Heterogeneous Expression and Activity of Endothelial and Inducible Nitric Oxide Synthases in End-Stage Human Heart Failure

Their Relation to Lesion Site and β-Adrenergic Receptor Therapy

Mitsumasa Fukuchi, MD; Sabah N.A. Hussain, PhD; Adel Giaid, PhD

Background—Recent reports have suggested that excessive amounts of endogenous NO may contribute to the myocardial dysfunction and injury in heart failure. In the present report, we investigate the cellular expression and activity of endothelial (eNOS) and inducible (iNOS) NO synthase in failing human hearts with special reference to the underlying lesion and drug therapy.

Methods and Results—Myocardial tissues were obtained from 28 failing human hearts with various pathogeneses and 4 nonfailing hearts as controls. Only weak or focal expression of both eNOS and iNOS was seen in ventricles of nonfailing hearts. In failing hearts, immunoreactivity and hybridization signals for eNOS were increased only in cardiac myocytes of subendocardial areas. Signals for iNOS in cardiac myocytes were consistently seen in heart failure of various pathogeneses and were apparent in both infarcted and noninfarcted regions of ischemic cardiomyopathy. Apparent signals for iNOS were also seen in infiltrating macrophages in infarcted regions of ischemic cardiomyopathy, myocarditis, and septic hearts. The expression of eNOS but not iNOS in the myocytes was intimately associated with β-adrenergic therapy before the operation, being more abundant in patients on β-blockers compared with diminished presence in patients on β-agonists. In contrast to immunohistochemical data, iNOS activity was more variable than constitutive NOS activity and correlated significantly with the density of infiltrating macrophages.

Conclusions—These results suggest that whereas increased eNOS and/or iNOS expression in failing cardiac myocytes may in general contribute to myocardial dysfunction, myocyte injury or death associated with inflammatory lesions may be caused in part by abundant iNOS expression within infiltrating macrophages rather than cardiac myocytes. **(*Circulation*. 1998;98:132-139.)**

Key Words: cells ■ biopsy ■ RNA ■ immunohistochemistry

Nitric oxide has been shown to modulate myocyte function mainly via formation of cGMP, which inhibits the sarcolemmal L-type calcium channel and reduces the myofilament response to calcium.[1] NO is synthesized from L-arginine by a family of enzymes, the NOSs, consisting of 3 isoforms: the constitutive types brain NOS and eNOS and the inducible type, iNOS.[2] In myocardial tissue, eNOS is constitutively expressed in myocytes, endothelial cells, and endocardial endothelium; iNOS is induced in multiple cardiac cells, including myocytes, vascular endothelial and smooth muscle cells, and inflammatory cells after stimulation with lipopolysaccharide and cytokines.[3] Recent experimental studies have shown that excessive amounts of NO produced within myocardial tissue may contribute to myocardial dysfunction and injury found in such pathological conditions as endotoxemia,[4] myocardial infarction,[5] myocarditis,[6] and cardiac allograft rejection.[7,8]

In the failing human heart, DeBelder et al[9] reported that iNOS activity was increased but cNOS activity was reduced in right ventricular subendocardial specimens from patients with dilated cardiomyopathy or myocarditis but not with other noninflammatory heart disease. Habib et al[10] also observed increased iNOS immunoreactivity in cardiac myocytes and endocardial endothelium of patients with dilated cardiomyopathy but not with ischemic heart disease. Conversely, several other studies have demonstrated apparent iNOS mRNA or protein expression in human heart failure of various underlying pathogeneses.[11,12] In addition, increased cNOS as well as iNOS activity has been isolated in cardiac myocytes and tissue homogenate from experimentally induced heart failure.[13] In a clinical setting, patients with heart failure are treated with drugs that are known to affect NOS expression, including ACE inhibitors and β-adrenergic agonists or blockers.[3,14] Indeed, increases in intracellular cAMP

Received December 12, 1997; revision received March 5, 1998; accepted March 12, 1998.

