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Identification of Protein Disulfide Isomerase as a Cardiomyocyte Survival Factor in Ischemic Cardiomyopathy

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Objectives The aim of the study was to analyze the molecular mechanisms activated during postinfarction remodeling in human hearts.

Background The molecular mechanisms of initial response to ischemic insult in the heart and the pathways involved in compensation and remodeling are still largely unknown.

Methods Up-regulation or down-regulation of gene expression in the human viable peri-infarct (vs. remote) myocardial region was investigated by complementary deoxyribonucleic acid array technology and confirmed at a single-gene/protein level with reverse transcriptase polymerase chain reaction and immunohistochemistry. An in vitro model of cardiomyocyte hypoxia in HL1 cells was used to validate anti-apoptotic effects of the candidate gene/protein and to assess the associated downstream cascade. Finally, a mouse model of myocardial infarction was used to test the in vivo effects of exogenous transfection with the candidate gene/protein.

Results Protein disulfide isomerase (PDI), a member of the unfolded protein response, is 3-fold up-regulated in the viable peri-infarct myocardial region, and in a postmortem model, its expression is significantly inversely correlated with apoptotic rate and with presence of heart failure (HF) and biventricular dilatation. Induced PDI expression in HL1 cells conferred protection from hypoxia-induced apoptosis. Adenoviral-mediated PDI gene transfer to the mouse heart resulted in 2.5-fold smaller infarct size, significantly reduced cardiomyocyte apoptosis in the peri-infarct region, and smaller left ventricular end-diastolic diameter versus mice treated with a transgene-null adenoviral vector.

Conclusions These results suggest that PDI promotes survival after ischemic damage and that zinc-superoxide dismutase is one of the PDI molecular targets. Pharmacological modulation of this pathway might prove useful for future prevention and treatment of HF. (J Am Coll Cardiol 2007;50:1029–37) © 2007 by the American College of Cardiology Foundation

Progressive remodeling in ventricular architecture occurs in infarcted and noninfarcted regions, after transmural myocardial infarction (MI). Initially, remodeling is required for maintenance of cardiac output but ultimately leads to left ventricular dysfunction (LVD) and heart failure (HF) (1). Substantial changes in gene expression are required to bring about the profound changes within the ischemic and nonischemic myocardium (2). Experimental studies in animal models of MI have identified several candidate genes whose expression is altered after MI. These include genes involved

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Abbreviations and Acronyms

HF = heart failure
LVD = left ventricular dysfunction
LVEDD = left ventricular end-diastolic diameter
LVESD = left ventricular end-systolic diameter
MI = myocardial infarction
PDI = protein disulfide isomerase
qPCR = quantitative reverse transcriptase polymerase chain reaction

in the cytoskeleton and contractile apparatus, connective tissue, and in the compensatory response to improve hemodynamics (3). However, owing to the difficulties in obtaining ribonucleic acid (RNA) from infarcted hearts, our knowledge of these events in humans is still limited. Different phenomena occur in MI and are important determinants of HF development: cardiomyocyte loss due to necrosis occurring early during acute MI, apoptosis that might occur in the subsequent days or weeks in the peri-infarcted region and that might play a significant role in expansion of dysfunctional area and therefore in HF development, and fibrosis occurring as replacement of lost myocardium and contributing to myocardial stiffness (4). Experimental animal studies revealing beneficial effects of inhibiting apoptosis support a key role of apoptosis in postinfarction remodeling (5). Therefore, understanding these apoptotic events might provide novel insights into HF and its management. We report that the protein disulfide isomerase (PDI) gene, a member of the unfolded protein response (UPR) regulon, is up-regulated in the viable peri-infarct myocardial region of human hearts within the first days after infarction. The functional consequences of this up-regulation, as demonstrated by in vitro and in vivo models of cardiac hypoxia-ischemia, are a significant decrease in the rate of cardiomyocyte apoptosis and, consequently, in a more favorable ventricular remodeling. These results, for the first time, strongly suggest that PDI plays a key protective role in the myocardium after an infarct and identify PDI as a potential target for future pharmacological treatments.

Methods

Postmortem analysis: characteristics of selected cases. Eighteen independent cases were selected at time of autopsy (Department of Pathology, University of Trieste, Italy) according to the following inclusion and exclusion criteria: 1) death occurring 10 to 60 days after acute MI; 2) no evidence of re-infarction at clinical and pathologic assessment; and 3) total occlusion of the infarct-related artery as defined by absence of residual lumen at pathologic examination. All subjects were hospitalized before death. Clinical and pathological characteristics of the subjects are shown Table 1.

Pathological examination and determination of apoptotic cardiomyocytes. Autopsy was performed within 24 h after death in all cases (median 16 h). Gross examination of the hearts and determination of apoptotic cardiomyocytes by co-staining for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL), caspase-3, or muscle-actin were performed as described (6). Tissue specimens (125 to 1,000 mm^3) were obtained at sites of MI and in regions of the right ventricle remote from the infarcted area supplied by a patent coronary artery. Specimens were either snap-frozen for RNA extraction or fixed in 10% paraformaldehyde in 0.1 mol/l phosphate-buffered saline. The PDI expression was detected with a rabbit polyclonal antibody anti-PDI H-160 (Santa Cruz Biotechnology, Santa Cruz, California) at 1:200 dilution. Immunohistochemistry and TUNEL scores were performed by 2 pathologists (F.F. and A.B.) unaware of clinical and PDI data.

