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Increased Myocardial NADPH Oxidase Activity in Human Heart Failure

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OBJECTIVES This study was designed to investigate whether nicotinamide adenine dinucleotide 3-phosphate (reduced form) (NADPH) oxidase is expressed in the human heart and whether it contributes to reactive oxygen species (ROS) production in heart failure.

BACKGROUND A phagocyte-type NADPH oxidase complex is a major source of ROS in the vasculature and is implicated in the pathophysiology of hypertension and atherosclerosis. An increase in myocardial oxidative stress due to excessive production of ROS may be involved in the pathophysiology of congestive heart failure. Recent studies have suggested an important role for myocardial NADPH oxidase in experimental models of cardiac disease. However, it is unknown whether NADPH oxidase is expressed in the human myocardium or if it has any role in human heart failure.

METHODS Myocardium of explanted nonfailing (n = 9) and end-stage failing (n = 13) hearts was studied for the expression of NADPH oxidase subunits and oxidase activity.

RESULTS The NADPH oxidase subunits $p22^{phox}$, $gp91^{phox}$, $p67^{phox}$, and $p47^{phox}$ were all expressed at messenger ribonucleic acid and protein level in cardiomyocytes of both nonfailing and failing hearts. NADPH oxidase activity was significantly increased in end-stage failing versus nonfailing myocardium (5.86 ± 0.41 vs. 3.72 ± 0.39 arbitrary units; $p < 0.01$). The overall level of oxidase subunit expression was unaltered in failing compared with nonfailing hearts. However, there was increased translocation of the regulatory subunit, $p47^{phox}$, to myocyte membranes in failing myocardium.

CONCLUSIONS This is the first report of the presence of NADPH oxidase in human myocardium. The increase in NADPH oxidase activity in the failing heart may be important in the pathophysiology of cardiac dysfunction by contributing to increased oxidative stress. (J Am Coll Cardiol 2003;41:2164–71) © 2003 by the American College of Cardiology Foundation

Considerable evidence supports a role for oxidative stress due to excessive production of reactive oxygen species (ROS) such as superoxide (O_2^-) in the pathophysiology of congestive heart failure (CHF). Increased oxidative stress may contribute to contractile dysfunction, endothelial dysfunction, myocyte apoptosis and necrosis, remodeling of the extracellular matrix, and the progressive downward spiral of heart failure (1,2). Patients with CHF have elevated plasma levels of thiobarbituric acid-reactive substances (3) and pericardial levels of 8-isoprostaglandin F2-alpha (4), and these markers of increased oxidative stress correlate with functional severity of CHF (4). Aspects of the CHF phenotype, such as the impairment of nitric oxide-dependent vasodilation, can be restored to normal by acute treatment with antioxidants (5).

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Experimental studies also support a role for increased oxidative stress in failing myocardium (1,6). In isolated cardiac myocytes, a moderate increase in ROS induces a phenotype characterized by hypertrophy and apoptosis (7). In experimental pressure-overload cardiac hypertrophy, the transition to heart failure is associated with increased oxidative stress and can be suppressed by treatment with the antioxidant vitamin E (8). Likewise, progressive myocardial remodeling following experimental myocardial infarction could be inhibited by antioxidant treatment (9).

Because these studies suggest an important role for oxidative stress in ventricular remodeling and the progression to CHF, it is important to define the nature and sources of ROS production in the failing heart. Recent animal studies have suggested that both mitochondria and xanthine oxidase may be significant contributors to ROS production in experimental heart failure (6,10,11). However, there is still a paucity of data regarding potential ROS sources in the failing human myocardium (12).

