Activation of the Poly(ADP-Ribose) Polymerase Pathway in Human Heart Failure

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Poly(ADP-ribose) polymerase (PARP) activation has been implicated in the pathogenesis of acute and chronic myocardial dysfunction and heart failure. The goal of the present study was to investigate PARP activation in human heart failure, and to correlate PARP activation with various indices of apoptosis and oxidative and nitrosative stress in healthy (donor) and failing (NYHA class III-IV) human heart tissue samples. Higher levels of oxidized protein end-products were found in failing hearts compared with donor heart samples. On the other hand, no differences in tyrosine nitration (a marker of peroxynitrite generation) were detected. Activation of PARP was demonstrated in the failing hearts by an increased abundance of poly-ADP ribosylated proteins. Immunohistochemical analysis revealed that PARP activation was localized to the nucleus of the cardiomyocytes from the failing hearts. The expression of full-length PARP-1 was not significantly different in donor and failing hearts. The expression of caspase-9, in contrast, was significantly higher in the failing than in the donor hearts. Immunohistochemical analysis was used to detect the activation of mitochondrial apoptotic pathways. We found no significant translocation of apoptosis-inducing factor (AIF) into the nucleus. Overall, the current data provide evidence of oxidative stress and PARP activation in human heart failure. Interventional studies with antioxidants or PARP inhibitors are required to define the specific roles of these factors in the pathogenesis of human heart failure.

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INTRODUCTION

Acute and chronic heart failure are major causes of hospitalization, morbidity, and mortality worldwide. The mechanisms leading to cardiac pump failure may have various origins and include acute and chronic ischemic heart diseases that can develop on the basis of an altered coronary artery circulation or infarction, cardiomyopathies, myocarditis, a pressure overload and defects in the genes encoding the contractile apparatus, the intercellular matrix, the cytoskeleton, and the mitochondrial proteins, among many others. These defects result in a mismatch between the load applied to the heart and the energy needed for contraction, leading to mechanoenergic uncoupling (1-3).

The pathomechanism of heart failure is complex and can include the activation of numerous secondary pathways (involving neurohormones, neuropeptides, cytokines, inducible nitric oxide synthase [iNOS], and oxidative/nitrosative stress), leading to abnormalities in various signaling processes and cardiac receptors, calcium homeostasis, contractile protein alterations, and endothelial dysfunction. The resultant structural alterations give rise to cardiac and vascular remodeling with hypertrophy, fibrosis, cardiac dilation, and myocardial necrosis. The adverse remodeling and increased peripheral resistance further aggravate heart failure (1,2).

Recent work, mainly on various animal models of heart failure, has provided evidence of the pathogenetic role of oxidative and nitrosative stress and downstream mechanisms, including the activation of matrix metalloproteinases (MMPs) and poly(ADP-ribose) polymerase (PARP), in various forms of heart failure (1-6). These studies have also suggested that these pathways may allow potential novel therapeutic possibilities. To validate these pathways and targets, it is essential to assess the activation of these pathways in human samples. In the present study, using clinical tissue material, we have compared healthy donor myocardial samples and myocardial samples from failing hearts, to obtain evidence for oxidative and nitrosative stress and PARP activation.

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MATERIALS AND METHODS

Left Ventricular Tissue Samples

Healthy human hearts were obtained from 5 general organ donor patients whose hearts were explanted to obtain

pulmonary and aortic valves for transplant surgery (donor hearts). The donors did not show any sign of cardiac abnormalities and did not receive any medication except plasma volume expanders, dobutamine, and furosemide. The causes of death included cerebral contusion due to accidents and cerebral hemorrhage or subarachnoid hemorrhage due to stroke. Failing hearts were obtained from 8 explanted end-stage failing hearts (NYHA class III-IV). Both the failing and the donor hearts were kept in cardioplegic solution (110 mM NaCl, 16 mM KCl, 1.6 mM $MgCl_2$, 1.2 mM $CaCl_2$, and 5 mM $NaHCO_3$) until their arrival to the laboratory. A detailed summary of the pretransplant data and drug therapy is shown on Table 1. The experiments complied with the Helsinki Declaration of the World Medical Association and were approved by the Albert Szent-Györgyi Medical University Ethical Review Board (no. 51-57/1997.OEj). Left ventricular wall samples were obtained from the base. All biopsies were stored in cardioplegic solution and kept at 4 °C for approximatelly 6 to 8 h before being frozen in liquid nitrogen. Subsequently, the tissue samples were stored at –80 °C.

Immunohistochemical Analysis of Poly(ADP-Ribose) (PAR) and Apoptosis Inducing Factor (AIF)

Human ventricular tissue samples were frozen in Tissue-Tek OCT compound (Electron Microscopy Sciences, Hatfield, PA, USA). Cryostat sections of 5 μm were placed on adhesive slides and fixed in acetone for 10 min. Endogenous peroxidase activity was suppressed by treating the slides with 0.3% H_2O_2 in methanol for 30 min. Sections were washed 3 times in 0.01 M PBS (pH 7.4) and blocked with 1.5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS containing 0.2% Triton X-100 (blocking serum) for 60 min. Primary antibody against PAR polymers (anti-PAR; Calbiochem, San Diego, CA, USA) and AIF (anti-AIF; Chemicon International, Temecula, CA, USA) were applied at a dilution of 1:100 in blocking serum and sections were incubated overnight at 4 °C. After washing in PBS containing 0.2% Triton X-100 (wash buffer), sections were incubated with biotinylated anti-rabbit antibodies (1:200 in blocking serum; Vector Laboratories, Burlingame, CA, USA) for 30 min. Sections were washed in wash buffer and incubated with ABC peroxidase reagent (Vectastain Elite ABC kit; Vector Laboratories) for 30 min. After washing in wash buffer, sections were rinsed in PBS, and peroxidase activities were revealed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 (DAB substrate kit; Vector Laboratories). Slides were washed and counterstained with Gill's hematoxylin (Accustain; Sigma Diagnostics, St. Louis, MO, USA), dehydrated in an ascending alcohol series, cleared in xylene, and coverslipped with Permount (Fischer Chemicals, Fairlawn, NJ, USA). As a negative control, sections were simultaneously stained with omission of the primary antibodies (7). Figures show representative staining patterns from 4 to 6 separate sections.