RNA extraction, complementary deoxyribonucleic acid array analysis and reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from myocardial autoptic samples by using RNeasy Fibrous-Tissue-Extraction-kit (Qiagen, Hilden, Germany). Total RNA was purified with RNeasy mini-columns (Qiagen). Five micrograms of pooled RNAs (from border and remote zones) were used to perform an Atlas Human Cardiovascular array (Clontech, Mountain View, California) analysis. The choice of pooled remote myocardium as comparison was considered essential in order to have an internal (rather than external) control and reduce to the minimum the possibility

Table 1 Clinico-Pathological Characteristics of the Patients

Age (yrs)	72 (63–78)
Male gender	13 (72%)
Time to death after MI (days)	14 (10–50)
Fibrinolysis	6 (33%)
Transmural infarction	15 (83%)
Infarct extent	
Small (<10%)	2
Moderate (10%–20%)	8
Extensive (>30%)	8
Anterior AMI	7 (38%)
Previous additional AMI	9 (50%)
Pathological characteristics	
Heart weight (g)	510 (470–578)
Transverse diameter (mm)	125 (121–136)
Longitudinal diameter (mm)	104 (100–115)
LV free wall thickness (mm)	13 (11–16)
Diameter-to-wall thickness ratio	9.8 (7.9–11.6)
Right ventricular enlargement	8 (47%)
Multivessel CAD	8 (47%)
Associated conditions	
Diabetes	5 (28%)
Heart failure	11 (61%)
Trauma	1 (6%)
Respiratory failure (including sepsis)	12 (67%)
Gastrointestinal bleed	3 (17%)
Renal failure	5 (28%)
Stroke	2 (11%)
Pulmonary embolism	1 (6%)

n = 18. Quantitative results are expressed as median and interquartile range.
AMI = acute myocardial infarction; CAD = coronary artery disease; LV = left ventricular; MI = myocardial infarction.

of postmortem autolysis-related changes. Arrays were scanned with a PhosphorImager and analyzed by ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, California). The experiment was performed in duplicate, with 2 independent RNA preparations. The PDI up-regulation in the border zone was confirmed by semi-quantitative reverse transcriptase polymerase chain reaction (qPCR) assay on the same pooled RNAs. Two micrograms of total RNA were treated with M-MLV reverse transcriptase (Invitrogen, Carlsbad, California) and amplified with Taq DNA Polymerase (Promega, Madison, Wisconsin). The following oligonucleotides were used: PDI-forward:5′-GGACCACGTCTTGGTGCTGCG-3′, PDI-reverse:5′-GATGACGGCCACCTCGCTGCTGGA-3′, β-actin-forward: 5′-AAGAGAGGCATCCTCACCCT-3′, β-actin-reverse:5′-ATCTCCTGCTCGAACTCCAG-3′.

Culture of HL1 cells. HL1 cells are differentiated and proliferating atrial cardiomyocytes derived from mouse AT-1 cells (7). Cells were cultured as described in Claycomb-Medium (JRH Biosciences, Andover, United Kingdom).

Treatment with hypoxic stress. Subconfluent HL1 cultures were kept at 37°C in a humidified sealed chamber (Billups-Rothenburg, Del Mar, California) gassed with 5% carbon dioxide- 95% gaseous nitrogen (5% oxygen) for 24 and 48 h.

Western blot analysis. Immunoblot analysis was performed with rabbit polyclonal antibody anti-PDI H-160 (Santa Cruz Biotechnology) or mouse monoclonal antibody anti–β-actin C-2 (Santa Cruz Biotechnology) at 1:200 dilution.

Isolation of PDI cDNA. Full-length human PDI cDNA was isolated from Phoenix cell line total RNA by RT-PCR. The cDNA was subcloned in P-dominant cDNA (pcDNA) 3.1(+) expression vector with the following oligonucleotides: PDI-forward-BamHI:5′-GATCGGATCCATGCTGCGCCGCGCTCTGCTGT-3′, PDI-reverse-EcoRI:5′-GATCGAATTCTTACAGTTCATCTTTCACAGCTTTCTG-3′.

Site-directed mutagenesis. The point mutants PDI-N (C36/40S) and PDI-C (C383/S387) were generated with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, California) on the basis of the long-PCR method. The wild-type PDI (PDI-wt) pcDNA3 was used as a template. The following oligonucleotides were used: PDI-N(1):5′-GGCCAGAGCCTTGGAGTGGCCAGACCAGGGGGCATG-3′, PDI-N(2): 5′-CATGCCCCCTGGTCTGGCCACTCTAAGGCTCTGGCC-3′; PDI-C(1):5′-GAGCCAACTGTTTGGAGTGACCAGACCATGGGGCATAG-3′, PDI-C(2):5′-CTATGCCCCATGGTCTGGTCACTCCAAACAGTTGGCTC-3′.

Stable overexpression of PDI-wt and PDI mutants. Transfection of PDI-wt, PDI-N, and PDI-C into HL1 cells was carried out by Lipofectamine (Invitrogen). The PDI-transfected or pcDNA3-transfected cells were selected in the presence of 700 μg/ml G418 for 2 weeks and then maintained in the presence of 300 μg/ml G418. Finally, individual colonies were isolated, expanded, and assayed for the expression of the transfected gene by Western blotting.

Construction of adenoviral vectors. Recombinant adenoviral vectors were constructed with the Ad-Easy system (Stratagene). Viral titers were estimated by serial dilution of the viral stocks in transduced 293 cells and expressed as plaque-forming units/ml (PFU/ml). Viral stocks were negative for presence of replication-competent adenovirus as tested on A549 cells.

