Protein disulfide isomerase increases in myocardial endothelial cells in mice exposed to chronic hypoxia: a stimulatory role in angiogenesis

Fei Tian,1* Xianghua Zhou,1,2* Johannes Wikström,3 Helen Karlsson,4 Helén Sjöland,5 Li-Ming Gan,5 Jan Borén,1,5§ and Levent M. Akyürek1,2§

1Sahlgrenska Center for Cardiovascular and Metabolic Research, Wallenberg Laboratory, and 2Institute of Biomedicine, Department of Medical Biochemistry and Cell Biology, University of Gothenburg, Göteborg, 3Bioscience, AstraZeneca Research and Development, Mölndal, 4Department of Molecular and Clinical Medicine, Linköping University, Linköping, 5Institute of Medicine, Department of Molecular and Clinical Medicine, University of Gothenburg, Göteborg, Sweden

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Protection of the heart against ischemic injury is one of the most important goals in cardiovascular research. Ischemic preconditioning has been shown to powerfully protect the heart against myocardial infarction (MI) (19, 24), and a number of studies have addressed the underlying mechanisms (23, 43). In addition to ischemic preconditioning, adaptive responses to chronic hypoxia increase cardiac protection against MI, as demonstrated both in experimental studies (25, 38) and in human populations living at high altitudes (28). Although this form of protection is less powerful and less understood than ischemic preconditioning, it persists for a much longer period of time (16, 25).

The enhancement of coronary blood flow by the induction of angiogenesis is a potential approach to preserve myocardial function (7). The increased blood supply might contribute to salvaging ischemic myocardium, limiting infarct size and improving cardiac function. There is considerable evidence to show that chronic hypoxia can stimulate angiogenesis in the brain (3, 12). However, there are conflicting results regarding the development of myocardial capillaries in animals exposed to hypoxia, with reports of both decreased (4, 36) and increased (22, 46) capillary density in chronically hypoxic animals. Thus it is not clear whether and to what degree chronic hypoxia induces myocardial angiogenesis.

We used a model of MI in mice exposed to chronic hypoxia to investigate the role of hypoxia-mediated angiogenesis in myocardial protection and to identify novel proteins that are upregulated in this process. Using a proteomics approach [two-dimensional gel analysis (2-DE)] to investigate proteins with an increased expression in the hypoxic myocardium, we identified protein disulfide isomerase (PDI), which is known to play a role in protein folding. Here we observed that chronic hypoxia for 3 wk resulted in improved survival of mice (from 64% to 83%), reduced infarction size (from 0.76 to 10.34%), and arteriolar density (from 162 to 22.0). In addition, the increased cardiac ejection fraction (from 19 ± 4% to 32 ± 4%, P < 0.05), increased coronary flow velocity under adenosine-induced hyperemia (from 58 ± 2 to 75 ± 5 cm/s, P < 0.05), myocardial capillary density (from 3,772 ± 162 to 4,760 ± 197 capillaries/mm², P < 0.01), and arteriolar density (from 8.04 ± 0.76 to 10.34 ± 0.69 arterioles/mm², P < 0.05) 3 wk after myocardial infarction. With two-dimensional gel electrophoresis, we identified that protein disulfide isomerase (PDI) was highly upregulated in hypoxic myocardial capillary endothelial cells. The loss of PDI function in endothelial cells by small interfering RNA significantly increased the number of apoptotic cells (by 3.4-fold at hypoxia, P < 0.01) and reduced migration (by 52% at hypoxia, P < 0.01) and adherence to collagen I (by 42% at hypoxia, P < 0.01). In addition, the specific inhibition of PDI by PDI small interfering RNA (by 46%, P < 0.01) and bacitracin (by 72%, P < 0.001) reduced the formation of tubular structures by endothelial cells. Our data indicate that chronic hypoxic exposure improves coronary blood flow and protects the myocardium against infarction. These beneficial effects may be partly explained by the increased endothelial expression of PDI which protects cells against apoptosis and increases cellular migration, adhesion, and tubular formation. The increased PDI expression in endothelial cells may be a novel mechanism to protect the myocardium against myocardial ischemic diseases.

