Ventricular Assist Device
Therapy Normalizes Inducible Nitric Oxide Synthase Expression and Reduces Cardiomyocyte Apoptosis in the Failing Human Heart

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OBJECTIVES
We examined the effect of mechanical unloading with ventricular assist device (VAD) therapy on myocardial inducible nitric oxide synthase (iNOS) expression and cardiomyocyte apoptosis in patients with end-stage heart failure (HF).

BACKGROUND
Despite advances in medical therapy, HF continues to be a progressive and ultimately fatal disorder. High levels of iNOS expression are present in the myocardium of failing hearts, suggesting a potential role for iNOS in HF progression.

METHODS
Inducible NOS protein expression was analyzed by Western blotting and cardiomyocyte apoptosis by terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) in myocardial samples from failing hearts. Included in these analyses were tissues from 9 patients at the time of transplantation (HF-transplant group), 10 patients at the time of VAD insertion (pre-VAD group), and 11 patients undergoing transplant after VAD support (post-VAD group). Seven control samples were obtained at autopsy.

RESULTS
Low or undetectable levels of iNOS were present in controls (0.005 ± 0.002). The HF-transplant and pre-VAD myocardial specimens exhibited a marked increase in iNOS expression (1.48 ± 0.34 and 1.29 ± 0.26, respectively; p < 0.01 for both vs. controls). The increase in iNOS expression was reversed in the post-VAD group (0.36 ± 0.16; p < 0.01 vs. HF-transplant and pre-VAD groups). The rate of TUNEL-positive cardiomyocytes was high in the pre-VAD group and significantly lower in the post-VAD group (0.64 ± 0.15% in pre-VAD group and 0.16 ± 0.07% in post-VAD group; p < 0.01). The iNOS levels correlated significantly with cardiomyocyte apoptosis (r = 0.66, p < 0.01).

CONCLUSIONS
Therapy with VAD normalizes iNOS expression in association with diminished cardiomyocyte apoptosis in the failing heart. Further work is required to define whether a causal relationship exists between iNOS and cardiomyocyte apoptosis. (J Am Coll Cardiol 2005; 45:1419–24) © 2005 by the American College of Cardiology Foundation
able or detected at only low levels in healthy human hearts, but it is expressed at high levels in the myocardium of failing hearts (8,9).

In severe, end-stage HF, ventricular assist device (VAD) therapy is an accepted treatment modality to bridge critically ill patients to heart transplantation. Ventricular assist device support reverses the hemodynamic aberrations present in end-stage failing hearts. In this setting, HF-related end-organ dysfunction often improves, and patients demonstrate significant improvement in clinical and functional status (10,11). In this study, we hypothesized that the hemodynamic improvements that accompany VAD support of patients with severe, end-stage HF will decrease myocardial iNOS expression. Moreover, we sought to explore whether VAD therapy influences the level of cardiomyocyte apoptosis in failing hearts and to examine the relationship between apoptosis and iNOS protein abundance.

METHODS

Patients. Myocardial tissue was obtained from the hearts of consecutive patients who had undergone heart transplantation because of severe HF due to LV systolic dysfunction. Myocardial samples were obtained from 9 patients without previous VAD support (HF-transplant group), 10 patients during VAD placement (pre-VAD group), and 11 patients at the time of heart transplantation after VAD support (post-VAD group). Normal myocardial control samples were obtained at the time of autopsy from seven subjects with no evidence of cardiac disease. This protocol was approved by the Institutional Review Board for Human Studies at Tufts-New England Medical Center.

Tissue processing. During VAD placement, a portion of LV myocardium was harvested from the segment removed to facilitate insertion of the apical cannula. Adipose and fibrous tissue was carefully trimmed, and segments were aliquotted into small transmural pieces that were snap-frozen in liquid nitrogen and stored at −70°C. One piece was fixed overnight in 10% buffered formalin and stored in 70% ethanol until processing. At the time of heart transplantation, a segment of myocardium was removed, typically from the high lateral wall, to avoid the infarct zone. The segment was trimmed, aliquotted, and stored as described earlier.

