muscle cells (VSMCs) were isolated from thoracic aorta of control rabbits. After cleansing of connective tissues, aorta was digested in collagenase type-IV (165 U/mL),
elastase (15 U/mL) and trypsin inhibitor (0.375 mg/mL) for 2-3 h at 37°C in full DMEM. Next, pre-digested aorta was cut in small pieces, endothelium was gently removed and fragments were put in gelatin (0.1%)-treated plates with luminal side facing the plate and cultivated with full DMEM. When VSMCs reached local confluence, fragments were removed and cells were trypsinized and passed to bottles for cell expansion.

**VSMC cyclic stretch protocol**

Briefly, 1x10^5 cells were cultivated in 6-well plates on collagen I flexible membranes (Flexcell plates) during 24 h in full DMEM. Thereafter, cells were starved (FBS 0.1% for 12 h), medium was changed to DMEM without FBS with anti-PDI or anti-IgG 1µg/mL for 1 h before stretch/static period. Finally, cells were submitted to each condition during 24 h (static; or stretch 12%, 1 Hz). At the end, medium was collected, centrifuged (2000 rpm, 10 min 4°C) and cells were lysed in specific buffers or fixed (paraformaldehyde 4%) for immunofluorescence studies.

**F-actin alignment**

For analysis of actin alignment in phalloidin-labeled injured sections, the whole media and intima were selected and the plugin Orientation J from image J software calculated the directional coherence of the actin fibers, in which a value close to 1, representing slender ellipse, indicates stronger coherence coefficient of alignment in the direction of the ellipse long axis.

**Actin fiber analysis**

Fiber analysis methodology was based as previously reported. F-actin from arteries were labelled with phalloidin, imaged by confocal detection and selected for analysis using ImageJ’s “line tool” through the region of interest (ROI). Pixel Intensity along the line was plotted using the “Plot Profile” tool. In tissue samples we conducted six series of scans for each image. The scans were performed perpendicular to the actin fiber orientation. Fluorescent labeled fibers can be represented as curvilinear structures with local intensity variations (minima or maxima). Such intensities can be detected using a Hessian matrix, which describes the second-order information or curvature of these intensity variations. Using an image-filtering based on a Hessian matrix allows to extracts line-like information from the input image. Fluorescence 8-bit images information were extracted through a Hessian-based filter in NIH ImageJ (version 1.44) software plugin, FeatureJ, using the following parameters “Largest eigenvalue of Hessian tensor” option, “Absolute eigenvalue comparison” option, and set the “Smoothing scale” factor to 0.5. This setting generates a resulting image of largest eigenvalues after comparing the absolute values of the Hessian matrices. Separation of the actin fibers was determined following nearest-neighbor deconvolution. Intensity matrices were processed through a slope peak-detection formula. The total number of peaks was then divided by the length of each line to yield the average number of peaks per mm scan. Additionally, fiber intensity histogram was plotted based on fiber/peak intensities measured in each line scan normalized to maximum values obtained in a particular image. The bin size for the intensity histogram was 2 a.u. (arbitrary unit of normalized fluorescence intensity).

**Cell-surface thiols**

Immediately after excision or tissue culture protocol, the arterial rings were washed in OR2 buffer (NaCl 92 mmol/L, KCl 2.5 mmol/L, MgCl₂ 1 mmol/L, HEPES 10 mmol/L, pH 7.2) and incubated with membrane-impermeable thiol-chelating fluorescent probe AlexaFluorMaleimide (ALM 3 µmol/L for 20 min at 4°C). After 3 washes (OR2 buffer), segments were included in OCT. Cryosections were collected under protection from light and slides mounted in medium containing DAPI. For method validation, controls with DTT or catalase were performed.
Statistical Analyses

Data are presented as mean±standard error(SEM). Comparisons were performed by paired Student t test, one-way ANOVA with Newman-Keuls post-hoc or two-way ANOVA with Bonferroni post-hoc test using GraphPad Prism 6.0 (GraphPad Software Inc., CA, USA). Significance level was 5%.