To quantify the immunohistochemical data in cases of PAR staining, the positive and negative nuclei were counted in the view field of the microscope. At least 3 different failing or donor samples were analyzed. Data are shown as percentages of positive nuclei in the case of PAR staining.

Detection of Poly-ADP-Ribosylation

Human left ventricular tissue samples weighting 0.21 to 0.37 g were placed into prechilled small glass tissue homogenizers with about 0.5 mL isolating

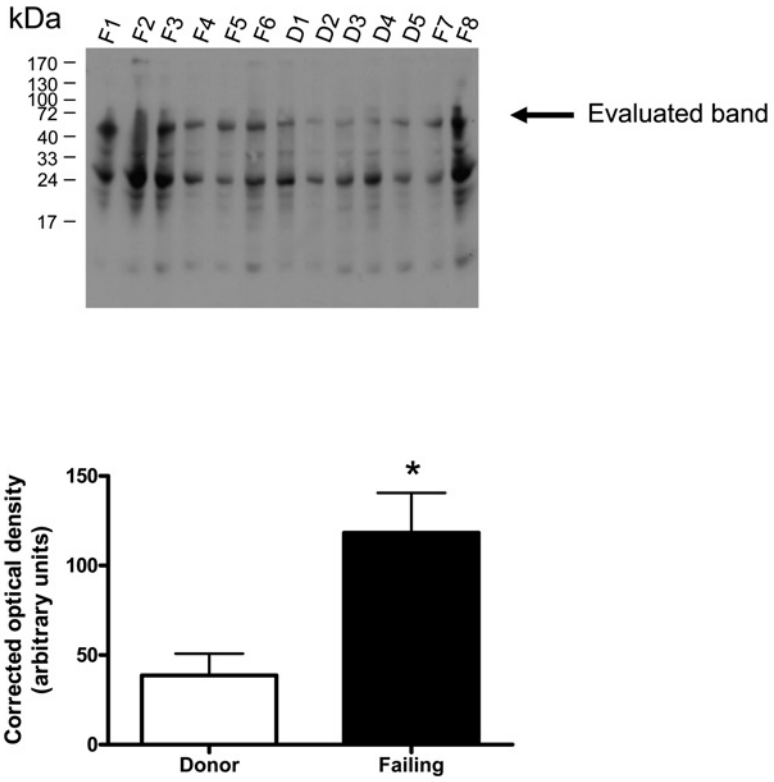

Figure 1. Detection of oxidized proteins. About 0.3 g (wet weight) of ventricular tissues was homogenized in RIPA buffer; 3 μg protein was used for the experiments. Samples are indicated above the lanes (F, failing; D, donor). The optical densities of oxidized protein–derived bands (the evaluated band is indicated) were corrected to the loading, and mean values and SEM are shown in the bar graph (donor, $n = 5$; failing, $n = 8$; $P = 0.02$).

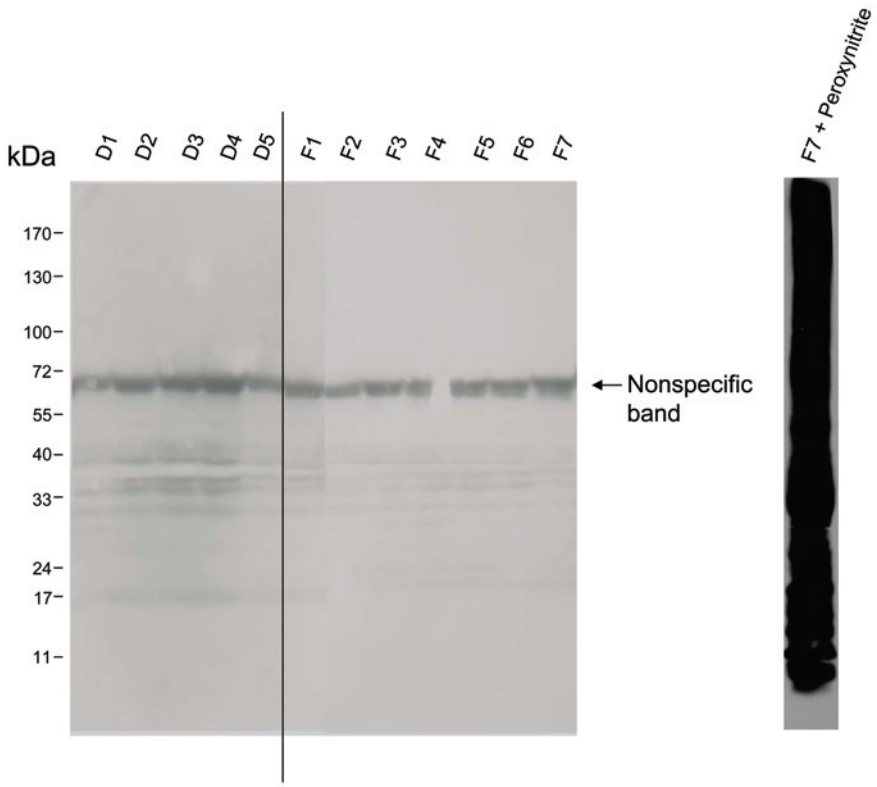

Figure 2. Detection of protein nitration. Human ventricular tissue homogenates (F, failing; D, donor) were loaded (30 μg of protein in each lane) onto 5% to 20% gradient SDS-polyacrylamide gels and probed with a nitrotyrosine-specific antibody (1:10,000; Calbiochem). It should be noted that the major band (indicated) is recognized by the secondary antibody alone and is therefore not specific to nitrotyrosine. A positive control for nitrotyrosine staining (sample F7 treated with 500 μM peroxynitrite for 5 min) is shown on the right.