Western blotting. Myocardial segments were homogenized using a Bio-Spec tissue homogenizer (Bio-Spec Inc., Bartlesville, Oklahoma) at high speed in tissue lysis buffer containing: 50 mmol/l NaCl, 50 mmol/l NaF, 20 mmol/l Tris-HCl, 10 mmol/l EDTA, 20 mmol/l Na3P2O7, 1 mmol/l Na2VO4, 1% Triton, 1 mmol/l phenylmethylsulfonyl fluoride, and protease inhibitors. Samples were centrifuged, and the protein concentration of the supernatants measured (BioRad Inc., Hercules, California). Samples (80 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% to 12.5% gels). Proteins were transferred to nylon membranes using a standard transfer buffer (Tris/glycine) at 100 V for 90 min. Successful transfer was confirmed by ponceau stain. After blocking with 5% nonfat milk in 0.05% Tween in phosphate-buffered saline (PBS), membranes were incubated with 1° antibodies to iNOS (rabbit polyclonal, BD Biosciences, Palo Alto, California) followed by the appropriate 2° antibody (horse-radish peroxidase-conjugated; Amersham Biosciences, Piscataway, New Jersey). Membranes were then incubated with enhanced chemiluminescence reagent (Amersham) and exposed to film. Bands were quantified by computerized densitometry. To normalize iNOS levels, the same membranes were re-probed for the control protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using a mouse monoclonal antibody (Research Diagnostic Inc., Flanders, New Jersey) and processed as described. The iNOS levels were expressed as a ratio to GAPDH.

Terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL). Five-micrometer sections were cut from paraffin-embedded tissue. Sections were post-fixed by immersion in EtOH/acidic acid (V:V of 1:1) for 5 min. Sections were then incubated with terminal deoxynucleotidyltransferase (TdT) in reaction buffer containing digoxigenin-labeled uridine triphosphate for 60 min at 37°C, followed by fluorescein isothiocyanate-tagged, anti-digoxigenin antibody (Serologicals Corp., Norcross, Georgia). Sections were counterstained with an anti-desmin antibody (Sigma, St. Louis, Missouri) and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI). To diminish background immunofluorescence, sections were immersed in 0.1% Sudan black following DAPI staining and washed once in 70% EtOH. Positive controls were treated with DNAsel. Negative controls were incubated in reaction buffer not containing TdT. A total of ~3,000 nuclei (10 medium power fields) per section were counted by a blinded observer.

Immunohistochemistry. Paraffin-embedded tissues were re-hydrated and treated with proteinase K for 30 min at room temperature. Endogenous peroxidases were blocked by incubation of sections in 1% H2O2 in PBS for 30 min. Sections were blocked with 10% goat serum in PBS and then incubated with a rabbit polyclonal anti-iNOS antibody at 1:400 dilution (Transduction Laboratories) overnight at 4°C and washed in PBS. Samples were then incubated in the secondary biotinylated antibody and stained with the
with a left VAD only. Symptom duration was shorter in the group were supported with a biventricular device and eight an ischemic etiology. Three of the patients in the post-VAD tions of males and females and of patients with HF due to the patient group, although this difference was only statis-

Statistics. All data are presented as the mean value ± SEM. All statistical analyses were performed using commercially available software (SigmaStat, version 2.03, SPSS Inc., San Rafael, California). Chi-square analyses were used to compare distribution values among the baseline clinical characteristics. Normalized iNOS levels from multiple groups were compared by one-way analysis of variance (ANOVA), with the Student Neuman-Keuls test for post-hoc multiple pairwise comparisons. The time course of iNOS expression in post-VAD samples was analyzed using a curve-fitting algorithm (Sigma Plot, version 8.0); a polynomial, non-linear, inverse second-order regression equation ($y = y_0 + ax + bx^2$) provided the best-fitting curve and was applied to these data. Correlations between normalized iNOS levels and hemody-

RESULTS

Table 1 summarizes the clinical characteristics of patients and control subjects. The control group was younger than the patient group, although this difference was only statistically significant when compared with the HF-transplant group. Among the HF patients, there were similar proportions of males and females and of patients with HF due to an ischemic etiology. Three of the patients in the post-VAD group were supported with a biventricular device and eight with a left VAD only. Symptom duration was shorter in the post-VAD group than in the HF-transplant group ($p < 0.05$), but not compared with the pre-VAD group. Seven of the patients from whom myocardial samples were obtained before VAD implantation also supplied samples at the time of transplant after VAD support. The mean duration of VAD support in the post-VAD group was 113 days (range 12 to 280). Symptoms in the post-VAD group improved during the period of mechanical support. The three patients who required biventricular assist were ambulating to a limited extent. Among the other eight VAD patients, one required continued inotropic treatment for right ventricular (RV) dysfunction, and the remaining seven patients had no overt symptoms of HF.