E. F. Tian and X. Zhou equally contributed as first authors.
§ J. Borén and L. M. Akyürek equally contributed as senior authors.
Address for reprint requests and other correspondence: J. Borén, Sahlgren ska Ctr. for Cardiovascular and Metabolic Research, Wallenberg Lab, Bruna stråket 16, SE-413 45 Göteborg, Sweden (e-mail: jan.boren@wlab.gu.se).

Materials and Methods

Animals. Male C57BL/6 mice aged 6–8 wk (M&B, Ejby, Denmark) were housed with free access to standard laboratory food and water for at least 1 wk before the experiments. Animal experiments were approved by the Institutional Animal Ethics Committee in Gothenburg, Sweden.

MI following exposure to systemic hypoxia. Mice were placed in a normobaric Plexiglas chamber ventilated with nitrogen to gradually

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reduce the level of oxygen to 10% during an adaptation period of 30 min as described (33). The oxygen concentration was continuously monitored with an oxygen sensor (Model 600, Engineered Systems & Designs, Newark, DE) and maintained at 10%. Subgroups of mice were euthanized after 1 wk (n = 7) or 3 wk (n = 9) of exposure to hypoxia. A further subgroup underwent a ligation of the left anterior descending coronary artery (LAD) to produce MI, as described (21), after exposure to hypoxia for 3 wk (n = 23). Control mice were kept in room air and were euthanized before surgery (n = 15) or randomly assigned to undergo either sham surgery (n = 5) or coronary artery ligation (n = 33). All mice, regardless of systemic hypoxic or normoxic exposure before the ligation of LAD, were then kept at normoxia after surgery. Mice were euthanized with an overdose of pentobarbital sodium at the end of the experimental procedures. The left ventricles were separated, weighed, rapidly frozen in liquid nitrogen, and stored at −80°C until assayed or fixed in 4% paraformaldehyde (PFA) neutral-buffered solution at 4°C.

Echocardiography. Transthoracic color-Doppler echocardiography was performed on isoflurane-anesthetized mice as described (8) 1 day before and 1 day and 3 wk after MI. Ultrasound scanning was performed as described earlier (20). Coronary flow velocity (CFV) was recorded in the first diagonal branch of the ligated LAD before and after the infusion of adenosine (140 μg·kg⁻¹·min⁻¹ iv; ITEM Development, Stocksund, Sweden).

Cell culture studies. Human umbilical vein ECs (HUVECs) were cultured in EGM-2 medium (Cambrex, Karlskoga) that contained a mixture of hydrocortisone, human FGF-B, VEGF, R3-IGF-1, ascorbic acid, heparin, human EGF, and GA-1000. The cells were regularly passed to maintain exponential growth. All experiments were carried out between passages 3 and 7. After 48 h incubation, the cells were 90–95% confluent and suitable for transfection with small interfering RNA (siRNA). HUVECs were transfected with either scrambled negative control siRNA (Qiagen, Solna, Sweden) or PDI siRNA (Applied Biosystems, Foster City, CA) using electroporation previously reported (9). Peptides obtained after tryptic digestion were mixed with α-cyano 4-hydroxycinnamic acid (20 μg/ml) in 70% acetonitrile/0.3% trifluoroacetic acid as matrices and then spotted on a stainless steel target plate. Analyses of peptide masses were performed as previously described (15).

Histology and immunostaining. Measurements of the infarcted left ventricular areas were carried out by light microscopy coupled with a computerized imaging system (Axiovision 3.0, Carl Zeiss, Jena, Germany). Three tissue sections (5 μm thick) were taken from the apex to the base of the left ventricle and stained with Masson’s trichrome (13), staining viable areas red and necrotic tissues green. To account for the thinning of the infarcted myocardium, we applied the most widely used method for measuring infarct size: we measured the epicardial and endocardial circumference of the infarction for each section and divided this value by the total epicardial and endocardial circumference (13). Infarct size was then expressed as the percentage of the total left ventricular circumference.