From the Departments of Medicine and Pathology, The Montreal General Hospital and McGill University, and GenPath Laboratories, Montreal, Quebec, Canada H3G 1A4.

Correspondence to Dr Adel Giaid, Suite L3–314, The Montreal General Hospital, 1650 Cedar Ave, Montreal, Quebec, Canada H3G 1A4. E-mail mdga@musica.mcgill.ca

Selected Abbreviations and Acronyms

cNOS = constitutive NOS
eNOS = endothelial NOS
iNOS = inducible-type NOS
L-NAME = N^G-nitro-L-arginine methyl ester
NOS = NO synthase

levels by β-adrenergic stimulation has been shown to diminish eNOS expression in rat cardiomyocytes.[14] To date, however, the relationship between the cellular expression of NOS and its activity in end-stage heart failure and their relationship to the type of myocardial lesion and medical therapy remain to be elucidated.

In the present study, we investigated the cellular expression of eNOS and iNOS mRNAs and protein in the myocardium of patients with end-stage heart failure and correlated their cellular expression with NOS enzyme activity in the adjacent myocardial tissue, with special reference to the lesion sites (ie, myocardial infarction or inflammatory cell accumulation). In addition, we examined the relationship between NOS expression in left ventricular myocytes of failing hearts and medical treatment, including β-agonist and β-blocker therapy.

Methods

Patients

The study was approved by the ethical committee of the Montreal General Hospital. Myocardial tissues from 25 patients with end-stage heart failure were collected at surgery for heart transplantation. Thirteen patients had ischemic cardiomyopathy, 8 had idiopathic dilated cardiomyopathy, 2 had myocarditis, and 2 had aortic valvular stenosis/regurgitation. All patients had severe congestive heart failure classified as New York Heart Association functional class III to IV. Other clinical data from these patients are shown in the Table. Hemodynamic variables measured within 6 months before heart transplantation were used. All patients were treated with a combi-

Biographical and Hemodynamic Data of Patients With Heart Failure

Diagnosis, Sex	Age, y	CI, L · min^{-1} · m^{-2}	EF, %	PCWP, mm Hg	LVEDD, mm	Complications
ICM						
M	51	3.0	19	38	72	HT
M	47	NA	25	NA	74	HT
F	55	1.7	16	35	65	HT, DM
M	32	NA	NA	NA	62	
M	57	2.0	20	NA	54	HT
M	58	NA	24	NA	74	Hyperlipidemia
M	49	2.0	15	32	65	
M	61	NA	20	NA	100	ASO
M	63	NA	25	NA	66	Af
F	59	1.8	22	26	64	HT
M	50	2.2	15	24	76	HT, DM
M	48	NA	15	NA	81	DM
M	61	NA	22	19	67	
DCM						
F	31	NA	14	24	NA	
F	54	1.4	11	18	77	
M	52	1.1	15	32	NA	HT
M	53	0.92	41	27	61	
M	58	NA	25	NA	65	HT
M	23	2.2	10	22	89	
F	44	1.5	20	34	54	Hyperthyroidism
M	41	2.1	10	30	60	
Myocarditis						
M	34	NA	30	NA	46	
F	55	2.7	13	19	71	DM
ASR						
M	30	1.5	25	46	NA	
M	63	2.9	20	13	75	

CI indicates cardiac index; EF, ejection fraction; PCWP, pulmonary capillary wedge pressure; LVEDD, left ventricular end-diastolic dimension; ICM, ischemic cardiomyopathy; DCM, dilated cardiomyopathy; ASR, aortic valvular stenosis/regurgitation; NA, not available; HT, hypertension; DM, diabetes mellitus; ASO, arteriosclerosis obliterance; and Af, atrial fibrillation.