solution (5 mM Na_2ATP, 6 mM $MgCl_2$, 15 mM Na_2-creatine phosphate, 20 mM imidazole, 91 mM KCl, 2 mM EGTA, and 1% vol/vol protease inhibitor cocktail) (Sigma-Aldrich). The tissue samples were mechanically disrupted at medium speed. The homogenates were centrifuged for 20 min at 14,000*g*. The pellets were discarded and the protein concentrations of the supernatants were determined according to the BCA method (Sigma-Aldrich). Subsequently, aliquots of 20 μg supernatant boiled in SDS loading buffer (total volume 40 ul) were used for Western immunoblot analysis. Electrophoresis was performed on 10% SDS-polyacrylamide gels, followed by transfer to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). For the detection of poly-ADP-ribosylated proteins, membranes were probed with an anti-PAR antibody (developed in rabbit, dilution 1:10,000; Calbiochem) followed by an anti-rabbit secondary antibody (dilution 1:10,000; Sigma-Aldrich), and the bands were detected by enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA, USA).

Oxidized Protein, PARP-1, Caspase-9, and Nitrotyrosine Detection

Human left ventricular tissue samples were homogenized as mentioned above, except that RIPA buffer (1% Igepal CA-630 [Sigma-Aldrich], 0.5% sodium deoxycholate [Reanal Finechemical, Budapest, Hungary], 0.1% sodium dodecylsulfate, 2% vol/vol protease inhibitor cocktail [Sigma-Aldrich], 0.5 mM PMSF [Fluka, Buchs, Switzerland], and 1 mM benzamidine hydrochloride hydrate [Sigma-Aldrich]) was used instead of isolation buffer. The homogenates were centrifuged for 20 min at 14,000*g* and supernatants were used for further determinations.

To detect the carbonyl groups caused by oxidation of protein side-chains, an OxyBlot oxidized protein detection kit (Oncor, Gaithersburg, MD, USA) was used according to the manufacturer's instructions(8). The protein contents of the samples were determined by the BCA method. Each sample (3 mg) was diluted to 5 mL with water, and an equal volume of 12% SDS was added. DNP (10 mL) was added, and the reaction was allowed to proceed at room temperature for 15 min. Neutralization solution (7.5 mL of 0.74 M 2-mercaptoethanol) was added to stop the reaction. The supernatant treated in the absence of DNP served as a negative control. The derived samples and standards were loaded onto 10% SDS-polyacrylamide minigels, and the proteins were transferred to nitrocellulose membranes. The membranes were then blocked with 1% BSA in PBS-T (0.1% Tween-20 containing PBS [150 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4] [Sigma-Aldrich]) for 1 h at room temperature under continuous agitation on an orbital shaker. The blocked membranes were incubated with rabbit anti-DNP 1:150 in 1% BSA/PBS-T for 1 h, washed, and incubated with goat anti-rabbit IgG-HRP 1:300 in 1% BSA in PBS-T for 1 h. The positive bands were detected with an ECL chemiluminescent reagent.

For the detection of PARP-1, caspase-9, and nitrotyrosine, 15 to 30 mg/lane protein (supernatants of the homogenates) was loaded onto 10% SDS-polyacrylamide minigel (for PARP-1 and caspase-9) or 5% to 20% SDS-polyacrylamide gradient gel (for nitrotyrosine). After separation, proteins were transferred to nitrocellulose membrane. The membranes were blocked with 1% BSA in PBS-T for 1 h on an orbital shaker and then probed with