Superoxide dismutase activity measurements. Cells were resuspended in TSS (145 mmol/l sodium chloride; 2.5 mmol/l potassium chloride; 10 mmol/l Hepes; 10 mmol/l Glucose; 1.5 mmol/l calcium chloride; 1.2 mmol/l magnesium chloride; pH 7.4) and incubated for 1 min at 37°C. Hydrogen peroxide contents were measured by horseradish peroxidase-enhanced chemiluminescence as described (8).

Flow cytometric analysis. The HL1 cells were fixed in ice-cold 70% ethanol. After resuspension, cells were incubated with propidium iodide (50 μg/ml) and RNaseI (1 mg/ml) for 30 min. Flow cytometry and Expo32 ADC software (Beckman Coulter, Fullerton, California) were used for analysis of sub-G1 DNA content.

qPCR. Quantitative PCR was done with the Platinum SYBR Green qPCR-superMix-UDG-Kit (Invitrogen) with Rotor-Gene RG3000 from Corbett Research as described (9). The following oligonucleotides were used: mPDI-forward-CAAGTTCTTCAAGAATGGAGACACAGC; mPDI-reverse-TTCTTCAGCCAGTTGACAATGTCATC; mATF6alpha-forward- CGACGTTGTTTGCTGAACTTGGCTA; mATF6alpha-reverse-CTGACTCCCAAGGCATCAAATCC; mBIP-forward-CGATAATCAGCCAACTGTAACAATCAAGG; mBIP-reverse-GGAATTCCAGTCAGATCAAATGTACCC; mGRP94-forward-CACACTAGGTCGTGGAACAACAATTAC; mGRP94-reverse-TCTTGCTACTCCACACGTAGATGG; mEro1-forward-GGTGAACTTGAAGAAGCCTTGTCC; mEro1-reverse-CTTGTAGCTCGCAGACTTAATTCCATCAGG; mPERK-forward-GCGACGGAGCCCGATGACG; mPERK-reverse-GCATCCAGTGCAGCGATTCGTCC; mIRE1a-forward-TCACTGCCGGAAGACGACGTGG; mIRE1a-reverse-CCACAGGACATCCCCAGATTCACTGTCC; mβ-actin-forward-GATTACTGCTCTGGCTCCTAG; mβ-actin-reverse-ACTCATCGTACTCCTGCTTGC.

The housekeeping gene β-actin was used to normalize the results, and cDNA from untreated HL1 cells was used as a reference.

Surgical procedures for in vivo experiments. Experimental procedures complied with the Guidelines of the Italian National Institutes of Health, with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences), and were approved by the Institutional Animal Care and Use Committee. Myocardial infarction was induced in 34

C57BL/6 mice (male, 8 weeks of age, Charles-River, Italy) as previously described (10). We set the protocol for in vivo adenoviral-mediated gene transfer as follows: 4 injections, 2 for each side flanking the left coronary artery of adenoviral vectors, were performed into the ventricular wall. The volume of each injection was 2.5 μl (approximate titer 1×10^{10} PFU/ml). The size of the heart of animals (8-week-old C56/bl6 mice) hampered both increments in the number of injections and volume for single administration. The chest was closed and mice were allowed to recover. After 48 h animals were re-operated and the left coronary artery was ligated. Sham-operated mice were treated similarly (without adenovirus injection), except that the ligature around the coronary artery was not tied. Animals were killed 1 week after surgery, after echocardiography.

Evaluation of myocardial function in vivo. Echocardiography was performed in 14 conscious mice (7 AdPDI/green fluorescent protein [GFP]-injected and 7 Adnull/GFP control subjects) with a Sequoia 256c echocardiography device (National Ultrasound, Duluth, Georgia) equipped with a 13-MHz linear transducer as described (10). Left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were measured.

Histological and immunohistochemical analysis. After echocardiography, hearts were recovered for histology as described (10). Adenoviral-mediated gene transfer was determined with rabbit polyclonal antibodies for GFP (ABCAM, Cambridge, United Kingdom) and PDI (anti-PDI H-160, Santa Cruz Biotechnology). Apoptosis was detected with co-staining for TUNEL (Apoptag-Oncor, Gaithersburg, Maryland) and muscle actin.

Statistical analysis. Statistical analysis was performed with SPSS 10.1 for Windows (SPSS, Chicago, Illinois). For data obtained from human subjects, quantitative results are expressed as median (interquartile range), because of potential deviations from assumptions of normality. The uncorrected chi-square test or the Fisher exact test was used for categorical variables, when appropriate. Continuous variables were analyzed with Mann-Whitney *U* or Wilcoxon rank sum tests for unpaired and paired data, respectively. Correlation between continuous variables was analyzed with the Spearman test. For animal data, quantitative results are expressed as mean ± SE. Continuous variables were analyzed with analysis of variance to compare 3 or more groups and, when $p < 0.05$, the Student *t* test for unpaired data (with Bonferroni's correction) was used to compare 2 of 3 groups at a time. Two-tailed statistical significance was at the 0.05 level.

Results

Apoptosis in human postmortem specimens. Apoptotic cardiomyocytes were detected both in the peri-infarct and remote areas in 18 infarcted hearts. The apoptotic index was significantly higher in the peri-infarct versus remote areas (8.9% [5.1 to 12.6] vs. 0.7% [0.5 to 0.9], $p < 0.001$). The apoptotic rate both in the peri-infarct and remote areas significantly correlated with increasing cardiac transverse diameter ($r = +0.51$, $p = 0.022$; $r = +0.48$, $p = 0.048$, respectively) and cardiac weight ($r = +0.47$, $p = 0.042$; $r = +0.44$, $p = 0.050$, respectively).