nation of drugs including diuretics, digoxin, ACE inhibitors, nitrates, Ca^{2+} channel blockers, low-dose aspirin, warfarin, and antiarrhythmic drugs. These drugs were discontinued on the day of transplantation. In addition, 6 patients received intravenous dopamine (3 to 6 $\mu g \cdot kg^{-1} \cdot min^{-1}$) and/or dobutamine (3 to 10 $\mu g \cdot kg^{-1} \cdot min^{-1}$) for 3 to 10 days before the heart transplantation. Specimens were also obtained at autopsy (within 10 hours of death) from 2 patients with septic death (men 42 and 48 years old) and 4 patients with nonfailing hearts who died as a result of motor vehicle accident (n=2), of metastatic cancer (n=1), and of a cerebrovascular attack (n=1) (22, 53, 59, and 69 years old; 3 men and 1 woman). Transmural myocardial tissues were isolated (within 20 minutes after hearts were harvested) from left and right ventricles and left atrial appendages and were either immediately fixed in 4% paraformaldehyde or snap-frozen in liquid N_2.

Immunohistochemistry

Cryostat sections (6 μm thick) were immunostained with monoclonal anti-human eNOS antibody and polyclonal anti-mouse macrophage iNOS antibody by a modification of the avidin-biotin-peroxidase method.[15] Briefly, sections were incubated serially with the following solutions: (1) 2% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity, (2) 0.3% Triton-X 100 for 15 minutes to permeabilize the membrane, (3) 10% normal goat serum for 60 minutes to reduce nonspecific binding of the antiserum, (4) primary antisera for 16 hours at 4°C, (5) biotinylated goat anti-mouse or goat anti-rabbit IgG at a dilution of 1:200 for 45 minutes, and (6) avidin-biotinylated horseradish peroxidase complex (Vectastain, Vector Laboratories) at a dilution of 1:100 for 45 minutes. Immunoreactive sites were visualized by incubation with 0.025% 3,3-diaminobenzidine and 0.01% hydrogen peroxide for 3 minutes. PBS, pH 7.4, was used to dilute each solution and to wash the sections 3 times between each step. To identify the cell types, serial sections were immunostained for the endothelial cell marker von Willebrand factor and macrophage-specific marker CD68 (both Dako). The specificity of immunostaining was assessed by preabsorption of the NOS antisera with NOS antigens and incubation with the nonimmune serum instead of the primary antiserum. Immunostaining intensity was semiquantitatively graded independently by 2 observers without prior knowledge of case history. In cases in which there was a discrepancy between the 2 observers, the final score was determined by joint observation. Grades for several transmural sections from different regions of the same heart were averaged to give a single value. The numbers of infiltrating inflammatory cells (CD68-positive) were counted in 100 to 200 fields at a magnification of ×400, and the cell density was expressed as the cell number per field.