To quantify the capillary and arteriolar density, the left ventricles were fixed in 4% PFA for 24 h, embedded in paraffin, sectioned to 5 μm thickness, and used for immunohistochemistry as described previously (44, 47). The cardiac sections were incubated overnight at 4°C with either rat anti-platelet EC adhesion molecule-1 (PECAM-1; Pharmingen, San Diego, CA) or mouse anti-α-smooth muscle actin (Zymed, San Francisco, CA). Negative control sections were incubated with the diluted blocking solution in the absence of primary antibodies. The capillary density was calculated by computer in five randomly areas captured from PECAM-stained sections from the endocardium through the epicardium of the midportion of the left ventricular free wall. In ligated coronary samples, the capillaries were counted similarly but in peri-infarct regions. The arterioles were counted in α-smooth muscle actin-stained sections in the entire left ventricular wall by two investigators blinded to the experimental setting. Arterioles were defined as vessels with an internal diameter of <50 μm that had at least one layer of smooth muscle cells; vascular structures without the presence of smooth muscle cells and a diameter of <10 μm were considered capillaries (45).

To colocalize PDI expression in myocardial ECs, serial frozen sections of infarcted left ventricles were immunofluorescently double labeled with rabbit anti-PDI (Stressgen, Ann Arbor, MI) or rat anti-PECAM-1 antibodies. An anti-rabbit IgG antibody served as a negative control for PDI staining. Secondary antibodies coupled with fluor dyes, donkey anti-rabbit Alexa 488 (green), and rabbit anti-rat Alexa 594 (red) were applied. Stained cells were fixed with 3% PFA, mounted with Vectashield mounting medium with 4,6-diamino-2-phenylindole (Vectorstain, Vector, Burlingame, CA), and analyzed using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss).

To study whether PDI was expressed by ECs, HUVECs were grown in slides with eight chambers to subconfluence before treatment. After 24 h exposure to hypoxia, the cells were fixed in 4% PFA for 30 min at room temperature, blocked with 1% BSA for 30 min at room temperature, and incubated for 1 h with an anti-PDI antibody and secondary Alexa-568 antibody. The images were captured using light microscopy (Axiovision 3.0).

Two-dimensional gel electrophoresis. Frozen left ventricle (50 mg) from mice exposed to hypoxia for 3 wk without undergoing MI was ground to a fine powder using liquid nitrogen and a cooled mortar and pestle. Powdered tissue was solubilized in 1 ml of 2-DE sample solution containing 4 mM Pefabloc (Roche, Basel, Switzerland) and sonicated for 3 × 10 s. Solubilized samples were incubated for 2 h at 4°C and then centrifuged for 1 h at 4 × 10⁵ g at 15°C. The supernatant was then saved for 2-DE analysis or stored at −70°C.

2-DE was performed using a horizontal 2-DE setup as described in detail previously (11). Separated proteins (400 μg) were fixed and detected by fluorescent staining according to the manufacturer’s protocol (SYPRO Ruby, Molecular Probes). 2-DE images were evaluated by spot detection, spot intensities, and pattern matching as previously reported (9). Peptides obtained after tryptic digestion were mixed with α-cyano 4-hydroxycinnamic acid (20 μg/ml) or 2,5-dihydroxybenzoic acid (40 μg/ml) in 70% acetonitrile/0.3% trifluoroacetic acid as matrices and then spotted on a stainless steel target plate. Analyses of peptide masses were performed as previously described (15).