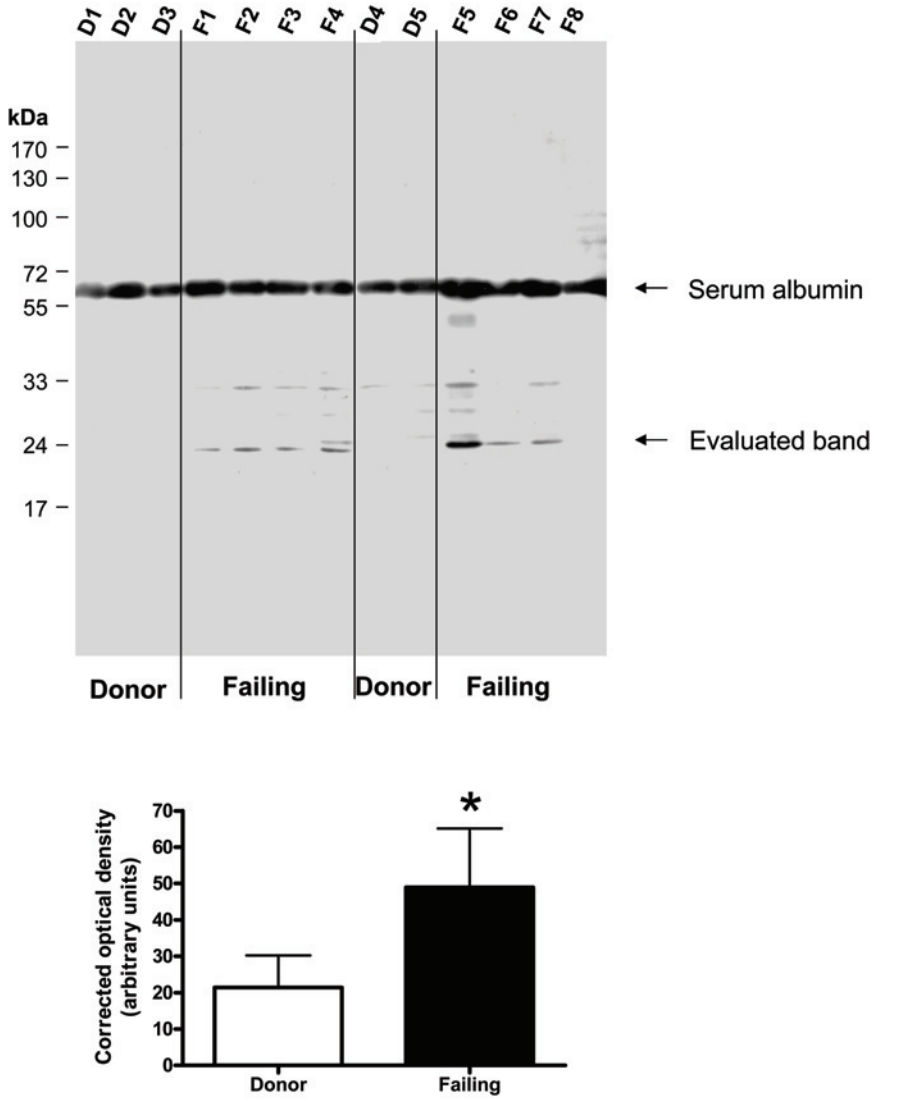

Figure 3. Detection of poly(ADP-ribosyl)ation. Human ventricular tissue homogenates (D, donor; F, failing) were loaded onto 5% to 20% gradient SDS-polyacrylamide gels (20 μg of protein in each lane) and probed with a poly-ADP-ribose-specific antibody (1:10,000; Calbiochem). According to the manufacturer's data sheet, this antibody recognizes poly-ADP-ribosylated proteins specifically and serum albumin nonspecifically (indicated). The most prominent nitrotyrosine-specific band (indicated) was further evaluated for purposes of densitometry. Mean values and SEM are shown in the bar graph (donor, $n = 5$; failing, $n = 8$; $P = 0.005$).

rabbit anti-PARP-1 antibody (Calbiochem) at a dilution of 1:10,000, rabbit anti-caspase-9 antibody (Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:1,000, or rabbit anti-nitrotyrosine antibody (Calbiochem) at a dilution of 1:10,000 in 1% BSA in PBS-T for 1 h. The blots were then incubated with a HRP-conjugated anti-rabbit secondary antibody (Sigma-Aldrich) or with a biotinylated secondary antibody (for nitrotyrosine staining, Vectastain ABC kit; Vector Laboratories), and the immunoreactive proteins were visualized using an ECL detection reagent (PerkinElmer Life Sciences) and X-ray autoradiographic film (Agfa-Gevaert, Belgium).

For nitrotyrosine detection, one of the failing samples (F7) was treated with 10 to 500 μM peroxynitrite for 5 min at room temperature (5 mL of 10 mM peroxynitrite [Calbiochem] in 150 mM KCl, pH 11, was added to 95 mL of tissue homogenate) and was used as positive control for nitrotyrosine staining (see Figure 2).

Densitometric Analysis of the Bands

X-ray films were scanned and evaluated with ImageJ v 1.32 software (www.nih.gov). The densities of the bands of interest (shown in the figures) were measured and corrected for the density of the actin band on the same membranes visualized by Ponceau (Sigma-Aldrich) staining (loading control). The corrected optical densities from the same or parallel membranes were calculated, and the differences between the donor and the failing samples were evaluated with Student t test. The data presented in the graphs are means ± SEM.

RESULTS

Overall, we set out to investigate some of the pathological events occurring during human heart failure that lead to apoptosis of the ventricular myocytes, and therefore, to progression of the disease. To determine the levels of oxidized proteins in the donor and failing heart samples, tissues were screened for carbonyl adducts (8). Compared with the donor heart samples, strong immunoreactivity for oxidized protein end-products was readily detected in the failing hearts (Figure 1). As assessed by semiquantitative densitometry, the level of carbonyl adducts was increased approximately 3-fold (38.7 ± 12.1, $n = 5$, in the donor hearts and 118.3 ± 22.3, $n = 8$, in the failing hearts; $P = 0.02$).

The higher production of superoxide (probably resulting in the oxidation of myocardial proteins, as shown in Figure 1) and NO leads to the formation of peroxynitrite, which reacts with protein tyrosine residues to generate nitrotyrosine (9). Earlier, it was found that myocardial