In Situ Hybridization

Cryostat sections (10 μm thick) were hybridized with digoxigenin-labeled riboprobes, and the hybridization products were visualized by an immunological detection method. The labeled RNA was generated as transcripts from linearized template DNA for human eNOS (provided by Dr Kenneth D. Block, Massachusetts General Hospital, Boston) and human iNOS (provided by Dr M. Yanagisawa, University of Texas Southwestern Medical Center, Dallas) in the presence of digoxigenin-11-UTP (Boehringer Mannheim). Sections were hydrated in PBS and treated with 0.3% Triton X-100 in PBS for 15 minutes. After 3 washes in PBS, sections were incubated in 2 μg/mL proteinase K in Tris buffer (0.1 mol/L Tris, 5 mmol/L EDTA, pH 8.0) for 30 minutes at 37°C and immersed in 4% paraformaldehyde for 3 minutes to stop the reaction. After 3 washes in PBS, sections were acetylated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine buffer (pH 8.0) for 10 minutes. The sections were dehydrated with ethanol and hybridized for 16 hours at 42°C with the digoxigenin-labeled riboprobe (2 to 4 ng/μL) in hybridization buffer. Unbound riboprobes were removed by incubation with 20 μg/mL RNase A for 30 minutes at 37°C in a solution of 0.5 mol/L NaCl, 10 mmol/L Tris (pH 7.5), and 1 mmol/L EDTA. Sections were further washed in decreasing concentrations of SSC, pH 7.0: 4×SSC for 10 minutes at 42°C, 2×SSC for 10 minutes at 42°C, and 0.1×SSC for 10 minutes at room temperature. To detect digoxigenin-labeled riboprobe, the sections were incubated for 45 minutes in Tris buffer (pH 7.5) containing 3% BSA followed by incubation with an alkaline phosphatase–conjugated Fab fragment of a sheep anti-digoxigenin polyclonal antibody (Boehringer Mannheim) for 4 hours at room temperature. The hybridization products were visualized with 4-nitro blue tetrazolium (340 μg/mL) and 5-bromo-4-chloro-3-indolyl-phosphate (175 μg/mL) in a solution of 0.1 mol/L NaCl, 0.1 mol/L Tris (pH 9.5), 50 mmol/L $MgCl_2$, and 10% wt/vol polyvinyl alcohol. Negative control experiments included hybridization with the sense probe, RNase A pretreatment before hybridization, and omission of the antisense probe.

Measurement of NOS Activity

NOS activity was quantified as described previously.[16] Briefly, frozen tissues from both infarcted and noninfarcted regions were homogenized in 6 volumes (wt/vol) of ice-cold buffer (pH 7.4, 10 mmol/L HEPES buffer, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mg/mL PMSF, 0.32 mmol/L sucrose, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin A). After centrifugation at 4°C for 15 minutes at 10 000 rpm, the supernatant (50 μL) was added into 100 μL of reaction buffer containing 50 mmol/L KH_2PO_4, 60 mmol/L saline, 1.5 mmol/L NADPH, 10 mmol/L FAD, 1.2 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 1 mg/mL BSA, 1 μg/mL calmodulin, 10 μmol/L BH_4, and 25 μL of 120 μmol/L stock [2,3-^{3}H]L-arginine (150 to 200 cpm/pmol). The samples were incubated for 30 minutes at 37°C, and the reaction was stopped by the addition of ice-cold HEPES buffer (pH 5.5, 100 mmol/L HEPES, 12 mmol/L EDTA). Excess [2,3-^{3}H]L-arginine in the reaction mixture was eliminated with Dowex 50w resin (2 mL). The supernatant was assayed for [^{3}H]L-citrulline by liquid scintillation counting. Enzyme activity was expressed in pmol L-citrulline produced $\cdot$ $min^{-1} \cdot$ mg total $protein^{-1}$. Protein was measured as described above. NOS activity was also measured in the presence of 1.5 mmol/L each of EGTA and EDTA, which replaced $CaCl_2$ and calmodulin in the reaction buffer and in the presence of 1 mmol/L L-NAME (NOS inhibitor). Ca^{2+}/calmodulin-dependent NOS (cNOS) activity was calculated as the difference between that measured in the presence of $CaCl_2$ and that measured in EDTA/EGTA buffer. Ca^{2+}/calmodulin-independent NOS (iNOS) activity was calculated as the difference between samples assayed in the presence of EGTA/EDTA and those measured in the presence of L-NAME.

Statistical Analysis

Values of continuous variables were expressed as mean±SEM and were compared between 2 groups by paired or unpaired Student's *t* test. Immunohistochemical grades for eNOS and iNOS in cardiac myocytes were categorized into ≤1, ≤2, ≤3, and ≤4 and compared among patient groups with β-agonist or β-blocker treatment by the Kruskal-Wallis rank test and between 2 groups by the Mann-Whitney *U* test. A relationship between immunohistochemical grades for eNOS or iNOS and cAMP contents was analyzed by Spearman's rank correlation. A relationship between iNOS activity and the density of infiltrating macrophages was assessed by linear regression analysis. Values of $P<0.05$ were considered significant.