In the vasculature, accumulating evidence from experimental and clinical studies indicates that a phagocyte-type nicotinamide adenine dinucleotide 3-phosphate (reduced form) (NADPH) oxidase complex is a major source of ROS in several disease settings (13). This enzymatic complex was initially discovered in neutrophils, where it is activated during phagocytosis, resulting in high-output ROS (O_2^-) production, and plays an essential role in nonspecific host defense against infective microbes (14). A similar or identical enzyme complex is also expressed in many non-

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Abbreviations and Acronyms

CHF	= congestive heart failure
DPI	= diphenyleneiodonium
L-NAME	= Nω-nitro-L-arginine methyl ester hydrochloride
mRNA	= messenger ribonucleic acid
NADPH	= nicotinamide adenine dinucleotide 3-phosphate (reduced form)
O_2^-	= superoxide
ROS	= reactive oxygen species

phagocytic cells. Reactive oxygen species production by the NADPH oxidase in smooth muscle and endothelial cells of the vessel wall has been implicated in the genesis of hypertensive vascular smooth muscle hypertrophy (13,15,16), atherosclerosis (17), and endothelial dysfunction (18). Importantly, NADPH oxidase is known to be activated by several stimuli of relevance to cardiovascular pathophysiology, including angiotensin II, noradrenaline, tumor necrosis factor-alpha (TNF-alpha), and increased mechanical forces (13,14).

Recent experimental studies suggest a role for NADPH oxidase in cardiac pathophysiology. We have reported an increased myocardial expression and activity of NADPH oxidase in experimental pressure-overload cardiac hypertrophy (19) and suggested a role for ROS derived from this oxidase in the genesis of cardiac contractile dysfunction in this setting (20). The development of experimental angiotensin II-induced cardiac hypertrophy was found to be inhibited in mice lacking a functional NADPH oxidase (21). Likewise, ROS production by NADPH oxidase was implicated in alpha-adrenergic agonist-induced cardiomyocyte hypertrophy (22).

The aims of the present study were to investigate whether NADPH oxidase is expressed in the human heart, and if so, whether its expression or activity are altered in the failing heart.

METHODS

Patients. All studies conformed to the Declaration of Helsinki and institutional ethical regulations. Explanted failing hearts were obtained from patients undergoing cardiac transplantation for end-stage CHF secondary to idiopathic dilated cardiomyopathy (n = 13; mean age 53 ± 3.2 years). All patients had New York Heart Association class IV CHF, with a mean pretransplant left ventricular ejection fraction of 22% ± 4%. None had been treated with left ventricular assist devices or had received chronic intravenous inotropic support over at least seven days immediately before transplantation. Heart failure therapy consisted of angiotensin-converting enzyme inhibitors and diuretics in all patients. Nonfailing hearts (n = 9; mean age 47 ± 4.4 years) were obtained from prospective multiorgan donors who had died from head trauma or intracranial bleeds; these hearts were unsuitable for transplantation for technical reasons. All tissues were stored at −80°C until further analyses.

Myocardial NADPH oxidase activity. Specific left ventricular myocardial NADPH oxidase activity was measured in total tissue homogenates of explanted failing and nonfailing hearts by lucigenin-enhanced chemiluminescence (23,24). Experiments were performed on a microplate luminometer (Anthos "Lucy 1," Labtech; 37°C) using 100 μg protein/well, NADPH 300 μmol/l, and a non-redox cycling dose of lucigenin (5 μmol/l). Oxidase activity was also measured in the presence of a cell-permeable O_2^- scavenger 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron, 20 mmol/l) to confirm that the measured signal was attributable to superoxide. Experiments were also performed with the following pharmacologic inhibitors to confirm that the measured activity was attributable to NADPH oxidase: a flavoprotein inhibitor, diphenyleneiodonium (DPI, 10 μmol/l); a nitric oxide synthase inhibitor, Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μmol/l); a mitochondrial site I electron transport inhibitor, rotenone (20 μmol/l); or a xanthine oxidase inhibitor, oxypurinol (100 μmol/l) (22). A buffer blank (<2% of the homogenate signal) was subtracted from each reading. Chemiluminescence readings were expressed as integrated light units over 40 min, a period over which maximal chemiluminescence was achieved; this method of quantifying activity (area under the curve) correlates well with enzyme rates measured by other methods such as O_2 consumption or cytochrome c reduction (23).