Western blot analysis. Protein lysates were obtained from total cellular extracts of left ventricles from mice exposed to hypoxia for 3 wk without undergoing MI. HUVECs were washed twice with cold PBS before lysis in radioimmunoprecipitation assay buffer supplemented with protease inhibitor. Immunoblotting and densitometric analysis were performed as described (47). Signal intensities of the PDI protein were normalized to those of mouse actin protein (Sigma) as ratios to provide arbitrary densitometric units of relative abundance.

Detection of apoptosis. The quantification of apoptotic cells was carried out by annexin V binding assays according to the manufacturer’s protocols (Becton Dickinson Biosciences, Franklin Lakes, NJ). Briefly, the cells transfected with siRNAs were seeded in 60-mm culture plates and incubated at 37°C at either normoxia or hypoxia. After 24 h, the cells were collected in a centrifuge tube, washed with PBS, and double-stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide using the annexin V-FITC Apoptosis Detection kit. The stained cells were later processed by flow cytometry analysis (FACScan and LYSIS II software, Becton Dickinson). The cells that were annexin V positive and propidium iodide negative were considered apoptotic.

Migration assay. A migration assay was performed in a transwell Boyden chamber (Becton Dickinson) using a 1% gelatin-coated poly-carbonate filter with 8-μm pores. HUVEC suspension (0.1 ml; 5 × 10⁵ cells/ml diluted in serum-free EGM-2 medium) transfected with either scrambled negative control siRNA or PDI siRNA or pretreated with adenosine (140 μM) was added as a cell source to the upper compartment. The bottom compartment contained 0.5 ml of EGM-2 medium. The migration was allowed to proceed for 24 h, followed by a 3-h incubation with fluorescently labeled fibronectin. The migrated cells were then captured and analyzed by fluorescence microscopy.
with 3 mM bacitracin was added to the upper compartment of the chamber. The lower compartment contained 0.6 ml of EGM-2 medium with 2% of FBS. After 4 h incubation at 37°C in normoxia or hypoxia, nonmigrant cells were removed from the upper face of the transwell membrane with a cotton swab and the cells that migrated to the lower face of the membrane were fixed with methanol and stained with 4',6-diamidino-2-phenylindole. Migration results are presented as the mean number of cells per microscopic field at ×20 magnification.

**Adhesion assay.** The cell adhesion assay followed the manufacturer’s protocols (ECM cell adhesion kit, Chemicon, Temecula, CA). HUVECs transfected with either negative control siRNA and PDI siRNA were seeded in 96-well plates (Chemicon) coated with collagen I, II, and IV; fibronectin; laminin; tenascin; or vitronectin and incubated at normoxia or hypoxia for 90 min. The culture medium was then discarded, and the adhered cells were gently washed with assay buffer, stained for 5 min, and followed by three washes with deionized water. Absorbance was determined at 570 nm on a microplate reader. Three replicates were performed for each ligand.

**Matrigel assay.** Each well of Lab-Tek 8-well chamber slides (Nunc) was coated with a thin layer of 5 µl 100% Matrigel (BD Biosciences). HUVECs were either transfected with siRNA PDI or pretreated with bacitracin at a dose of 3 mM. The cell suspensions in EGM-2MV medium were mixed with an equal volume of 4% Matrigel in EGM-2MV to make a final concentration of 1 × 10^6 cells/ml. Cell suspension (400 µl) was added into each well and cultured overnight in quadruplicates. The cells were fixed with Diff-Quick fixative solution (Labex) and stained with 1:1 prediluted Diff-Quik solution II. Photos were taken by light microscopy. Four different high-power fields that nearly covered the majority of the structures were blindly counted for the number of circular HUVEC networks formed. Results are presented as the mean number of networks per viewing field at ×5 magnification.

**Statistical analysis.** Results are expressed as means ± SE. Differences between the groups were tested for statistical significance by two-way ANOVA. P values of <0.05 were considered statistically significant.