Results

Localization of eNOS and iNOS Proteins and mRNAs

Typical examples of immunohistochemistry for eNOS and iNOS in nonfailing and failing human hearts are shown in Figure 1. In nonfailing hearts, only weak or focal immunoreactivity for both eNOS and iNOS was seen in left ventricular myocytes and endocardial endothelium (Figure 1A through 1C). In left ventricles of failing hearts, apparent iNOS immunoreactivity was seen in cardiac myocytes of most failing left ventricles (Figure 1F, 2C, and 2F). Immunoreactivity for eNOS was variably increased in cardiac

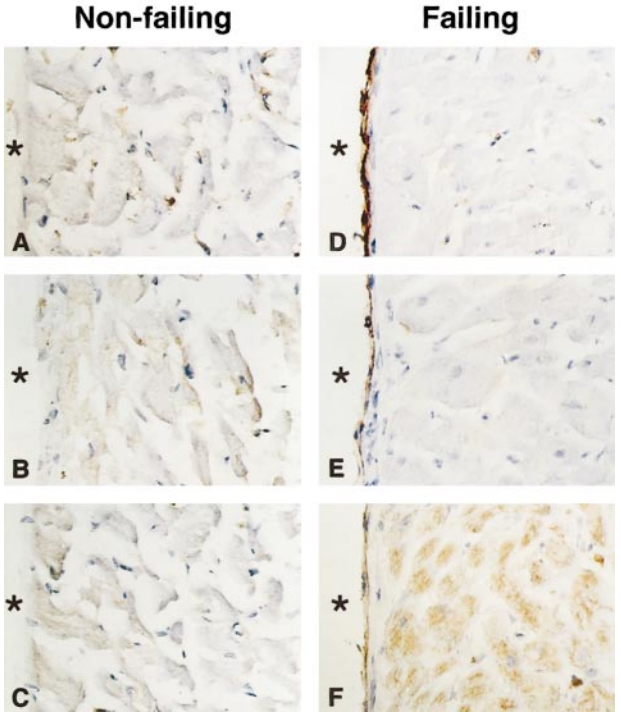

Figure 1. Immunohistochemical localization of eNOS and iNOS in left ventricles of nonfailing and failing human hearts. A through C, Immunoreactivity for von Willebrand factor (A), eNOS (B), and iNOS (C) in serial sections of nonfailing heart. Note only weak or focal immunoreactivity for both eNOS and iNOS in myocytes and endocardial endothelium. D through F, Immunoreactivity for von Willebrand factor (D), eNOS (E), and iNOS (F) in serial sections of failing heart. Note presence of apparent immunoreactivity for eNOS and/or iNOS in myocytes and endocardial endothelium. Asterisk indicates intracardiac cavity. Magnification ×400.