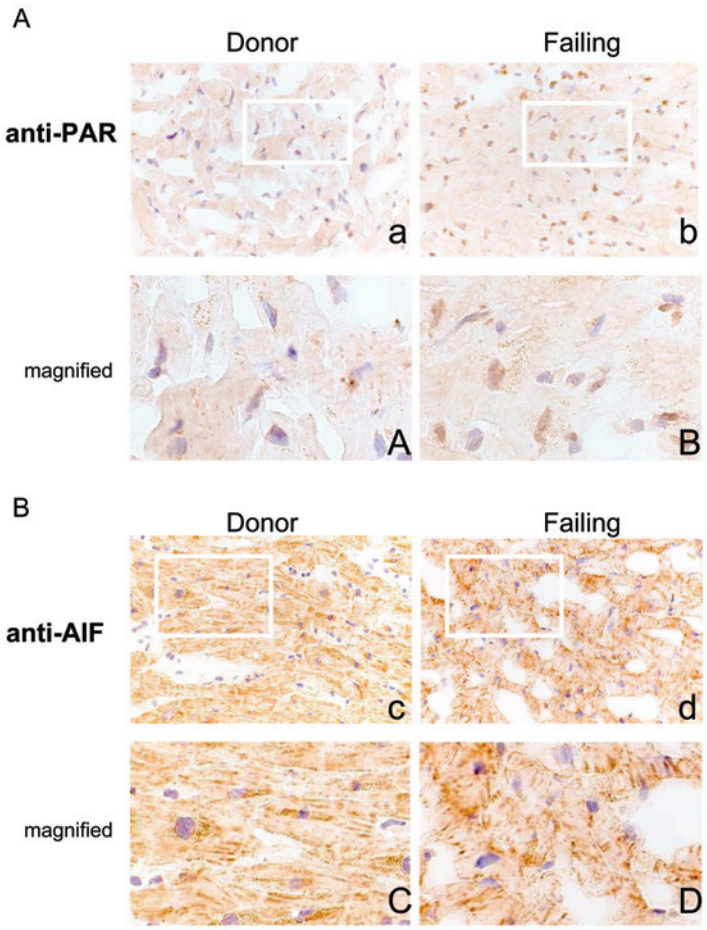

Figure 4. Immunohistochemical analysis of poly-ADP-ribosylation. Poly-ADP-ribosylated proteins (A) or AIF (B) were detected on frozen sections from donor (a and c) and failing (b and d) left ventricular tissue samples with antibodies against poly-ADP-ribose polymers (1:100; Calbiochem) or against AIF (1:100; Chemicon), using the ABC method and DAB as chromogen. Sections were counterstained with hematoxylin. Control sections from donor and failing hearts were simultaneously stained with omission of the primary antibody and no significant staining was observed (data not shown). Three samples were evaluated from both donor and failing hearts and representative pictures are shown. The indicated regions on the pictures recorded with low magnification (squares in pictures a, b, c, and d) are also shown in higher magnification, labeled by capital letters (A, B, C and D), respectively.

proteins (such as alfa-actinin) could be nitrated by peroxynitrite treatment, in vitro, with a consequent decrease in the contractile force (10). Via the same approach, failing and donor hearts were tested for nitrated proteins: no difference was found (Figure 2). It should be noted that in vitro peroxynitrite treatment resulted in a robust signal (Figure 2), indicating that the experimental system was sensitive to protein tyrosine nitration. Additionally, no significant nitrotyrosine staining was found in donor or failing hearts (data not shown), confirming the absence of nitrotyrosine-producing (nitrosative) stress.

Oxidative stress is associated with the activation of PARP in many cells and tissues (11). To determine the level of PARP activation, donor and failing heart samples were screened for poly-ADP ribosylated proteins (Figure 3). As assessed by semiquantitative densitometry, the level of poly-ADP ribosylated proteins was increased 2.3-fold in the failing hearts compared with the donor hearts (49.0 ± 16.2, $n = 8$, in the donor hearts and 21.4 ± 8.8, $n = 5$, in the failing hearts; $P = 0.005$). Investigation of the subcellular pattern of poly-ADP ribosylated proteins demonstrated an obvious nuclear localization in the failing hearts (Figure 4). Relative to the donor hearts, the PAR-positive nuclei were 3.1-fold more abundant (20.8% ± 7.1% in the donor hearts and 64.0% ± 2.2% in the failing hearts). The nominal difference between the western immunoblot technique (a 2.3-fold elevation in poly-ADP ribosylated proteins) and the immunohistochemical data (a 3.1-fold increase in PAR-positive nuclei) may suggest a higher sensitivity of the latter approach.

Activation of PARP-1 often results in AIF-mediated apoptosis. To test the initiation of the apoptotic program, the localization of AIF was also tested (Figure 4); no translocation (indicative of AIF activation) was found in donor or failing hearts.

An elevation in poly-ADP ribosylation may be due either to a higher expression of PARP-1 or to catalytic activation of the enzyme (12). PARP-1 expression was tested in the donor and in the failing heart samples with a PARP-1–specific antibody (Figure 5), and no significant difference was found (in the donor hearts 13.1 ± 6.7, $n = 5$, and 19.9 ± 3.1, $n = 7$ in the failing hearts; $P = 0.33$). Interestingly, a high level of degradation of PARP-1 was detected in both the failing and the donor hearts. This is in accordance with the finding that PARP-1 activation is often followed by the poly-ADP ribosylation of PARP-1 and by proteolytic degradation and inactivation (11). It is not clear what is the relevance of the variability in expression (for example, donor 1 and 2 compared with donor 3 and 4) or in degradation of PARP-1 (for example, samples 3, 4, and 5) in the donor samples (Figure 5). It is possible that variability in the donor sample pool or differences in the collection of the samples may contribute to the diversity in both the donor and the failing hearts.