PDI is up-regulated after infarction. A human Cardiovascular Array was used to compare the RNA transcripts from 2 different pools: 1 from samples obtained in the peri-infarct region and 1 from a remote area. The PDI gene was up-regulated 3-fold in the RNA extracted from the peri-infarct areas. Semiquantitative RT-PCR was performed on the same pooled RNAs to confirm differential expression (Fig. 1A). Immunohistochemical analysis of PDI expression on all human samples confirmed the up-regulation of PDI at the protein level in the peri-infarct areas (Figs. 1B and 1C). Finally, co-staining for PDI and TUNEL showed that most of the cardiomyocytes over-expressing PDI were TUNEL negative (Fig. 1D).

PDI expression, cardiomyocyte apoptosis, and cardiac remodeling. The PDI was expressed in the peri-infarct areas in all 18 patients investigated but only in 7 in the remote regions (100% vs. 39% of remote regions, $p < 0.001$). Expression in peri-infarct regions was mild in 3 cases (17%), moderate in 5 (28%), and intense in the remaining 10 cases (55%). The intensity of PDI expression was significantly inversely correlated with the apoptotic rate ($r = -0.55$, $p = 0.021$). In the 10 cases with intense PDI expression the apoptotic rate was significantly lower (7.1% [1.4 to 9.0] vs. 12.0% [9.1 to 14.1], $p = 0.012$) (Fig. 1E) than in the 8 cases with mild or moderate expression (Fig. 1E) (Table 2). Interestingly, subjects with intense PDI expression were less likely to be suffering from stage-C New York Heart Association functional class IV congestive HF (30% vs. 88%, $p = 0.025$) (Fig. 1F) and to have biventricular enlargement at postmortem examination (20% vs. 88%, $p = 0.015$) (Fig. 1G).

PDI is over-expressed in vitro under hypoxic conditions and protects cardiomyocytes from apoptosis. Murine HL1 cells were subjected to hypoxic stress, and proteins were extracted at various time points. The PDI protein level increase was detected by Western blot analysis (Fig. 2A). These data suggest that the over-expression of PDI is physiologically relevant and might be beneficial for cells to survive hypoxic conditions. To test this hypothesis, we investigated the functional consequences of PDI over-expression after hypoxic stress in cardiomyocytes. Wild-type *PDI* as well as 2 enzymatically inactive point mutants, *PDI-N* [C36/40S] and *PDI-C* [C383/S387], mapped in 2 thioredoxin-like domains located in the N-terminal and C-terminal regions of the protein (Fig. 2B), were over-expressed in HL1 cells. Increased levels of PDI in transfected cells were verified by Western blot analysis. Stable transfectants were subjected to hypoxic stress, and apoptotic rates were measured by flow cytometric (FACS) analysis. Over-expression of PDI was able to significantly decrease

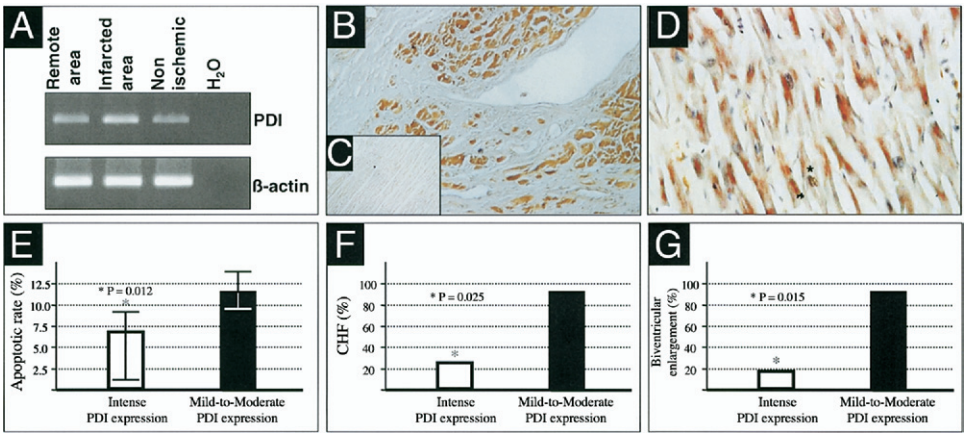

Figure 1 **Analysis of PDI Expression on Autoptic Human Infarcted Hearts**

(A) Semiquantitative reverse transcriptase polymerase chain reaction showing higher expression of protein disulfide isomerase (PDI) transcript in the ribonucleic acid (RNA) extracted close to the infarcted area, with respect to the RNA extracted in the area remote from the infarct. The PDI expression in RNA extracted from a nonischemic heart as well as a reaction made with no RNA were used as controls. **(B)** Immunohistochemical expression of PDI protein in the infarcted area (original magnification ×40). **(C)** Immunohistochemical expression of PDI in the area remote from the infarct (original magnification ×40). **(D)** Double staining for PDI protein and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) in the infarcted area: only a few cardiomyocytes expressing high levels of PDI were TUNEL positive, as indicated by an **asterisk** (original magnification ×40). **(E)** Correlation between PDI expression and apoptotic rate in the peri-infarct region. **(F)** Correlation between PDI expression and congestive heart failure (CHF) signs. **(G)** Correlation between PDI expression and biventricular enlargement.

the apoptotic rate in normoxic and hypoxic cardiomyocytes at 24 h and 48 h, respectively ($p < 0.001$). Over-expression of both enzymatically inactive point mutants rather increased the number of apoptotic cells compared with the control in normoxic and hypoxic conditions at 24 h and 48 h ($p < 0.001$) (Fig. 2C). These data indicate that the enzymatic activity of PDI specifically protects cells from undergoing apoptosis in hypoxic conditions.