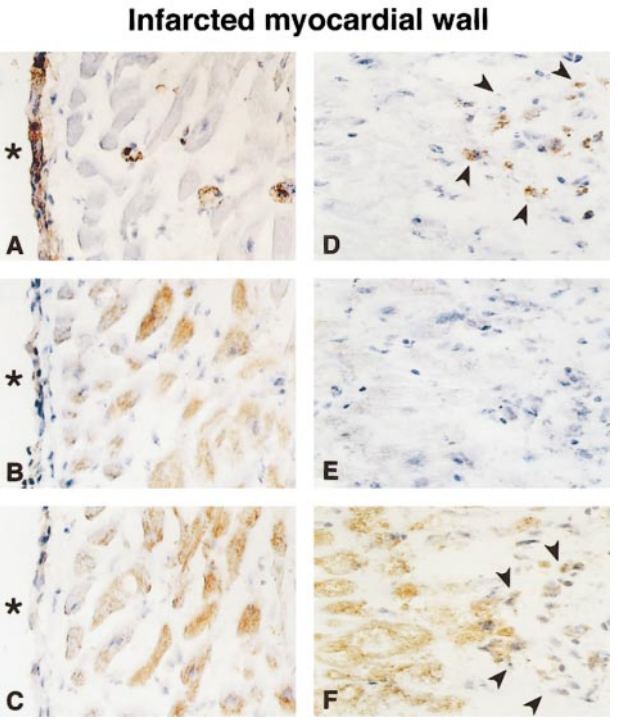

Figure 2. Immunohistochemical localization of eNOS and iNOS in infarcted myocardial wall of ischemic cardiomyopathy. A through F, Different areas of same infarcted myocardial wall: subendocardial area (A through C) and scarred area of deep myocardial layer (D through F) immunostained for von Willebrand factor (A), macrophage-specific marker CD68 (D), eNOS (B and E), and iNOS (C and F) in serial sections. Note that increased immunoreactivity for eNOS was localized only in subendocardial myocytes, whereas apparent iNOS immunoreactivity was found in most surviving myocytes and infiltrating macrophages (arrowheads) of infarcted myocardial wall. Asterisk indicates intracardiac cavity. Magnification ×400.

myocytes in subendocardial areas (5 to 10 cell layers) (Figure 1E versus 2B) but was consistently weak or focal in the remaining myocardium (Figure 2E). The variability of eNOS immunoreactivity in cardiac myocytes of subendocardial areas was seen irrespective of the underlying pathogenesis of heart failure and irrespective of the site of infarction in ischemic cardiomyopathy (grades: infarcted, 2.9 ± 0.30 versus noninfarcted, 2.6 ± 0.53). Endocardial endothelium of failing left ventricular chambers showed variably increased eNOS and iNOS immunoreactivities among individual hearts (Figures 1E, 1F, 2B, and 2C) despite the presence of strong immunoreactivity for von Willebrand factor (Figures 1D and 2A). In right ventricular and left atrial tissues of both failing and nonfailing hearts, cardiac myocytes showed variable eNOS and apparent iNOS immunoreactivities as seen in subendocardial myocytes of failing left ventricles. Apparent eNOS immunoreactivity in the endothelium of intramyocardial vessels was seen in nonfailing hearts, but it was inconsistent in failing hearts. Apparent iNOS immunoreactivity was also seen in infiltrating macrophages (positive to CD68) (Figure 2D and 2F). Preabsorption of the antiserum with NOS antigen or incubation with nonimmune serum abolished the immunostaining (data not shown). No cross-reaction between anti-eNOS antibody and anti-iNOS antibody was evident by different staining patterns (Figures 1E versus 1F and 2E versus 2F).

In situ hybridization in failing human hearts showed apparent signals for eNOS in cardiac myocytes of subendocardial areas and endothelial cells of intramyocardial vessels (Figure 3A and 3D). Apparent hybridization signals for iNOS were consistently seen in cardiac myocytes of most myocardial areas and infiltrating inflammatory cells (Figure 3B and 3E). Control sections incubated with the sense probe and pretreated with RNase A or omission of antisense riboprobe showed no apparent signals (Figure 3C and 3F).

Clinical Relevance of NOS Expression in Failing Hearts

Immunoreactivities for NOS, particularly for iNOS, in cardiac myocytes were significantly increased in patients with heart failure compared with patients with nonfailing hearts (Figure 4). Among patient groups with heart failure of different underlying pathogeneses, there was no significant difference in immunoreactivity for eNOS or iNOS in cardiac myocytes. In ischemic cardiomyopathy, immunoreactivity for both enzymes in myocytes was similar in infarcted and noninfarcted regions of the same hearts. Immunoreactivities for eNOS and iNOS in cardiac myocytes were not significantly related to any clinical variables, including age, sex, complications, cardiac index, ejection fraction, pulmonary