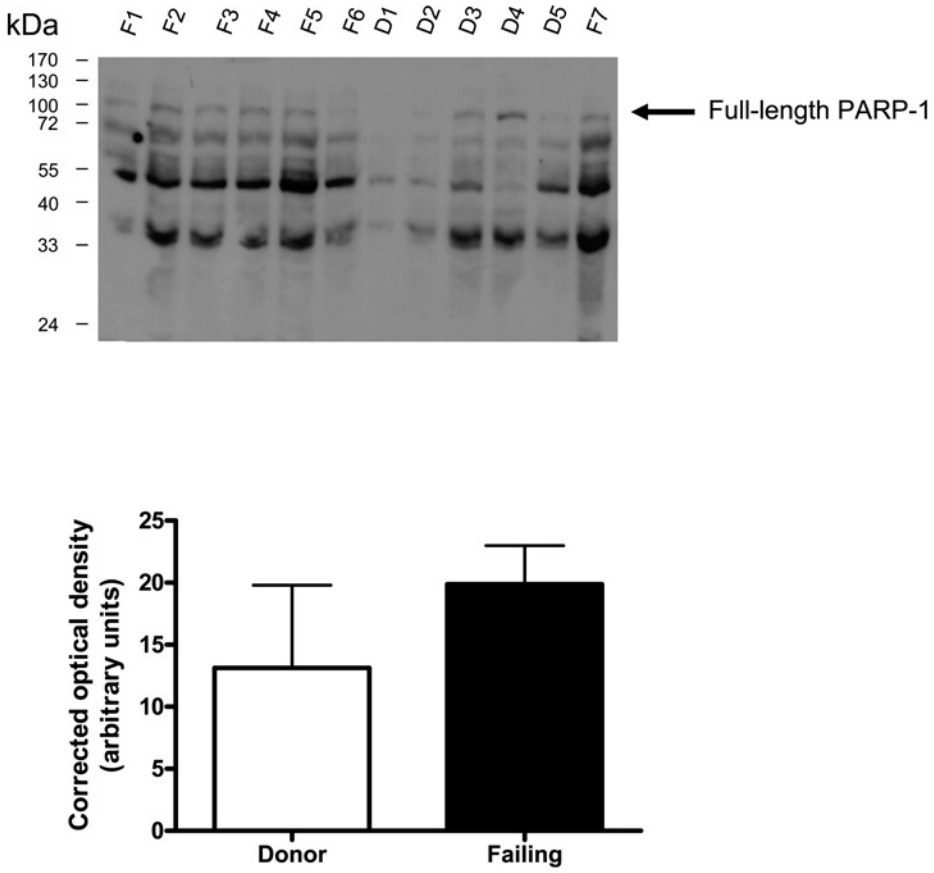

Figure 5. Detection of poly(ADP-ribose) polymerase (PARP-1) expression. Human ventricular tissue samples (F, failing; D, donor) were loaded (15 μg of protein in each lane) onto 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with a PARP-1–specific antibody (1:10,000; Calbiochem) and also stained with Ponceau. In the densitometric analysis, the optical density of the band representing full-length PARP-1 (indicated) was corrected with the loading, and the mean values ± SEM are shown in the bar graph (donor, $n = 5$; failing, $n = 7$; $P = 0.33$).

Caspases are well-known proteases capable of PARP cleavage and the promotion of apoptosis (11,13). The activation of one of the upstream caspases (caspase-9) was analyzed in our samples. Procaspase-9 (not cleaved, inactive caspase-9) expression was approximately 3-fold lower in the donor hearts than in the failing hearts (3.7 ± 0.6, $n = 8$, in the donor hearts and 12.3 ± 2.2, $n = 5$, in the failing hearts; $P = 0.009$), as determined by densitometry of caspase-9–specific bands using Western immunoblot (Figure 6).

DISCUSSION

Experimental and clinical studies have demonstrated the increased production of reactive oxygen species (ROS) (superoxide, H_2O_2, and hydroxyl radical) in the pathogenesis of acute and chronic heart failure. Plasma malondialdehyde-like activity, a marker of lipid peroxidation, is increased in patients with ischemic and nonischemic dilated cardiomyopathy; it correlates with the severity of the symptoms, and exhibits an inverse relationship with the ejection fraction and the exercise capacity (14). The pericardial concentration of 8-iso-PGF_2 (a marker for ROS production) correlates closely with the end-systolic and end-diastolic diameters of the left ventricle and with the functional severity of heart failure (15). Furthermore, there is a significant positive correlation between myocardial ROS production and left ventricular contractile dysfunction in experimental models (16).

Myocardial ROS generation is triggered by repetitive episodes of ischemia and reperfusion, by inflammatory cytokines, by catecholamine auto-oxidation, and by prostaglandin biosynthesis (1). Impaired antioxidant defense mechanisms (superoxide dismutase, catalase, and glutathione peroxidase) or reduced concentrations of endogenous antioxidants (vitamin E, ascorbic acid, and cysteine) can contribute to an increased ROS production within the myocardium (17). Sources of ROS in the failing myocardium include xanthine oxidoreductases, cyclooxygenases, the mitochondrial electron transport chain (complex I), activated neutrophils, NOS, and the auto-oxidation of certain tissue metabolites and NAD(P)H oxidoreductases (1-6,17). The current results are consistent with these findings and demonstrate the presence of significant oxidative stress in failing human heart samples, whereas the control donor hearts displayed low levels of oxidative stress. The latter finding is consistent with the view that the collection of the hearts and the storage and the processing of the samples during our procedures do not trigger a significant degree of oxidant generation.