Table 2 **Differential Characteristics of the Patients in the 2 Groups (High and Low PDI Expression in the Peri-Infarct Region)**

	Intense PDI Expression (PDI = 3) n = 10	Mild-to-Moderate PDI Expression (PDI <3) n = 8
Age (yrs)	70 (62–72)	77 (72–80)
Male gender	7 (70%)	6 (75%)
Time to death after MI (days)	16 (12–16)	18 (10–52)
Heart failure	3 (30%)	7 (88%)*
Infarct extent		
Small (<10%)	2	2
Moderate (10%–20%)	4	3
Extensive (>30%)	4	3
Pathological characteristics		
Heart weight (g)	510 (500–550)	480 (470–550)
Transverse diameter (mm)	124 (123–130)	130 (126–138)
Longitudinal diameter (mm)	110 (101–114)	103 (102–115)
LV free wall thickness (mm)	14 (12–16)	12 (12–14)
Diameter-to-wall thickness ratio	9.1 (7.9–10.8)	9.8 (9.6–11.0)
Biventricular enlargement	2 (20%)	7 (88%)*

Quantitative results are expressed as median and interquartile range. *$p < 0.05$ with Fisher exact test.

LV = left ventricular; MI = myocardial infarction; PDI = protein disulfide isomerase.

Influence of hypoxia on various genes involved in endoplasmic reticulum stress response. We used total RNA of hypoxia-treated HL1 cells to analyze by qRT-PCR the expression levels of PDI as well as of selected folding catalysts, transmembrane kinases, chaperones, and transcription factors known to be involved in the unfolded protein response of the endoplasmic reticulum (ER) (Fig. 3A). The PDI, Ero1, GRP94, and ATF6-alpha were significantly up-regulated ($n = 4$, $p < 0.05$) after exposure to hypoxia. For Ero1 and ATF6-alpha the expression level increased with duration of hypoxia. The expression level of IRE1-alpha increased significantly ($p < 0.05$) after 48-h exposure to hypoxia but not after 24 h. The messenger ribonucleic acid (mRNA) level of PERK did not significantly change, whereas the level of binding protein (BIP)-mRNA decreased under influence of hypoxia. These results provide independent confirmation for the observed up-regulation of PDI during hypoxia. In addition, the co-upregulation of Ero1 supports the notion that the enzymatic activity of PDI protects cardiomyocytes after hypoxic stress. We have also repeated the qPCR experiments with cells over-expressing PDI. These data indicated that over-expression of PDI did not significantly change the expression of the tested genes, suggesting that the detected effects of PDI over-expression are direct (data not shown).

Superoxide dismutase-1 is a target of the anti-apoptotic action of PDI. To obtain initial insights into protective mechanisms supported by PDI, we investigated whether the anti-apoptotic effects of PDI were caused by its protective

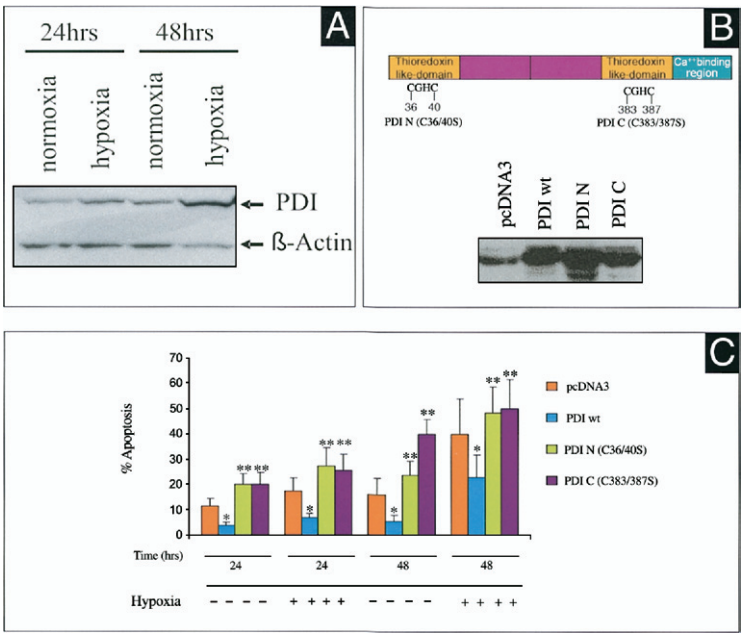

Figure 2 **PDI Over-Expression Protects HL1 Cardiomyocytes From Apoptosis Under Hypoxic Conditions**

(A) Endogenous protein disulfide isomerase (PDI) expression is induced under hypoxic conditions in mouse cardiomyocytes. HL1 cells were cultured under hypoxia conditions for 24 h and 48 h. The PDI expression was analyzed by Western blotting. **(B)** Schematic representation of PDI thioredoxin-like domains. The PDI variants have both cysteine-to-serine mutations in the N-terminal and/or C-terminal thioredoxin-like domains. Over-expression of wild-type PDI (PDI-wt), PDI-N, and PDI-C in HL1 stable individual clones were estimated by Western blot analysis. **(C)** Stable over-expression of PDI-wt but not of the N- and C-terminal mutants results in a significant protection from hypoxia-induced apoptosis. The HL1 cells were stable transfected with PDI-wt, PDI-N, PDI-C, or empty vector. Stable individual clones were incubated for the indicated periods in hypoxic conditions. The percentage of apoptotic cells was estimated by flow cytometric analysis. Values represent the mean ± SE of 5 independent experiments. *Significant differential apoptotic rate (Student *t* test, n = 5, $p < 0.001$) in normoxic and hypoxic cardiomyocytes at 24 h and 48 h. **Significant differential apoptotic rate (Student *t* test, n = 5, $p < 0.001$) in cells transfected with enzymatically inactive point mutants compared with the control in normoxic and hypoxic conditions at 24 h and 48 h.