References for supplemental methods section

## SUPPLEMENTAL TABLE

### Table S1. List of siRNAs against PDI (PDIsiRNA) or control scrambled (siScr) sequences

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>siSCR449</td>
<td>GACUUACAGUGGUCAGUCGAAGAGA</td>
<td>UCUCUUCGACUGACCACUGUAAGUC</td>
</tr>
<tr>
<td>siSCR1217</td>
<td>GAGCCGGGUUUAGCAGAAGAUGAGA</td>
<td>UCUCAUCUUCUGCUAAACCCGGCUC</td>
</tr>
<tr>
<td>PDIsiRNA205</td>
<td>CAAGCACCUGCUGUGGAGUUCUAU</td>
<td>AUAGAACUCCACCAGCAGGUCUUG</td>
</tr>
<tr>
<td>PDIsiRNA449</td>
<td>GACGACAUUGUGAACUGGCUGAAGA</td>
<td>UCUUCAGCCAGUUCACAUAUGUCGUC</td>
</tr>
<tr>
<td>PDIsiRNA1217</td>
<td>GAGCCGGGUUUAGCAGAAGAUGAGA</td>
<td>UCUCAUCUUCUGCUAAACCCGGCUC</td>
</tr>
</tbody>
</table>
**FIGURES S1 TO S14**

**Fig. S1.** PDI is overexpressed in rat model of vascular repair. Immunohistochemistry of PDI in control carotid artery and 14 days after injury with balloon catheter. Image magnification 10x, scale bar 200µm; n=4.

**Fig. S2.** Arterial pectorPDI does not colocalize with cortactin in control and injured arteries. Control intact arteries (upper panels) and arteries collected 14 days AI after in vivo perivascular application of control mouse-immunoglobulin (IgG, 1µg/mL, middle panels) or neutralizing mouse-monoclonal anti-PDI (PDIAb, clone RL90, 1 µg/ml, bottom panels) in pluronic gel (25%) at 12th day AI and analyzed 48h later. Artery segments were processed for immunofluorescence without permeabilization for detection of PDI (left panel, Alexa546 nm, red) and cortactin (middle panel, Alexa488 nm, green). Merging images are shown at right panels; n=3. Scale bar, 20 µm.
Fig. S3. Endoplasmic reticulum induction during vascular repair. Rabbit iliofemoral arteries undergoing angioplasty-type balloon overdistention were analyzed 7, and 14 days after injury (AI) for expression of KDEL-chaperones Grp78, Grp94, and calreticulin. Graphs show protein quantification as injured/control(Ctr) ratios. Data are mean±SEM; *p<0.05 vs.Ctr n≥5.
Fig. S4. Mechanisms of pecPDI externalization do not dependent of the classical route and occur in ER-stress conditions. (A) Control iliac arteries were treated with brefeldin (1 µg/ml, 24h) and immunofluorescence detection of PDI (red, 546nm) in non-permebilized slides were performed. Image magnification 40x, scale bar 20 µm. (B) Basal and 16h-tunicamycin control iliac arteries were biotinylated with MPB and extracellular PDI expression was detected by western blot. Similar results were observed at least in two independent experiments. (C) Control intact iliac arteries were treated with tunicamycin (Tn 2 ug/ml) and analyzed during different time points (1, 4 and 16h) by PDI immunofluorescence (Alexa546nm,red) and integrin alpha2 (Alexa488nm, green) in non-permebilized slides. Nuclei were labeled with DAPI (blue) and merged images as shown at right panels (Merge). Image magnification 40x, scale bar 20 µm.
Fig. S5. PDI{\textit{siRNA}} silencing increases markers of ER stress and proliferation and decreases differentiation in injured arteries. Rabbit iliofemoral artery segments collected at baseline or 14 days AI transfected with PDI{\textit{siRNA}}(siPDI) or scrambled control (siSCR), analyzed after 48h for: (A): PDI mRNA; (B): CHOP/GADD153 mRNA; or after 72h for: (C): calponin, (D): PCNA protein expression; (E): KDEL-chaperone protein expression; (G): analysis of TUNEL-positive cells. Quantitative data for western-blots from B, C and Dare provided in (F). mRNAs was normalized for GAPDH and protein for β-actin. All variables except TUNEL+ cells were expressed in relation to Ctr siScr; *p<0.05 vs. Control; **p<0.05 vs. Ctr siPDI; and #p<0.05 vs. Inj siScr n ≥3.
Fig. S6. Viability of vascular rings maintained in culture. Viability of vascular rings maintained in culture. Vascular reactivity in organ bath for rabbit carotid artery rings that were either freshly removed or cultured during 96h (Cult). Panel A: Contraction to phenylephrine $10^{-6}$ M. Graph depicts the maximal tension, expressed in grams per mg dry weight; n=3. Panel B: Phenylephrine-contracted rings were relaxed with cumulative concentrations of acetylcholine ($10^{-10}$ to $10^{-4}$ M); n=3. Graph depicts the maximal relaxation reached; n=3. There were no significant differences between freshly-removed and Cult with both agonists. Vascular reactivity in organ bath for rabbit carotid artery rings that were either freshly removed (FR) or cultured during 96h (Cult) in full DMEM as in other experiments. (A): Contraction to phenylephrine $10^{-6}$ mol/L. Graph depicts the maximal tension, expressed in grams per mg dry weight; n=3. (B): Phenylephrine-contracted rings were relaxed with cumulative concentrations of acetylcholine ($10^{-10}$ – $10^{-4}$ mol/L); n=3. Graph depicts the maximal relaxation reached; n=3. Non-significant differences were observed between FR vs. Cult with both agonists.