Immunoblotting. Left ventricular tissue homogenate was immunoblotted for the NADPH oxidase subunits $p22^{phox}$, $gp91^{phox}$, $p67^{phox}$, and $p47^{phox}$, as well as the myofilament protein cardiac troponin I, which was used as a loading control. Monoclonal anti-$p22^{phox}$ and anti-troponin I antibodies were kind gifts from Dr. A. Verhoeven (Amsterdam, Netherlands) and Dr. P. Cummins (Birmingham, United Kingdom), respectively. The polyclonal anti-$gp91^{phox}$ antibody was kindly provided by Dr. M. Quinn (Montana). Anti-$p67^{phox}$ and anti-$p47^{phox}$ polyclonals were purchased from Transduction Laboratories (Oxford, United Kingdom). Anti-mouse or -rabbit horseradish peroxidase-conjugated secondary antibodies were used. Immunoreactive bands were visualized by enhanced chemiluminescence and quantified by densitometry. Results are expressed relative to cardiac troponin I abundance.

Immunohistochemistry. Cryostat sections (7 μm thick) were postfixed in acetone/methanol (1:1) at −20°C. Nonspecific binding was prevented by preincubation in phosphate-buffered saline containing 5% BSA for 30 min at room temperature. The antibodies used were polyclonal anti-$p47^{phox}$ and anti-$gp91^{phox}$ antibodies (as described earlier), and monoclonal anti-vinculin and anti-alpha-actinin antibodies (Sigma, Poole, United Kingdom). Antibodies were applied to sections for 1 h at 25°C. FITC-conjugated sheep anti-rabbit immunoglobulin G or Texas Red-conjugated anti-mouse immunoglobulin G were the

Table 1. Information Regarding Oligonucleotide Primers Used in Study

Primer Target	PCR Product Size, Base Pairs	Number of Cycles	Primer Sequence (5′ - 3′)
gp91^{phox}	528	28	forward-ATGGTGGCGTGGATGATTGCA reverse-GAACATGGGACCCACGATCCA
p22^{phox}	316	26	forward-GTTTGTGTGCCTGCTGGAGT reverse-TGGGCGGCTGCTTGATGGT
p47^{phox}	767	32	forward-ACCCAGCCAGCACTATGTGT reverse-AGTAGCCTGTGACGTCGTCT
p67^{phox}	746	28	forward-CGAGGGAACCAGCTGATAGA reverse-CATGGGAACACTGAGCTTCA
cTnI	125	20	forward-TCCTCCAACTACCGCGCTTA reverse-CTCGCTCCAGCTCTTGCTTT

cTnI = cardiac troponin I; PCR = polymerase chain reaction.

secondary antibodies. Sections were washed and mounted in aqueous medium Vectashield (Vector) containing DAPI. Fluorescence was observed either with a DMRB Leica microscope equipped with epifluorescence optics or a Nikon E600FN upright microscope equipped with a Bio-Rad MRC-1024/2-P multiphoton imaging attachment.

Reverse transcription-polymerase chain reaction. Total RNA was extracted with Trizol Reagent (Roche). Moloney murine leukemia virus reverse transcriptase (200 U, Promega) was used for reverse transcription. C-deoxyribonucleic acid was amplified using 2.5 U Taq deoxyribonucleic acid polymerase (Roche) and 0.5 μmol/l of specific sense and antisense primers for gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, or cardiac troponin I (Table 1). The linear phase of amplification was determined for each primer set to allow semi-quantitative PCR analysis. All samples were run in duplicate. Gels were quantified by densitometry, and NADPH oxidase subunit expression level was normalized by cardiac troponin I. In preliminary experiments, we confirmed that myocardial messenger ribonucleic acid (mRNA) expression of troponin I did not vary significantly between normal and failing human myocardium (data not shown).

Data analysis. Data are expressed as mean ± SEM. Comparison of O_2^- production between nonfailing and failing tissue was performed by Student unpaired *t* test. Comparisons within groups were performed by one-way analysis of variance followed by Dunnett's test. A p value < 0.05 was considered statistically significant.

RESULTS

NADPH oxidase activity in nonfailing and failing myocardium. NADPH-dependent O_2^- production was readily detectable in both nonfailing and failing myocardium. However, oxidase activity was significantly higher in end-stage failing compared with nonfailing myocardium (5.86 ± 0.41 vs. 3.72 ± 0.39 arbitrary units, respectively; $p < 0.01$) (Fig. 1A).