**RESULTS**

**Chronic hypoxia results in reduced body weight and increased cardiac weight and hemoglobin levels.** In mice exposed to chronic hypoxia for 3 wk before MI surgery, the body weight was lower than normoxic controls but the right ventricular weight/body weight was higher (Table 1). There was no significant alteration in the left ventricular weight/body weight between the groups. The hemoglobin level was higher in the hypoxia group (Table 1). Three weeks after MI surgery, the relative weight of the left ventricle was increased compared with sham surgery, but there was no difference between mice exposed to either normoxia or hypoxia (Table 1).

**Chronic hypoxia improves survival and reduces infarct size.** In the hypoxia-pretreated group, 19 out of 23 mice survived 3 wk after MI, whereas only 12 out of 33 survived in the normoxia group (Fig. 1A). All LAD-ligated but no sham-operated mice developed transmural infarction and subsequent scar formation in the left ventricle as detected by Masson’s trichrome staining (Fig. 1B). All mice that died were confirmed to have MI on postmortem examination. Three weeks after MI, the mice exposed to hypoxia had a reduced infarction size compared with the mice kept at normoxia (Fig. 1, C and D).

**Chronic hypoxia improves myocardial function and coronary blood flow.** Before MI surgery, the ejection fraction was higher in mice exposed to hypoxia for 3 wk than in normoxic mice (Fig. 2A). The ejection fraction decreased in both groups 1 day after MI and was higher in hypoxic mice compared with normoxic mice 3 wk after MI (Fig. 2A). Following 3 wk of systemic exposure before the MI surgery, there was a tendency toward a lower baseline CFV in hypoxic mice compared with normoxic mice, but this failed to reach statistical significance (Fig. 2B). Before surgery, the hyperemic CFV induced by the infusion of adenosine was lower in hypoxic mice compared with normoxic mice (Fig. 2C). The hyperemic CFV in both groups decreased 1 day after MI and increased to the pre-MI level 3 wk after MI (Fig. 2C). However, the hyperemic CFV 3 wk after MI was significantly higher in hypoxia-pretreated mice compared with normoxic mice (Fig. 2C).

**Chronic hypoxic exposure increases number of myocardial capillaries and arterioles.** The capillary density was higher in the myocardium from mice exposed to hypoxia for 3 wk compared with normoxic mice before MI surgery (Fig. 3A, left 2 panels) and maintained increased 3 wk after MI (Fig. 3A, right 2 panels). The arteriolar density in the myocardium was not statistically different between the mice exposed to normoxia or hypoxia for 3 wk before surgery, but it was significantly higher in hypoxia-pretreated mice 3 wk after MI compared with mice kept at normoxia (Fig. 3B). As left ventricular hypertrophy occurred during MI and increases in capillary and arteriolar density could be related to myocyte cell size but not growth of vasculature, we measured cardiomyocyte cross-sectional areas. The cardiomyocyte cross-sectional area in the peri-infarction area of mice 3 wk after MI was not significantly different between mice pretreated with hypoxia or normoxia for 3 wk [247.7 ± 10.3 vs. 233.4 ± 14.2 µm² (n = 6), P = 0.42], indicating that the increase in capillary density in hypoxia-pretreated MI mice represents true capillary growth.

**Table 1. Characteristics of mice exposed to normoxia or hypoxia for 3 wk and either euthanized directly or surgically treated to induce MI and euthanized after a further 3 wk**