The cardiomyocytes, the endocardial endothelium, the coronary endothelium, and the cardiac nerves are sites of NO production by Ca^{2+}-dependent NOS. NO serves a number of important physiological roles in the regulation of cardiac function, including coronary vasodilation, inhibition of platelet and neutrophil adhesion and activation, modulation of the cardiac contractile function, and inhibition of cardiac oxygen consumption (18). Although NO is essential in cardiac physiology (18), at higher levels, or in the presence of reactive oxygen species, NO can also exert cytotoxic effects. Many of the toxic actions of NO are not due to NO directly, but are mediated via the production of the highly reactive oxidant

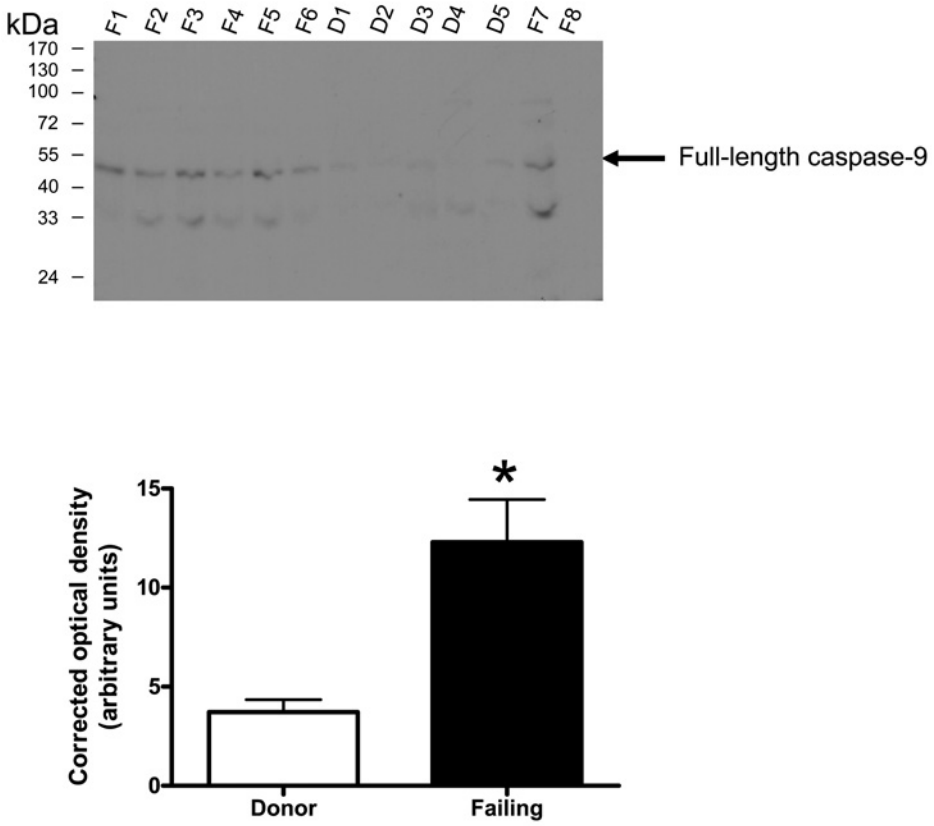

Figure 6. Detection of caspase-9 expression. Human ventricular tissue samples (F, failing; D, donor) were loaded (15 μg of protein in each lane) onto 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with a caspase-9–specific antibody (1:1000; Cell Signaling) and also stained with Ponceau. In the quantification, the optical density of the band denoting the full-length caspase-9 (procaspase-9, indicated) was corrected with the loading, and the mean values ± SEM are shown on the bar graph (donor, $n = 5$; failing, $n = 8$; $P = 0.009$).

peroxynitrite, the reaction product of NO and superoxide (9,19).

Animal models of heart failure have furnished ample evidence of both the overproduction of NO and the generation of peroxynitrite (at least as evidenced by its footprint, nitrotyrosine). NO is overproduced in the failing myocardium as a consequence of the increased expression and activity of iNOS (20). There is a correlation between the chronic overexpression of iNOS and peroxynitrite generation and cardiac enlargement, conduction defects, sudden cardiac death, and less commonly, heart failure in mice (21). Myocardial iNOS is induced in rats with volume-overload heart failure, and increased iNOS activity leads to a loss of myocardial contractility and β-adrenergic hyporesponsiveness (22). Neuronal NOS-derived NO production has also been demonstrated in the failing human heart (23). Although peroxynitrite generation has been reported in various forms of acute and chronic heart failure in many animal models (24-26) and in some human studies (27,28), we failed to detect differences in tyrosine nitration in the failing and donor hearts, even though the evidence of massive tyrosine nitration in the positive controls demonstrates that the assay used was adequate (Figure 2, data not shown for the immunohistochemistry). It is possible, however, that low, subthreshold levels of nitrotyrosine are present in our samples, and it is also conceivable that peroxynitrite formation and tyrosine nitration follow a particular time course and our samples were taken from patients where tyrosine nitration was no longer present.

Three particular pathways initiated by ROS and reactive nitrogen species in the failing heart are related to the activation of PARP: activation of caspases, activation of MMPs, and DNA strand breaks (12). As far as PARP activation is concerned, it has been demonstrated in ischemia-, banding-, diabetes-, and cardiotoxic drug–induced heart failure in murine studies (4,7,29-31). There is multiple evidence of the activation of caspases, and this issue has been widely investigated and debated in light of the relevance or nonrelevance of apoptosis in the context of chronic heart failure (32-34). The activation of MMPs also been revealed in heart failure (35,36). It is important that both caspases and MMPs have the ability to induce the cleavage of PARP-1 in heart failure (32-36).