NADPH-dependent chemiluminescence was virtually abolished by the O_2^- scavenger Tiron in both groups, confirming O_2^- as the measured species. Myocardial O_2^- production was abolished by the flavoprotein inhibitor DPI in both groups but was unaltered by a nitric oxide synthase inhibitor (L-NAME), an inhibitor of mitochondrial ROS production (rotenone), or a xanthine oxidase inhibitor (oxypurinol), supporting a phagocyte-type NADPH oxidase as the likely source (Fig. 1B).

NADPH oxidase expression and localization within the myocardium. All four major oxidase subunits were detectable at both mRNA (not shown) and protein level in end-stage failing as well as nonfailing human heart (Fig. 1C). However, there were no significant differences in protein expression level of oxidase subunits between the two groups (Fig. 1D). Likewise, the mRNA expression level of each subunit was similar in the two groups (data not shown).

To examine the cellular localization of NADPH oxidase subunits, immunohistochemical studies were undertaken on sections of nonfailing and failing myocardium ($n > 3$ hearts each; Figs. 2 and 3). These experiments were focused on gp91^{phox}, the subunit responsible for catalytic activity of the oxidase, and p47^{phox}, which is thought to play a key regulatory role in oxidase activation following its translocation to the cell membrane, where it associates with gp91^{phox} and p22^{phox} (14). In nonfailing hearts, there was a low amount of positive staining for gp91^{phox} in cardiac myocytes (Figs. 2A and 2C) as well as the endothelium of cardiac vessels (data not shown). In failing hearts, gp91^{phox} immunoreactivity was particularly prominent on sarcolemmal membranes and also intracellularly (Figs. 2B and 2D). Co-staining for alpha-actinin as a myocyte marker, which typically labels intracellular costamers and intercalated discs, confirmed that gp91^{phox} was expressed in cardiomyocytes (Figs. 2E to 2H).

Positive staining for p47^{phox} was also detected both in nonfailing and failing heart tissue. Figure 3A shows examples of nonfailing and failing heart sections co-stained for p47^{phox} and alpha-actinin, and confirms that p47^{phox} was expressed in cardiomyocytes. Interestingly, p47^{phox} immunoreactivity appeared to be significantly increased in the cardiomyocyte sarcolemma of failing hearts (Fig. 3A, panels d and f vs. a and c), suggesting a translocation of p47^{phox}