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<tr>
<td></td>
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<tr>
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<td>Hemoglobin, g/l</td>
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Values are means ± SE; n, number of mice. MI, myocardial infarction; RV, right ventricle; LV, left ventricle. *P < 0.01 and †P < 0.001 vs. normoxia; ‡P < 0.01 and $P < 0.001 vs. normoxia + sham.
Differentially expressed proteins in left ventricles of mice exposed to chronic hypoxia. Among selected differentially expressed proteins, six were upregulated and three were downregulated in the left ventricle from mice exposed to hypoxia for 3 wk (Table 2, and Fig. 4A). Immunoblot analyses confirmed an increased expression of PDI both in the left ventricle of mice exposed to hypoxia for 3 wk (Fig. 4B, left blots) and in the infarcted hearts 3 wk after MI (Fig. 4B, right blots). Serial cross-sections stained with anti-PDI and anti-PECAM antibodies indicated that capillary ECs are one cell type that expresses PDI under these conditions in infarct and peri-infarct areas of the myocardium (Fig. 4C). In addition, many inflammatory cells that invaded the infarct areas also expressed PDI. To confirm the endothelial origin of PDI expression, we demonstrated that HUVECs exposed to hypoxia expressed increased levels of PDI as detected by both immunofluorescence staining (Fig. 4D) and immunoblotting (Fig. 4E). We also identified angiopoietin-1 (Ang-1) to be upregulated in hypoxic myocardium, indicating that the angiogenic response is indeed induced in our mouse model (Table 2, and Fig. 4A). We confirmed this upregulated expression of Ang-1 both by immunoblotting of left myocardial tissues and its endothelial colocalization using serially sectioned left myocardium stained with anti-Ang-1 and anti-PECAM antibodies (data not shown).

Silenced PDI function increases apoptosis and inhibits migration and adhesion of ECs. Following the transfection of HUVECs with PDI siRNA, immunoblot analysis demonstrated an efficient inhibition of PDI expression in normoxia and hypoxia (Fig. 5A). To evaluate the functional importance of PDI in ECs, normoxic or hypoxic HUVECs transfected with either negative control siRNA and PDI siRNA were assayed for apoptosis (Fig. 5B), migration (Fig. 5C), and adhesion (Fig. 5D). PDI knockdown increased the number of apoptotic HUVECs more than threefold in both normoxia and hypoxia (P < 0.01). PDI knockdown also impaired the migratory properties of HUVECs at normoxia by 42% (P < 0.01) and hypoxia by 52% (P < 0.01), similar to the level of inhibition achieved by bacitracin (Fig. 5C). Following transfection with PDI siRNA, we also assayed the adhesive properties of HUVECs to collagen I, II, and IV; fibronectin; laminin; tenascin; and vitronectin. PDI knockdown significantly inhibited the adhesion of HUVECs to all tested ligands in both normoxia and hypoxia.
and hypoxia (Fig. 5D). Finally, we assayed HUVECs transfected with PDI siRNA or treated with bacitracin for the formation of tubular structures in Matrigel (Fig. 5E). These data indicated that the specific inhibition of PDI by either PDI siRNA ($P < 0.01$) or bacitracin ($P < 0.001$) remarkably impairs the ability of ECs to form tubular structures.

**DISCUSSION**

In this study, we found that the pretreatment of mice with hypoxia for 3 wk before MI improved myocardial function and coronary blood flow, prolonged survival, reduced infarction size, and increased capillary and arteriolar density in the myocardium in mice. As the molecular mechanisms causing remodeling after MI are still not fully understood, we used our model to screen for proteins with the altered expression in hypoxic myocardium and found that the expression of PDI was significantly increased in the myocardial endothelium from mice exposed to hypoxia. Furthermore, we showed that PDI knockdown in HUVECs increased apoptosis and reduced the migration, adhesion, and formation of tubular structures.

Mice exposed to chronic hypoxia were shown to have reduced body weight and increased cardiac weight and hemoglobin levels. We used relatively young animals in this study, and the angiogenic response may be different in older mice. However, elderly human patients have been shown to develop neovascularization following MI (6, 35). We used a high-resolution modification of color-Doppler echocardiography to evaluate serial changes in the coronary circulation of mice in vivo as described previously (8). This mode of echocardiography has been used in humans to assess coronary circulation (39a), but this technique is complicated in mice because of their high heart rates. Here we used it to determine changes in baseline and hyperemic CFV before and after MI. Before coronary artery ligation, hypoxia-pretreated animals displayed a decreased coronary flow, which could be due to increased hemoglobin and thereby increased blood viscosity. One day after MI, baseline and hyperemic CFV were diminished in both normoxic and hypoxic mice, which is in agreement with a previous study (29). However, 3 wk after MI, we observed an enhanced hyperemic CFV in hypoxic mice, which could be explained by the increased arteriolar density since hyperemic