Fig. S7. PecPDI neutralization with BD34PDIAb in vivo induces similar vascular caliber loss vs. RL90 PDIAb. In vivo perivascular delivery of pluronic gel loaded with either neutralizing anti-PDI antibody (PDIAb BD34, 1µg/mL) or control IgG between days 12 and 14AI. Graph depicts average of 5-8 measurements along all length of injury area; *$p<0.05$ vs. InjIgG, n=3.
**Fig. S8.** Inhibition of pecPDI promotes negligible changes of vascular caliber in control arteries. In vivo perivascular application of neutralizing mouse-mono-clonal anti-PDI (PDI Ab, clone RL90, 1 μg/mL) or control mouse IgG (1 μg/mL) in pluronic gel (25%) in non-injured control rabbit iliofemoral arteries analyzed after 48 h similarly to protocols used for injured arteries in the present study. Quantifications of optical coherence tomography measurement of lumen area (A), total vessel area (B) and wall to lumen ratio is shown in (C); expressed in relation to IgG-treated arteries n=4.

**Fig. S9.** Inhibition of pecPDI does not alter actin-cytoskeleton orientation in the neointima of injured arteries. F-actin alignment, quantified in equally-divided areas (yellow boxes) along whole neointima through the coherence coefficient and calculated using “orientation-J” plugin from Image-J. Vessel layers were identified by phase-contrast, and elastic laminae were excluded from quantifications. Graphs showing coherence coefficient in neointima.
Fig. S10. PecPDI supports cytoskeletal organization in injured arteries. Morpho-functional F-actin parameters were measured from phalloidin-labelled tissues using Hessian matrix-based analysis (for detailed description, please see methods). Graphs corresponding to quantitation of Figure 5D analysis depicting fiber density of medial (A) and neointimal (B) actin filaments from injured arteries exposed in vivo to mouse-IgG or PDIAb. Results are mean of six measurements from three independent experiments. *p<0.05 vs. InjIgG.

Fig. S11. PecPDI inhibition in vitro alters viscoelastic properties of injured arteries. PecPDI inhibition in tissue culture in experiments analogous to those of Fig. 4B. Arterial rings were mounted in organ bath system in physiological saline solution (0.9%) with papaverine 100 μmol/L. After short-term stabilization (0.5 g resting tension), 1 g-tension was manually applied and loss of isometric tension was recorded during 180s. *p<0.05 vs. InjIgG n=4.
Fig. S12. PecPDI inhibition in vitro decreases injury-induced upregulation of FAK phosphorylation. Phosphorylated/total FAK analyzed in tissue culture of control and injured arteries incubated with mouse-IgG or PDIAb (1µg/ml, 48h). Graph depicts p-FAK quantification corrected for β-actin and expressed in relation to Ctr IgG; n=3-4, p<0.05 vs. InjIgG.
Fig. S13. PecPDI inhibition in vitro decreases injury-induced upregulation of beta1 integrin and FAK mRNA. PecPDI inhibition in tissue culture in experiments analogous to those of Fig. 4B. Quantitative PCR analyzes of beta1 integrin, FAK, TGF-β, CTGF, collagen I and collagen III were corrected for GAPDH and expressed as ratio versus control IgG. N=4-5; p<0.05 vs. Inj PDI Ab.
Fig. S14. Intimal PDI expression in human coronary plaques inversely correlates with plaque stability. Immunohistochemical detection of PDI in left anterior descending or left circumflex coronary arteries of human specimens, with proximal segments assumed as reference controls (RC) were compared with atheromas presenting stable/fibrotic plaque (Fib) or vulnerable phenotype (Vul). Plaques without calcification and lipid deposits and with thicker fibrous caps were considered as fibrous plaques and plaques with thin fibrous cap and large lipid cores as vulnerable plaques. Top images were acquired through 1x objective and bottom images with 10x objective; scale bar, 300µm. Graphs depict quantitation of PDI expression corrected for area of media (B) or intima (C). *p<0.05 vs. RC; n=4-6.