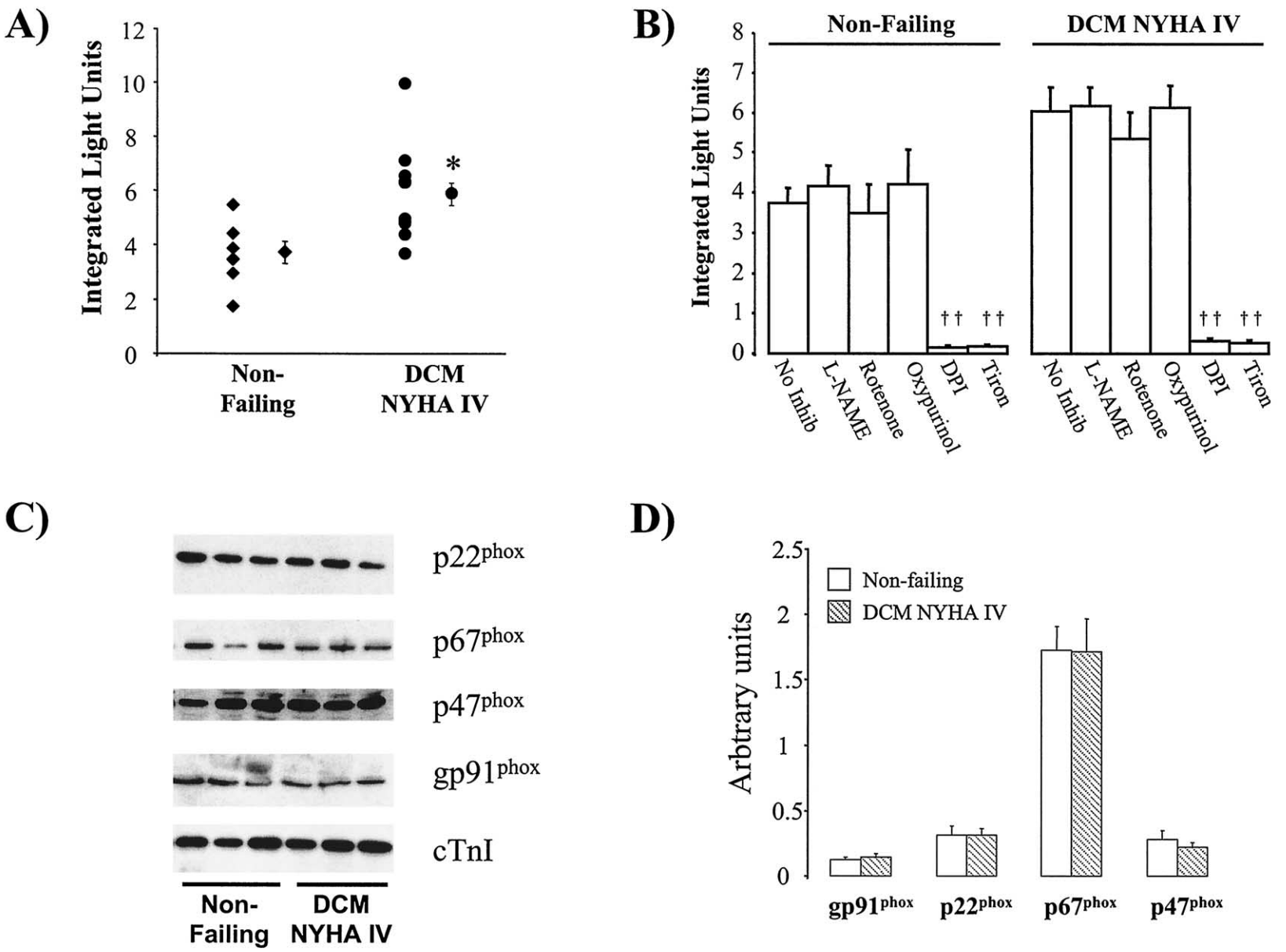

Figure 1. Myocardial nicotinamide adenine dinucleotide 3-phosphate (reduced form) (NADPH) oxidase expression and activity in nonfailing and end-stage failing human heart. **(A)** NADPH-dependent superoxide production in left ventricular homogenates measured by lucigenin-enhanced chemiluminescence. *$p < 0.01$ between groups. **(B)** Effect of specific inhibitors (Inhib) of enzymatic reactive oxygen species (ROS) production or of the superoxide scavenger Tiron. ††$p < 0.001$ for ROS production with and without inhibitor. **(C)** Representative immunoblots for NADPH oxidase subunits and for cardiac troponin I (cTnI). **(D)** Densitometric quantification of NADPH oxidase subunit expression relative to troponin I. DCM = dilated cardiomyopathy; DPI = diphenyleneiodonium; NYHA = New York Heart Association; L-NAME = Nω-nitro-L-arginine methyl ester.