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Fig. 3. Exposure to chronic hypoxia 3 wk before MI increases the myocardial capillary and arteriolar density 3 wk after MI. A: quantification of capillaries in LV sections ($n = 6$ hearts per group) and representative images of platelet endothelial cell adhesion molecule (PECAM)-stained capillary endothelial cells. B: quantification of arterioles in myocardial sections ($n = 7$ hearts per group) and representative images of α-smooth muscle actin (SMA)-stained arterioles. In A and B, left 2 panels represent myocardium sectioned before surgery, whereas right 2 panels demonstrate myocardium 3 wk after MI. All sections were counterstained with Mayer’s hematoxylin to stain nuclei blue. Data are means ± SE. *$P < 0.05$ and **$P < 0.01$ vs. normoxia.
coronary blood flow is known to be related to the total cross-sectional area of the coronary resistance vessels (14). Furthermore, our observed reduction in infarction size in response to chronic hypoxia potentially demonstrates the functional capacity of increased coronary blood flow. The potential clinical relevance of these findings is supported by a recent study that demonstrated an improved myocardial perfusion in patients with coronary disease following exposure to hypoxia (5).

Studies have previously shown that systemic hypoxia promotes angiogenesis by stimulating Ang-1, HIF-1α, and VEGF (30), but it is not known completely how these factors induce functional angiogenesis and protect against MI. In our study, hypoxic pretreatment for 3 wk resulted in an increased formation of myocardial capillaries. This finding is in agreement with observations in other organs (34). The increase in capillary density observed in mice pretreated with hypoxia was maintained at normoxia 3 wk after MI. By contrast, although arteriolar density was increased in hypoxia-pretreated mice 3 wk after MI, this increase was not apparent in mice that were exposed to hypoxia for 3 wk and euthanized before surgery. These data suggest that hypoxia induces angiogenesis and that some capillaries may remodel into stable arterioles. Arteriogenesis is an important change in response to MI, but the mechanisms responsible for this remodeling have not been fully characterized. Consistent with our finding, an increase in capillary density followed by an increase in arteriolar density during cardiac angiogenesis has been observed in other animal studies (40, 41). In this study, we illustrated a temporal and spatial association of PDI expression with capillary endothelial growth in hypoxic myocardium, but a definitive biological role of PDI in promoting angiogenesis in vivo requires further studies.

Complex cellular and molecular interactions between proapoptotic and prosurvival factors occur in the peri-infarct areas, and increased apoptotic activity worsens the clinical outcome (39). In our study, we identified PDI as a potential factor in the EC survival pathway. The upregulation of PDI in the hypoxic myocardium was demonstrated using 2-DE. We then observed that PDI is expressed by myocardial ECs in vivo and by hypoxic HUVECs in vitro. In addition, many inflammatory cells expressed PDI in the infarct areas. PDI is a ubiquitously expressed multifunctional protein, originally reported to be restricted in location to the endoplasmic reticulum.
and activated by stresses that lead to cell death via apoptosis by interfering with proteins in the ER (26). We inhibited the expression of PDI and tested its protective effect on EC apoptosis. Our data suggest a significant protective effect of PDI on hypoxic ECs by reducing the apoptotic rate. The expression of PDI may be activated to counteract ischemic heart insults in capillary ECs. Indeed, the overexpression of PDI in cardiac myocytes protects against heart ischemia (1). In addition, adenoviral-mediated PDI gene transfer to the mouse heart resulted in smaller infarct size by reducing cardiomyocyte apoptosis in the peri-infarct region, thereby improving cardiac function (32). An enzymatic redox function of PDI, such as the catalysis of disulfide bonds, may be responsible for the observed protective effects. As disulfide bond formation requires oxygen and is compromised by reactive oxygen species, protein folding problems in the ER may occur due to reduced oxygen supply during coronary occlusion and the oxidative stress (26).