action on superoxide dismutase (SOD)-1 (11). The SOD activity was determined by measuring the release of hydrogen peroxide (H_2O_2) generated during the dismutation of 2 superoxide anions. The HL1 cells over-expressing PDI showed a significantly higher H_2O_2 release than HL1 cells transfected with the vector alone (Fig. 3B). Therefore, PDI was able to protect SOD-1 activity, when over-expressed in HL1 cells.

Protective effects of PDI over-expression in the in vivo acute MI mouse model. The PDI was over-expressed in an in vivo mouse model of MI by injecting adenoviral vectors expressing both PDI and GFP (AdPDI/GFP) into the hearts, before the infarction. As a control, infarcted hearts injected with transgene-null, GFP-expressing adenoviral vectors (Adnull/GFP) or hearts from sham-operated animals were used. Thirty-one mice were killed at day 7: 13 in the AdPDI/GFP group, 12 in the AdGFP group, and 6 in the sham-operated group.

Efficiency of the viral infection by GFP expression and increased myocardial PDI expression in AdPDI/GFP-injected mice were verified by Western blots and immunohistochemistry (Figs. 4A and 4B). Interestingly, infarct size was significantly smaller in AdPDI/GFP-treated mice, determined as reduced circumferential extension (18 ± 1% vs. 43 ± 1%, $p < 0.001$) and as transmural extension (30 ± 2% vs. 89 ± 1%, $p < 0.001$) assessed in the mid-ventricular section (Figs. 4C and 5A to 5D). The apoptotic rate in the peri-infarct region was also significantly reduced (1.0 ± 0.1% vs. 3.0 ± 0.1%, $p < 0.001$; $p < 0.001$ vs. sham operated animals [apoptotic rate 0.01 ± 0.001%]) (Figs. 4D, 5E, and 5F).

In vivo assessment of the remodeling pattern with transthoracic echocardiography confirmed a more favorable pattern in AdPDI/GFP-treated mice. Compared with sham operated mice, Adnull/GFP-treated infarcted mice had significantly greater LVEDD ($p < 0.001$) and LVESD ($p < 0.001$). Compared with AdGFP animals, AdPDI/GFP mice had significantly smaller LVEDD (2.50 ± 0.02 mm vs. 3.11 ± 0.06 mm, $p = 0.009$), similar to that in sham-operated animals (2.49 ± 0.01 mm, $p = 0.95$) (Fig. 4E). A trend toward lower LVESD values in AdPDI/GFP versus Adnull/GFP-treated mice was also found (1.31 ± 0.01 mm vs. 1.54 ± 0.03 mm, $p = 0.054$) (Figs. 4E and 4F). Finally, we did not find significant differences in the left ventricular fractional shortening between AdPDI/GFP and Adnull/GFP-treated mice (data not shown).

These data clearly indicate that PDI is over-expressed after hypoxia due to infarction and that this over-expression

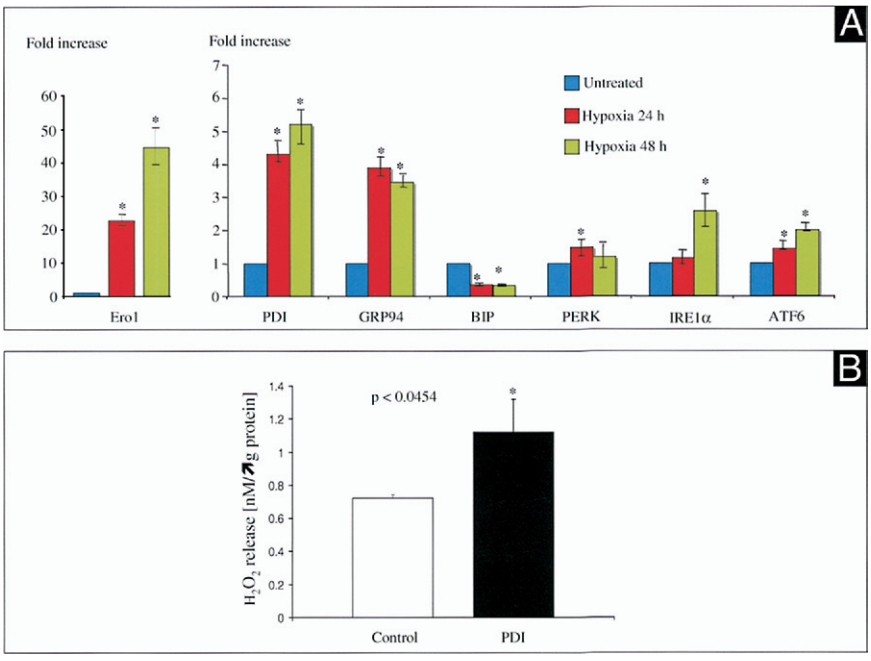

Figure 3 **Hypoxia Induces the Endoplasmic Reticulum Stress Response Genes in HL1 Cardiomyocytes, and Zinc-Superoxide Dismutase-1 Is a Target of the Anti-Apoptotic Action of PDI**