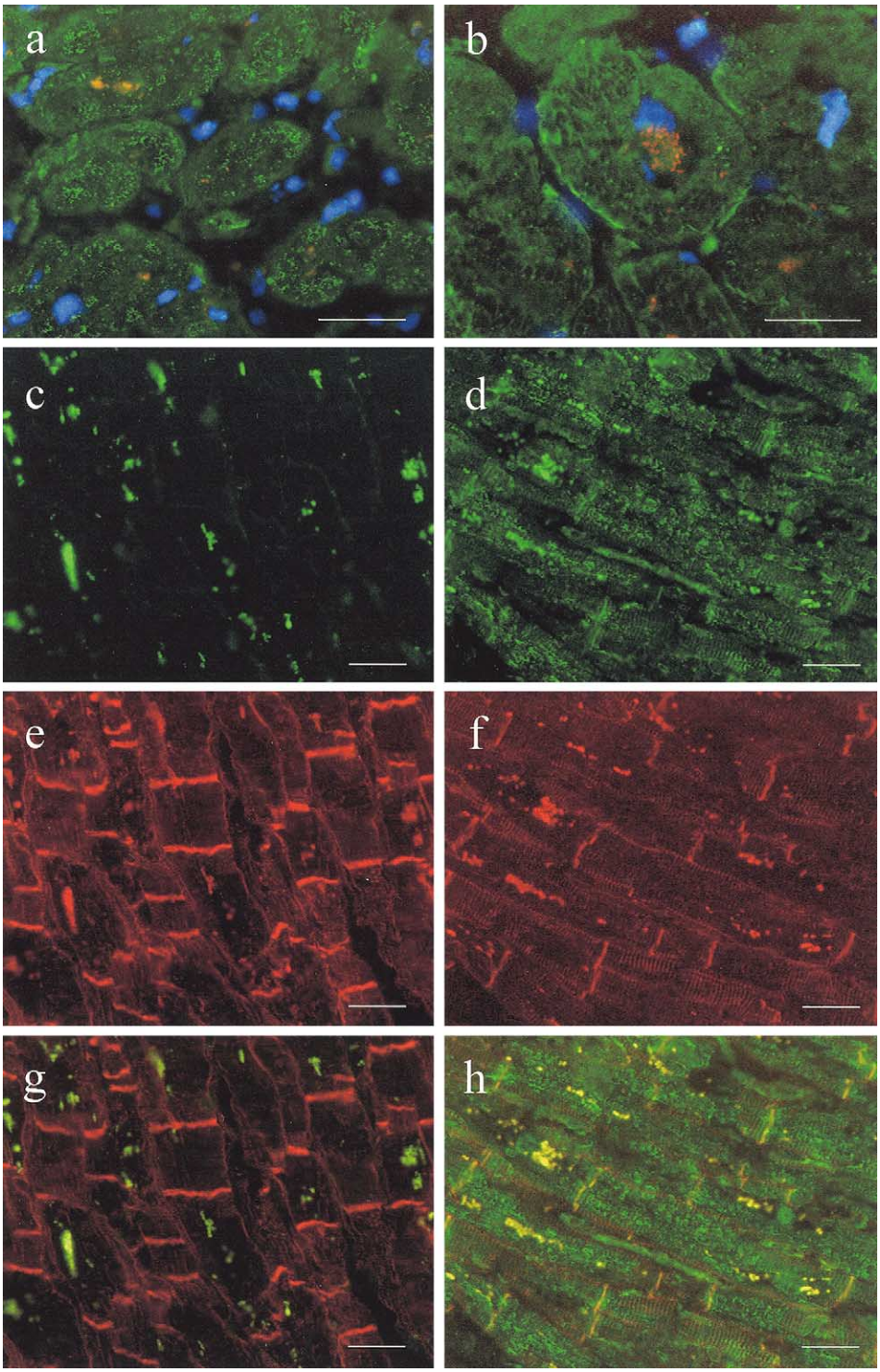

Figure 2. Representative immunofluorescence micrographs of human heart sections labeled for the nicotinamide adenine dinucleotide 3-phosphate (reduced form) oxidase subunit gp91^{phox}. **Panels a, c, e, and g** show nonfailing heart tissue and **panels b, d, f, and h** show end-stage failing tissue. Transverse **(a, b)** and longitudinal **(c, d)** sections labeled for gp91^{phox} show increased labeling in end-stage heart failure. Labeling for alpha-actinin **(e, f)** shows a typical intracellular pattern of myocyte costamer and intercalated disc labeling. **Panels g and h** show superposition of gp91^{phox} and alpha-actinin labeling. All **scale bars** = 20 μm.

from the cytosol to membrane in end-stage CHF. This was corroborated in sections co-stained for p47^{phox} and vinculin as a sarcolemmal marker (Fig. 3B), which demonstrated a distinctly sarcolemmal pattern of p47^{phox} labeling in failing human heart.