To compare normalized iNOS levels among all groups, one-way ANOVA was performed and was significant ($p < 0.001$). As shown in Figure 1A, iNOS protein levels were low in the control myocardial samples (ratio of densitometric units for iNOS/GAPDH protein: $0.005 \pm 0.002$). Levels of iNOS protein were markedly increased in the HF-transplant group ($0.48 \pm 0.34$) and pre-VAD group ($1.29 \pm 0.26; p < 0.01$ vs. control group for both) (Fig. 1). The increase in iNOS protein expression in failing hearts was observed in both ischemic and nonischemic dilated cardiomyopathies. After mechanical unloading with VAD support, myocardial iNOS protein abundance decreased significantly compared with the HF-transplant and pre-VAD groups, respectively ($0.36 \pm 0.16; p < 0.01$ vs. HF and pre-VAD groups, $p = NS$ vs. controls) (Fig. 1). A representative iNOS Western blot of myocardial lysates obtained from three patients from whom samples were obtained before and after VAD support (“matched” group) is shown in Figure 1B, and individualized data from all seven patients from whom matched samples were obtained both before and after VAD support are shown in Figure 1C. This graph demonstrates that iNOS protein levels decreased in six of the seven patients after VAD support. The level of iNOS protein increased in one of the seven patients with matched samples before and after VAD support; in this patient, the post-VAD sample was obtained only 12 days after device insertion when the patient underwent heart transplantation. Interestingly, the patient with a more modest decrease in iNOS abundance (Patient #3 in Figure 1A) was on continued inotropic therapy for RV dysfunction while awaiting heart transplantation. To determine whether the observed changes in myocardial iNOS

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*p < 0.05 vs. controls. †p < 0.05 vs. HF-transplant group. All data are expressed as the mean value ± SEM.

CAD = coronary artery disease; EF = ejection fraction; F = female; HF = heart failure; M = male; NYHA = New York Heart Association; VAD = ventricular assist device.
protein expression were occurring in cardiomyocytes, immunohistochemical localization was performed on three matched samples before and after VAD. As shown in Figure 2, iNOS expression was readily detected in pre-VAD samples, where it was localized to cardiomyocytes. In contrast, iNOS was barely detectable in the post-VAD section. This pattern was observed in all three matched specimens.

The time course of iNOS expression after VAD support was also analyzed. Out of all 11 patients in the post-VAD group, only three had a normalized iNOS level greater than the mean of 0.36. Two of these were from patients who had undergone transplantation within 12 and 21 days after VAD insertion. The third had a normalized iNOS level of 1.6 late after VAD insertion (280 days). Thus, with the exception of this sample, there was a rapid, time-dependent decrease in iNOS protein abundance after VAD support (p = 0.09 for non-linear regression) (Fig. 3).

We then focused specifically on data pertaining to the subjects from whom matched myocardial samples were obtained both before and after VAD support. Hemodynamics from these patients obtained immediately before and during VAD support are shown in Table 2. There was a significant rise in the cardiac index with VAD support, from $1.9 \pm 0.1 \text{l/min/m}^2$ to $2.5 \pm 0.2 \text{l/min/m}^2$, coupled with a significant decrease in all right heart pressure measurements, consistent with marked hemodynamic improvement during VAD support. Normalized iNOS levels correlated strongly with the pulmonary capillary wedge pressure ($r = 0.78$, $p < 0.01$) and pulmonary arterial systolic ($r = 0.62$, $p < 0.05$) and diastolic pressures ($r = 0.73$, $p < 0.01$). A non-significant, negative correlation existed between normalized iNOS protein abundance and the cardiac index ($r = -0.425$, $p = \text{NS}$).

We next explored the effect of VAD therapy on cardiomyocyte apoptosis, as assessed by TUNEL staining performed on matched pre- and post-VAD myocardial samples from these same seven subjects. The percent TUNEL-positive cardiomyocytes in the pre-VAD apical samples was $0.64 \pm 0.15\%$, the rate of which was similar to that observed in the lateral wall segments obtained in the HF-transplant group not supported with a VAD ($0.74 \pm 0.61\%$). The percent TUNEL-positive cardiomyocytes in the matched post-VAD samples decreased to $0.16 \pm 0.07\%$ ($p < 0.01$). The rate of TUNEL-positive cardiomyocytes within these samples correlated significantly with normalized iNOS levels ($r = 0.66$, $p < 0.01$) (Fig. 4).

**DISCUSSION**

In this study, we have confirmed the findings of other investigators (8,9), demonstrating markedly increased expression of iNOS within the myocardium of failing human hearts. We now extend these earlier observations further by demonstrating that ventricular unloading with a mechanical assist device markedly decreases iNOS abundance within cardiomyocytes of the failing heart. Furthermore, we observed a significant reduction in cardiomyocyte apoptosis during VAD therapy. Our findings therefore demonstrate that mechanical unloading of the heart normalizes myocardial iNOS expression in direct association with a reduction in cardiomyocyte apoptosis.