The present study has yielded evidence of PARP activation in myocardial samples from patients with heart failure. Thus, the current study adds a further example of a human disease in which PARP activation has been demonstrated. Previous work has demonstrated PARP activation, among others, in human diabetic microvessels (37). The current findings are also consistent with a recent report by Pillai, DiNapoli and colleagues of increased poly(ADP-ribosyl)ation in human failing hearts (31,32). However, in contrast with Pillai, DiNapoli and colleagues (31,32), we were unable to detect the upregulation of PARP-1 protein in the heart failure samples (Figure 4), which suggests that the increased poly(ADP-ribosyl)ation in our samples was most probably due to activation of the catalytic function of PARP. This can occur via two principal mechanisms: breaks in the DNA strands or increased intracellular calcium concentration (12,38). Both of these mechanisms have been demonstrated in failing hearts, and

Table 1. Summary of the pretransplant data and drug therapy for the patients

Case No.	Status	NYHA	Diagnosis	Age	Sex	Digoxin	ACE I	Diuretics	Dobutamine/ Dopamine	Syncumar	Other	Cause of death
1	Failing	IV	CAD	40	F			+	+		Salicylate, antidepressant, antiarrhythmic drug (class III), proton pump inhibitor, vasodilatator, heparin, lipid lowering	—
2	Failing	IV	HCM	49	M		+			+	β-blocker, H2-receptor blocker, lipid lowering	—
3	Failing	IV	DCM	54	M	+	+	+			β-blocker, H2-receptor blocker, NSAID	—
4	Failing	IV	CAD	49	F		+	+		+	Calcium channel antagonist, β-blocker, antidepressant	—
5	Failing	IV	DCM	50	M		+	+		+	β-blocker, antiarrhythmic drug (class III), antidepressant	—
6	Failing	IV	CAD	48	F		+	+		+	β-blocker, Ca/Mg supplements, anti-anginal drug	—
7	Failing	III-IV	DCM	49	M	+		+		+	—	—
8	Failing	IV	DCM	49	M			+		+	—	—
1	Donor			46	M			+	+		—	Ischemic stroke
2	Donor			56	M				+		—	Stroke
3	Donor			18	F			+	+		—	Trauma
4	Donor			37	F						—	Subarachnoid hemorrhage (stroke)
5	Donor			39	F				+		—	Apoplexia cerebri (stroke)

DCM indicates dilatative cardiomyopathy; HCM, hypertrophic cardiomyopathy; CAD, coronary artery disease; NYHA, class of failing according to the New York Heart Association; ACE-I, angiotensin convertase enzyme inhibitor.

in theory, both mechanisms could contribute to PARP activation.

The activation of caspases may potentially be triggered by two different mechanisms. In one of these, initiator caspases such as caspase-8 or -9 are activated in a multimeric complex (for example, caspase-8 in the death-inducing signaling complex and caspase-9 at the apoptosome). Alternatively, caspases are activated by catalytic processing of the zymogens at specific cleavage sites (13). Because of the central role of caspase-9 in the activation of PARP-1–degrading enzyme pathways (11), an effort was made to detect caspase-9 in our system; our data demonstrated that it is overexpressed (a higher expression of procaspase) and activated (a higher level of the proteolytically activated form). Activated caspases may trigger the cleavage of PARP (11), and may also act upon a variety of intracellular targets to promote apoptosis (13). The relationship between PARP activation and PARP cleavage, apoptosis vs. necrosis, is complex, but accumulating evidence suggests that PARP activation contributes to cell necrosis, while PARP cleavage (leading to a decreased PARP-1 activity) may serve as a protective mechanism (to prevent necrosis by cellular energy exhaustion) and thereby permit apoptosis (12). Interestingly, it was found that cardioplegic arrest induces apoptosis signaling pathways in myocardial endothelial cells and cardiac myocytes (39). In our case, however, we did not find significant translocation of AIF, suggesting that these apoptotic pathways were not activated in the donor or in failing hearts studied here.

Caspase-9 expression and the level of activated caspase-9 were higher in failing hearts than in donor hearts. The relevance of this finding is not clear. On the one hand, caspases can initiate the degradation of PARP-1, which degradation was indeed observed in the case of failing hearts. On the other hand, caspase activation can promote apoptosis, which is in contrast with the apparent lack of AIF translocation in both donor and fail-

ing hearts. It is therefore possible that caspase-9 overexpression and higher activation is a bystander effect of PARP-1 activation/degradation or apoptosis.

It is clear that further experiments (using biopsies obtained at different stages of heart failure) are required to clarify the relationship between apoptosis and PARP-1 activation. For example, such endocardial biopsies were taken and evaluated for apoptotic markers in the case of dilatative cardiomyopathy (40-44). Interestingly, controversial results were obtained, suggesting that the therapy of the patients or methodical difficulties (obtaining endocardial biopsies from the same locations) affect the results of the apoptotic tests. It should be also taken into account that a rather diverse set of human heart samples were analyzed. Therefore the etiology of the heart failure could have contributed to some of the differences seen (coronary heart disease, which is ischemic and would be expected to lead to enhanced oxidative stress, vs. a dilated cardiomyopathy, which could be from alcohol or a virus).

Our data suggest that PARP-1 activation did not result in the initiation of apoptosis in failing human hearts, according to the absence of AIF translocation. It is therefore possible that PARP-1 is activated by a limited number of DNA breaks as an important element of the DNA repair system. Additionally, it should be considered that our samples were from end-stage failing hearts, which may have successfully adapted to the higher level of oxidative stress and to the consequent PARP-1 activation. Altogether, it cannot be ruled out that acute PARP-1 activation results in apoptosis in the case of other pathologies (for example, in case of ischemia-reperfusion).

In animal studies, potent antioxidant compounds and potent PARP inhibitors exhibit significant efficacy in preventing myocardial dysfunction, reducing hypertrophy, and improving myocardial efficacy (38), suggesting that oxidative stress and PARP activation are related. It is certainly important to perform clinical studies to determine the potential causative role of the oxidative/nitrosative stress/PARP pathway in the pathogenesis of human heart failure. Overall, the current study has clearly provided evidence of oxidative stress and PARP activation in human failing heart samples, confirming earlier animal results in human tissue samples and supporting the design of future clinical trials.

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