DISCUSSION

This study provides the first evidence that a phagocyte-type NADPH oxidase is expressed in human myocardium and specifically in cardiomyocytes. We also show that NADPH

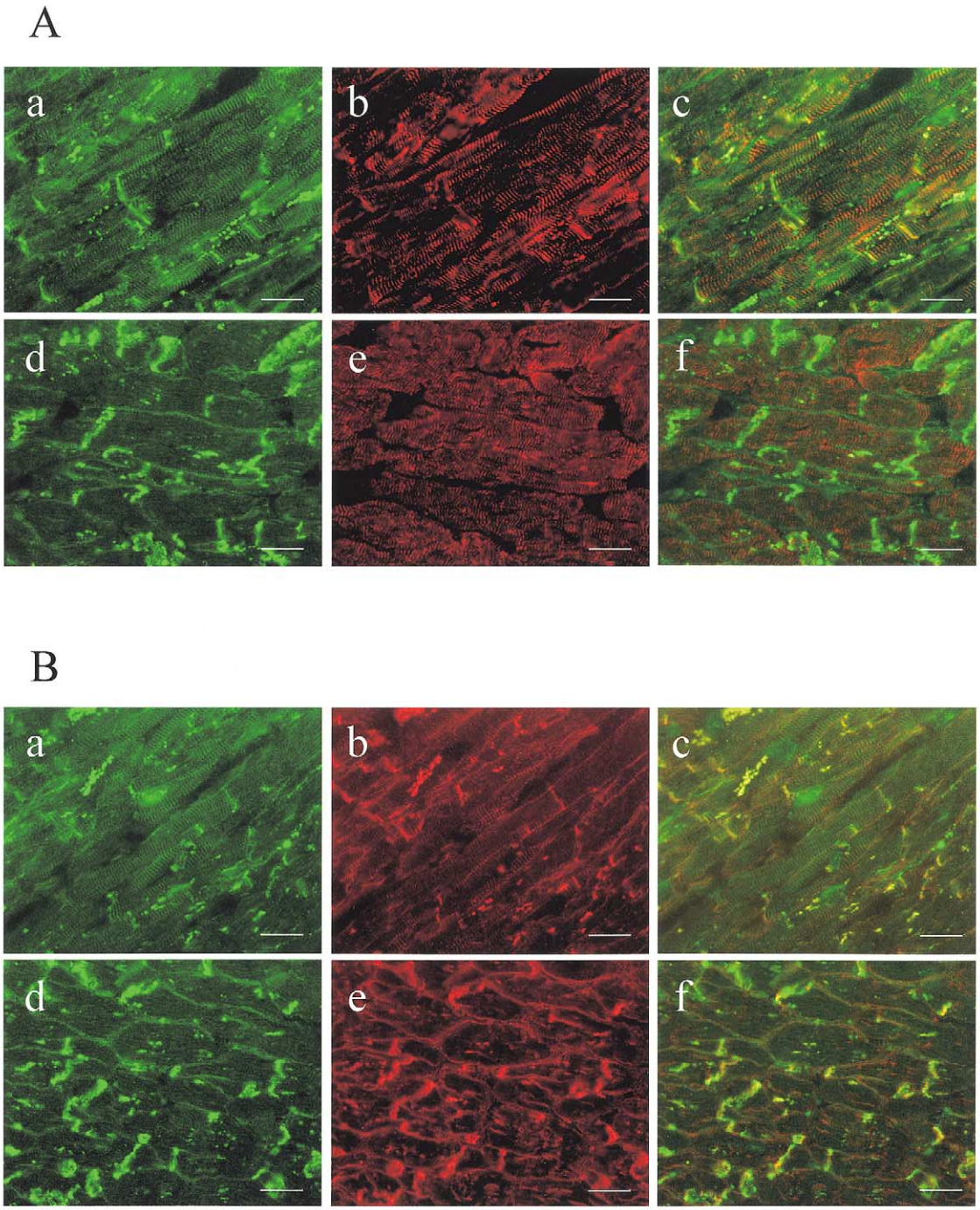

Figure 3. Representative immunofluorescence micrographs of human heart sections labeled for the nicotinamide adenine dinucleotide 3-phosphate (reduced form) oxidase subunit $p47^{phox}$. **(A)** Dual labeling for $p47^{phox}$ and alpha-actinin in nonfailing **(a–c)** and end-stage failing tissue **(b–f)**. **Panels a and d** show labeling for $p47^{phox}$; **panels b and e** show labeling for alpha-actinin; **panels c and f** show superimposed images. $p47^{phox}$ was detected in cardiac myocytes in both failing and nonfailing myocardium, with increased sarcolemmal labeling in the failing group **(d and f vs. a and c)**. All **scale bars** = 20 μm. **(B)** Dual labeling for $p47^{phox}$ and vinculin, as a sarcolemmal marker, in nonfailing **(a–c)** and end-stage failing tissue **(b–f)**. **Panels a and d** show labeling for $p47^{phox}$; **panels b and e** show labeling for vinculin. Superposition of the $p47^{phox}$ and vinculin images **(panels c and f)** demonstrates the sarcolemmal localization of $p47^{phox}$ in the end-stage heart tissue **(panel f, yellow color)**. All **scale bars** = 20 μm.

oxidase activity is increased in the end-stage failing human heart and that it is likely to be an important source of increased cardiac ROS in human CHF.

Numerous studies indicate an important role for NADPH oxidases in vascular pathologies such as hypertension (15) and atherosclerosis (17). Of relevance to heart failure and potential myocardial ROS production, NADPH oxidase are activated by angiotensin II, noradrenaline, tumor necrosis factor-alpha, and increased mechanical forces (13,14)—all of which may be implicated in CHF patho-

physiology. Indeed, recent studies in isolated cardiomyocytes and in-vivo animal models have implicated NADPH oxidase-derived ROS production in myocardial pathology (19–22,25). In the present study, NADPH oxidase rather than dysfunctional nitric oxide synthase, xanthine oxidase, or mitochondrial production was the source of NADPH-dependent ROS production because the latter was inhibited by DPI but not by L-NAME, oxypurinol, or rotenone. The evidence of oxidase subunit gene and protein expression in cardiac myocytes strengthened this conclusion, with no evidence of significant myocardial inflammatory infiltration. The extent of increase in NADPH oxidase activity observed in this study was comparable to that which was associated with significant biological effects in previous cardiovascular studies (15–17,20,21). Taken together, the present findings and data from recent experimental reports (19–22,25) suggest that NADPH oxidase-derived myocardial ROS may play a significant role in the pathophysiology of human CHF.