(A) Total RNA of hypoxia-treated murine HL1 cells was analyzed by quantitative reverse transcriptase polymerase chain reaction for the expression levels of PDI, Ero1, Grp94, binding protein (BIP), and the transcription factors PERK, IRE1-alpha, and ATF6-alpha. *Significant differential expression (Student *t* test, n = 4, $p < 0.05$) after exposure to hypoxia. **(B)** Hydrogen peroxide (H_2O_2) release was measured in HL1 cells by horseradish peroxidase-enhanced chemiluminescence. Given are the mean ± SD from 3 independent measurements. *Significant differential expression (Student *t* test, n = 5, $p < 0.05$). Abbreviations as in Figure 1.

reduces infarct size and adverse remodeling, thus suggesting that PDI plays a key role in myocardial response to ischemia and in postinfarction cardiac remodeling and dilatation.

Discussion

The molecular mechanisms causing remodeling after MI are still incompletely understood. As a consequence, most therapeutic approaches to limit injury after infarct, such as pharmacological manipulation and coronary revascularization, are based on the observation of a better clinical outcome associated with myocardial reperfusion or reduced cardiac work (1). Complex interactions between pro-apoptotic and pro-survival factors occur in the region bordering the infarct and remote myocardium for days to weeks after the ischemic event and the apoptotic rate is correlated with worse clinical outcome (12). In the current study, we have identified PDI as a key factor of the survival pathway. The PDI belongs to a family of proteins involved in the UPR system (13), which is activated by stresses that interfere with protein folding in the ER. Failure to eliminate misfolded proteins in the ER leads to cell death via apoptosis, and a variety of human disorders are related to ER stress (14). The role of UPR in ischemic diseases has been studied to a limited extent in the brain (15), tumor cells (16), and the ischemic heart, suggesting a contribution to counteract/repair ischemic damage (17–19).

Our data suggest a significant protective effect of PDI in human infarcted hearts mediated by a reduction of apoptotic rate and by prevention of cardiac remodeling. This association is in agreement with the hypothesis that also in humans the ER stress response is activated to counteract ischemic heart insults. Therefore, we tested the protective effects of PDI in in vitro and in vivo models of hypoxia and ischemia. The in vitro studies suggest that the enzymatic redox function of PDI (i.e., the catalysis for disulfide bond formation) is responsible for the observed protective effects. Accordingly, forced expression of enzymatically inactive point-mutants PDI C36/40S and PDI C383/S387 had no protective effects but rather resulted in an increase of apoptotic rate. These data indicate that both thioredoxin-like domains are critical for this protective function against hypoxic stress, which is in agreement with data obtained with glial cells (20). This model is further supported by the observation that Ero1, which re-oxidizes reduced PDI, is also strongly up-regulated after hypoxia. The induction of PDI and Ero1 is mediated by the transmembrane kinase Ire1 that is also up-regulated under these conditions. Because several other elements of the UPR, such as Grp94/Hsp90 and the transcription factor ATF6, are also up-regulated, we propose that this system plays an important role in the response to ischemia.

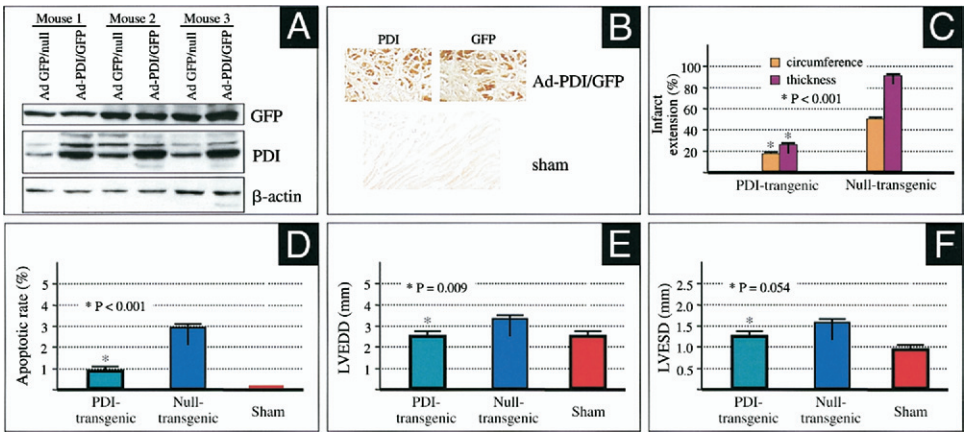

Figure 4 **PDI Over-Expression Has a Protective Effect in the In Vivo Acute MI Mouse Model**

Adenoviral vectors expressing both PDI and green fluorescent protein (GFP) (AdPDI/GFP) or GFP only (Adnull/GFP) were administered into the ventricular wall into C57/BL6 mice. A set of animals were killed 48 h after adenoviral administration to determine the amount of transgene expression. The remaining animals were subjected to left coronary artery ligation 48 h after viral administration. One week after the surgical procedure, mice were killed for analysis. **(A)** Adenovirus mediated over-expression of PDI-wt in C57/BL6 mice. Mice were killed 48 h after adenoviral injections. Western-blot analyses of GFP and PDI expression were performed with total lysates of homogenized myocardial tissue. **(B)** Immunohistochemical analysis of GFP and PDI expression in the injected hearts (original magnification ×20). **(C)** Analysis of infarct extension, both in terms of extension and thickness, in AdPDI/GFP-injected hearts with respect to the controls. **(D)** Analysis of the apoptotic rate in injected hearts with respect to the controls. **(E)** Echocardiographic analysis of the left ventricular end-diastolic diameter (LVEDD) parameter in AdPDI/GFP-injected hearts with respect to the controls. **(F)** Echocardiographic analysis of the left ventricular end-systolic diameter (LVESD) parameter in AdPDI/GFP-injected hearts with respect to the controls. Abbreviations as in Figures 1 and 2.