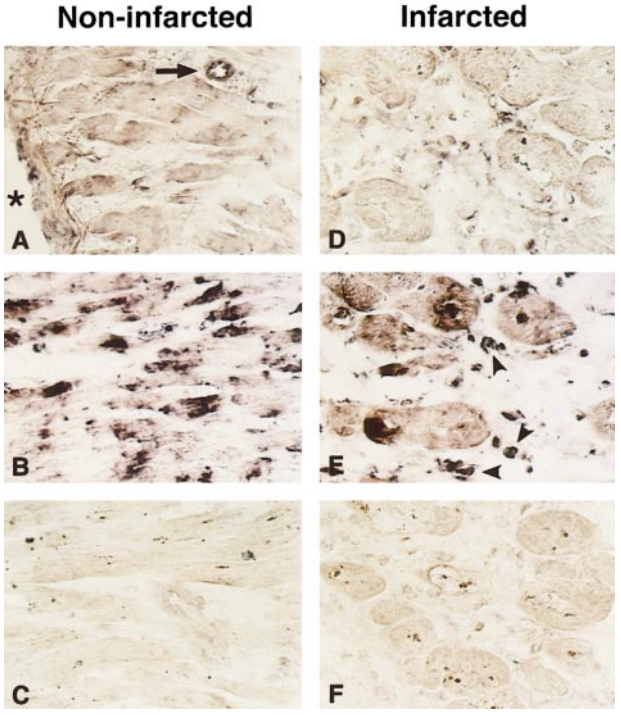

Figure 3. In situ hybridization for eNOS and iNOS mRNAs in noninfarcted (A through C) and infarcted (D through F) regions of ischemic cardiomyopathy. Myocardial sections were hybridized with antisense digoxigenin-labeled riboprobe for eNOS (A and D) and iNOS (B and E) and were visualized with an immunological detection method. Hybridization signals stained dark purple. Note that apparent hybridization signals for eNOS were seen in subendocardial myocytes and intramyocardial microvascular endothelium (arrow), whereas apparent signals for iNOS were seen in most myocytes and infiltrating inflammatory cells (arrowheads). C and F, Negative control sections incubated with sense probe, in which there is only background staining. No counterstain was used. Asterisk indicates intracardiac cavity. Magnification ×400.

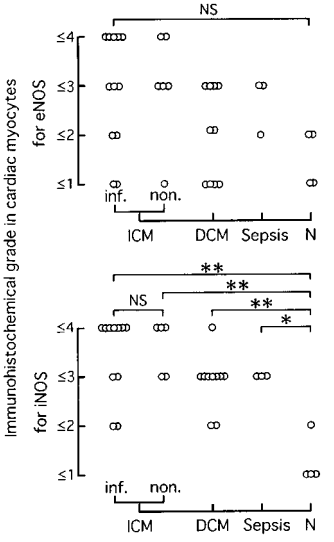

Figure 4. Distribution of immunohistochemical grades for eNOS and iNOS in cardiac myocytes of left ventricles according to underlying pathogenesis of heart failure. Immunohistochemical grades were evaluated in left ventricular subendocardial areas, in which immunoreactivity for NOSs, particularly for eNOS, in cardiac myocytes was selectively increased. Grades were averaged to give a single value to same lesion site and were categorized into 4 ranks (≤1, ≤2, ≤3, ≤4). A significant difference in iNOS immunoreactivity was seen between patients with heart failure of different underlying pathogeneses and patients without heart failure ($P<0.01$). ICM indicates ischemic cardiomyopathy; inf., infarcted; non., noninfarcted; DCM, dilated cardiomyopathy; and N, nonfailing heart. DCM group includes 2 patients with myocarditis. *$P<0.05$; **$P<0.01$.

capillary wedge pressure, or left ventricular end-diastolic dimension. In addition, no significant relationship was found between the immunohistochemical grades for NOS in cardiac myocytes and the medications used before heart transplantation, except for the usage of β-agonists or β-blockers. Figure 5 shows the immunohistochemical grades for eNOS and iNOS in the control group (patients without β-receptor therapy) and the groups treated with β-agonists and β-blockers. Immunoreactivity for eNOS in cardiac myocytes of left ventricular subendocardial areas was significantly lower in β-agonist–treated patients than in β-blocker–treated ($P<0.01$) or control untreated patients ($P<0.05$). A similar but not significant tendency among the 3 groups was seen in right ventricular and left atrial myocytes (data not shown).