The NADPH oxidase complex comprises a membrane-bound cytochrome b_{558} (consisting of one $p22^{phox}$ and one $gp91^{phox}$ subunit) and four cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and rac1), which translocate and associate with the cytochrome during acute oxidase activation (14). Chronic NADPH oxidase activation occurs through: 1) post-translational protein modifications and translocation of regulatory subunits (notably $p47^{phox}$), or 2) an increase in oxidase subunit expression (13,14). Interestingly, in the present study the increase in NADPH oxidase activity in end-stage failing heart appeared to be due to $p47^{phox}$ translocation to the cardiomyocyte sarcolemma rather than significant increases in oxidase subunit expression.

Increased oxidative stress may have several effects in the diseased heart. Activation of redox-sensitive signaling pathways leads to altered gene expression and has been implicated in the development of cardiac hypertrophy, fibrosis, and remodeling (7,9,19,21,22,25), as well as adaptive alterations such as an increase in catalase expression (26). Increased oxidative stress promotes myocyte apoptosis (7) and causes vascular endothelial dysfunction due to reduced nitric oxide bioavailability (5). Key proteins involved in myocardial excitation-contraction coupling, such as sarcolemmal ion channels, sarcoplasmic reticulum calcium release channels, and myofilament proteins, can also undergo redox-sensitive alterations in activity (27–29). Recently, it was suggested that ROS can modulate the inotropic response to beta-adrenergic stimulation (30).

A limitation of the present study is the source of "control" tissue used. The neurohumoral and hemodynamic status of "donor" subjects before organ retrieval is unlikely to be completely "normal," whereas true healthy control human tissue can rarely be ethically obtained for obvious reasons. However, there are no data to suggest that neurohumoral or cytokine activation downregulates oxidase activity; in fact, oxidase activity may be upregulated in the donors by these factors (13,22), which would have the effect of artifactually reducing the true difference between the two groups.

In summary, this study provides the first data implicating an increase in myocardial NADPH oxidase expression and activity in the pathophysiology of human CHF.

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