Both reduced oxygen supply during coronary occlusion and the oxidative stress associated with reperfusion cause protein folding problems in the ER, because disulfide bond formation requires oxygen and can be compromised by reactive oxygen species. Over-expression of PDI is expected to counteract such problems. Indeed, significant superoxide generation occurs in cardiomyocytes both during ischemia and during reperfusion (21). Over-expression of SOD-1, which efficiently dismutases superoxide, prevents postischemic injury in the heart, attenuating both apoptosis and the inflammatory response during ischemia (22). Interestingly, it has been demon-

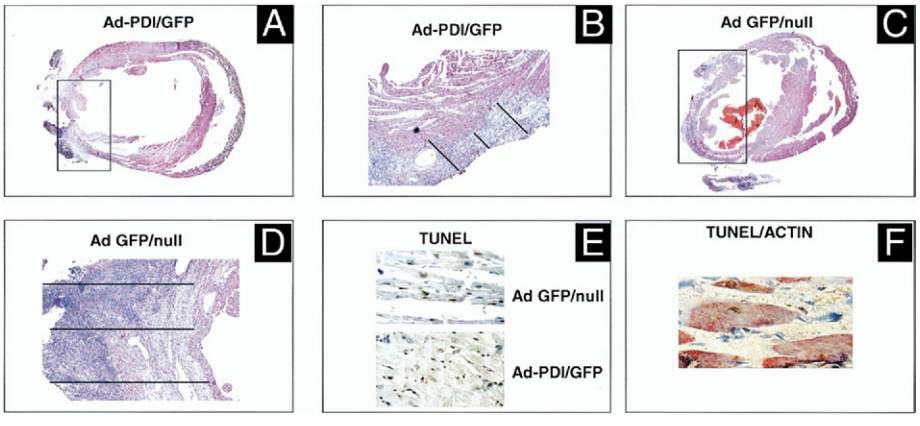

Figure 5 **Histopathological Analysis of the Infarcted Mice Hearts**

(A) Hematoxylin/eosin picture of the infarcted heart injected with AdPDI/GFP: the area of the wall of the left ventricle affected by the infarct is indicated by a **box** (original magnification ×10). **(B)** Hematoxylin/eosin picture of the infarcted heart injected with AdPDI/GFP: the involvement of the wall in terms of thickness is indicated by a **heavy black line** (original magnification ×20). **(C)** Hematoxylin/eosin picture of the infarcted heart injected with Adnull/GFP: the area of the wall of the left ventricle interested by the infarct is indicated by a **box** (original magnification ×10). **(D)** Hematoxylin/eosin picture of the infarcted heart injected with the vector alone: the involvement of the wall in terms of thickness is indicated by a **bold line** (original magnification ×20). **(E)** Examples of TUNEL staining in the infarcted hearts injected with AdPDI/GFP and with Adnull/GFP (original magnification ×40). **(F)** Examples of the double staining TUNEL-muscle actin to detect only the apoptotic cardiomyocytes (original magnification ×40). Abbreviations as in Figure 1.

strated that PDI is over-expressed in familial amyotrophic lateral sclerosis, acting as a survival factor through physical interaction with SOD-1 (11). This interaction prevents SOD-1 aggregation and consequently neuronal degeneration. Our findings of increased SOD activity in myocardiocytes over-expressing PDI are in line with these reports and suggest that over-expression of PDI might protect myocardial tissue from apoptosis, mediated by superoxide, through increased SOD activity.

We analyzed the effects of forced PDI expression in a mouse model of ischemic heart disease. The PDI over-expression was associated with a marked reduction of infarct size, with a significant reduction of cardiomyocyte apoptosis in the peri-infarct region, and prevention of cardiac dilatation, as determined in vivo both by transthoracic echocardiography and by pathological analysis of explanted hearts. Interestingly, we did not find significant differences in the ejection fractional shortening between AdPDI/GFP- versus Adnull/GFP-injected mice. This was probably due to the short window of time (1 week) after the infarction. Indeed, the initial increase of cardiac volumes serves the purpose of preserving the ejection fraction; only late cardiac dilation becomes a mechanism of disease resulting in a reduction of ejection fraction and development of HF (23).

The role of apoptosis in the pathophysiology of HF has been demonstrated, and the potential for preventing ischemic cardiomyopathy and HF by inhibiting apoptosis is promising. Our results from postmortem human hearts and in vitro and in vivo models of hypoxia and ischemia point out a pivotal role for PDI in the programmed survival pathway that promotes cell survival after infarction leading to more favorable cardiac remodeling. The resulting working model for cardiomyocytes close to the ischemic region proposes that PDI acts both on the hypoxic and oxidative stresses, by contributing to the correct protein folding in cooperation with ERO-1 and by modulating the activity of SOD-1. When this molecular mechanism is amplified in cardiomyocytes by over-expression of PDI, the programmed survival pathway is strongly ameliorated, resulting in effective prevention of adverse cardiac remodeling. Pharmacological modulation of this pathway might prove useful for future prevention and treatment of HF.

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