Several recent studies have demonstrated increased programmed cell death within cardiomyocytes of failing hearts (12,13), and both clinical and experimental evidence suggests
that cardiomyocyte apoptosis contributes to HF progression (14,15). The role of iNOS in the pathophysiology of HF progression and in cardiomyocyte apoptosis remains unclear. Experimental studies have shown that high levels of NO inhibit the cardiomyocyte-shortening velocity, in part by activating guanylate cyclase and increasing cyclic guanosine monophosphate (16,17). High concentrations of NO have also been shown to induce cardiomyocyte apoptosis (18). In this manner, elevations in iNOS expression within cardiomyocytes of the failing heart may contribute to the pathogenesis of progressive LV remodeling and HF. In transgenic mice, cardiac-specific overexpression of iNOS leads to cardiac fibrosis, dilation, and premature death (19), although another group of investigators reported no demonstrable phenotype accompanying iNOS overexpression in the mouse heart (20). Sam et al. (21) recently demonstrated that six months after a myocardial infarction, the extent of LV dysfunction and myocardial apoptosis were diminished in iNOS knockout mice, supporting a detrimental role of iNOS in this chronic HF model. Hence, the normalization of iNOS expression after VAD support observed here may contribute to improved cardiomyocyte survival and significant functional recovery of the myocardium noted in some patients after mechanical support (10,22–26). Indeed, our own data presented here show that iNOS abundance correlates directly to the percent TUNEL-positive cardiomyocytes, supporting a potential causal association between iNOS and cardiomyocyte apoptosis.

The mechanism by which VAD therapy normalizes myocardial iNOS expression cannot be deduced from our findings. However, within cardiomyocytes in vitro, tumor necrosis factor-alpha (TNF-alpha) and interleukin-6, both of which are elevated in the failing heart, induce iNOS transcription through the activation of nuclear factor kappa-B (NF-kappa-B) (27). A recent study by Torre-Amione et al. (28) demonstrated that the expression of TNF-alpha decreases after VAD support. Correspondingly, the level of NF-kappa-B activation was also recently shown to decrease within the myocardium of failing hearts after VAD support (22). Thus, alterations in iNOS expression may be a critical downstream event in these signaling and transcription factor alterations.

Study limitations. In this study, we did not measure iNOS enzymatic activity. Nonetheless, previous reports have demonstrated that high levels of NO inhibit the cardiomyocyte-shortening velocity, in part by activating guanylate cyclase and increasing cyclic guanosine monophosphate (16,17). High concentrations of NO have also been shown to induce cardiomyocyte apoptosis (18). In this manner, elevations in iNOS expression within cardiomyocytes of the failing heart may contribute to the pathogenesis of progressive LV remodeling and HF. In transgenic mice, cardiac-specific overexpression of iNOS leads to cardiac fibrosis, dilation, and premature death (19), although another group of investigators reported no demonstrable phenotype accompanying iNOS overexpression in the mouse heart (20). Sam et al. (21) recently demonstrated that six months after a myocardial infarction, the extent of LV dysfunction and myocardial apoptosis were diminished in iNOS knockout mice, supporting a detrimental role of iNOS in this chronic HF model. Hence, the normalization of iNOS expression after VAD support observed here may contribute to improved cardiomyocyte survival and significant functional recovery of the myocardium noted in some patients after mechanical support (10,22–26). Indeed, our own data presented here show that iNOS abundance correlates directly to the percent TUNEL-positive cardiomyocytes, supporting a potential causal association between iNOS and cardiomyocyte apoptosis.

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Figure 4. Regression plot of percent terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL)-positive cardiomyocytes (CMs) versus normalized inducible nitric oxide synthase (iNOS) levels (iNOS/GAPDH) in which a significant correlation was present (r = 0.66, p < 0.01).

strated that an increase in iNOS enzymatic activity coincides with increased protein expression in failing hearts (29). We also did not control for preoperative and postoperative medical regimens that may have altered iNOS expression in failing hearts. However, it is unlikely that drug regimens after VAD would have differentially influenced iNOS levels, as these regimens are standardized and nearly identical for all patients, with the exception of the one patient who remained on inotropic therapy for RV failure after LVAD placement.

Conclusions. This study demonstrates that iNOS protein expression is markedly increased in the hearts of patients with severe HF before heart transplantation. We extend previous observations by now showing that mechanical unloading with VAD therapy decreases iNOS protein abundance in association with a decrease in the rate of cardiomyocyte apoptosis. Moreover, we further show that there is a significant correlation between iNOS protein abundance and cardiomyocyte apoptosis. The extent to which a VAD-induced decrease in iNOS protein abundance contributes to a decrease in cardiomyocyte cell death and/or reversal of LV dysfunction seen in some patients with VAD support will require further investigation.

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REFERENCES