We also analyzed the effects of PDI knockdown on EC migration and adhesion at hypoxia. The silenced PDI expression was associated with a reduced number of migrating and adhering ECs. PDI has been demonstrated on the surface of vascular cells (18), including ECs, platelets, lymphocytes, and fibroblasts. Furthermore, the blockade of PDI function by bacitracin, a specific PDI inhibitor, or a PDI-neutralizing antibody inhibits the adhesion and invasion of tumor cells (10),

Fig. 5. Loss of PDI expression by RNA interference increases apoptosis and inhibits migration, adhesion, and tube formation in HUVECs at both normoxia and hypoxia. A: quantification of knockdown efficiency of PDI protein expression after transfection of HUVECs with PDI small interfering RNA (siRNA) as detected by immunoblotting at normoxia and hypoxia. Scrambled siRNA molecules are included as controls (Cntrl). B: fluorescence-activated cell sorting analysis of annexin V indicating apoptotic rate of HUVECs after transfection with PDI siRNA in normoxia and hypoxia. **P < 0.01 vs. respective controls; ¶ P < 0.01 vs. normoxic control. C: transwell migration of HUVECs as assayed by Boyden chambers after transfection with PDI siRNA or treatment with bacitracin. *P < 0.01 and **P < 0.01 vs. respective controls; ‡ P < 0.01 vs. normoxic control. D: silenced PDI function diminishes HUVEC adhesion onto indicated extracellular matrix substrates. Both normoxic and hypoxic HUVECs were plated onto various adhesion ligands and stained, and degree of cell adhesion was measured by absorbance at 550 nm. Results are normalized to collagen I controls and shown as fold changes in the number of adhered cells. *P < 0.05 and **P < 0.01 vs. normoxic controls; ‡ P < 0.05 and ‡‡ P < 0.001 vs. hypoxic controls. E: representative pictures and percentages of circular structures of endothelial tube formation in Matrigel following treatment with PDI siRNA or bacitracin. **P < 0.01 vs. scrambled siRNA controls; ***P < 0.0001 vs. controls without treatment with bacitracin.
indicating that PDI plays an important role in cellular migration. In agreement, bacitracin had an inhibitory effect on EC migration in our study. PDI on the cell surface acts as a chaperone, cleaving the disulfide bonds of proteins attached to the cell surface (27). It may also control the function of certain extracellular matrix proteins by regulating their redox state (31). Several integrins have been linked to EC migration and adhesion (2). PDI mediates the conformational changes in both \( \beta_1 \) and \( \beta_3 \)-integrins, which may lead to cell adhesion to particular substrates (17). A recent study showed that PDI physically interacts with \( \alpha_\beta_1 \)-integrin and that these two proteins colocalize at the surface of activated ECs (37), resulting in a disulfide exchange occurring in the integrin, which induces a conformational change that enables a sustained ligand binding. Finally, we demonstrated that the specific inhibition of PDI impairs formation of tubular structures by ECs.

In summary, we demonstrated a physiological adaptation mouse model in which we studied the beneficial effect of pretreatment with chronic hypoxia on myocardial blood flow and function in response to MI. In addition, using this model and proteomics screening technology, we identified PDI that is expressed by ECs. Moreover, the inhibition of endothelial PDI led to an increased apoptosis and a reduced cell migration, adhesion, and tubular formation. This indicates that PDI may play an integral role in apoptosis and angiogenesis by affecting EC survival, migration, adhesion, and tubular formation, contributing to the creation of new coronary arterioles to protect the heart against MI.

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