NOS Activity in the Failing Human Heart

NOS activity was measured in failing left ventricular tissues just adjacent to the specimens used for immunohistochemistry. cNOS activity ranged from 0.4 to 5.0 pmol · min^{-1} · mg $protein^{-1}$ among specimens examined, with a higher tendency in noninfarcted than infarcted regions (1.53 ± 0.43 versus 0.77 ± 0.19 pmol · min^{-1} · mg $protein^{-1}$) and in subendocardial than middle layers of noninfarcted regions (2.3 versus 0.8 pmol · min^{-1} · mg $protein^{-1}$). This is consistent with some characteristics of immunohistochemical data for eNOS in cardiac myocytes. However, there was no clear correlation between cNOS activity in the myocardium and eNOS immunoreactivity in cardiac myocytes of subendocardial areas. iNOS activity in failing human hearts was more variable than cNOS activity among specimens examined (0 to 40 pmol ·

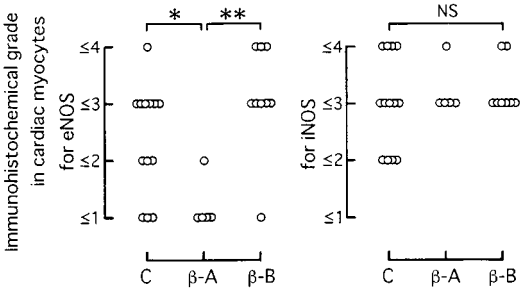

Figure 5. Distribution of immunohistochemical grades for eNOS and iNOS in cardiac myocytes of left ventricles in patient groups with and without β-agonist (β-A) or β-blocker (β-B) treatment. Immunohistochemical grades were averaged to give a single value to each patient and were categorized into 4 ranks (≤1, ≤2, ≤3, ≤4). Grades in myocytes were evaluated in left ventricular subendocardial areas as shown in Figure 4. A significant difference in eNOS immunoreactivity in left ventricular myocytes was found among 3 groups ($P<0.01$), with significantly lower grade in β-agonist–treated than control ($P<0.05$) or β-blocker–treated ($P<0.01$) groups. C indicates control (patients with end-stage heart failure who did not receive β-receptor therapy). *$P<0.05$; **$P<0.01$.

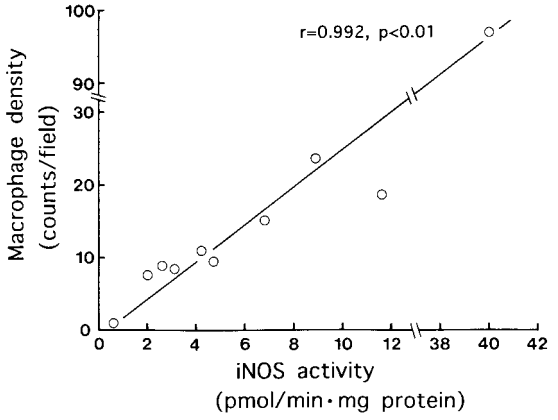

Figure 6. Plot showing significant correlation between iNOS activity and density of infiltrating macrophages in myocardium (r=0.992, P<0.0001). iNOS activity (pmol · min^{-1} · mg protein^{-1}) was measured by use of myocardial tissue adjacent to tissue used for immunohistochemistry. Density of infiltrating macrophages was expressed as number of CD68-positive cells per field at a magnification of ×400.

min^{-1} · mg protein^{-1}), which was not related to the underlying pathogenesis of heart failure (4.8±1.35 pmol · min^{-1} · mg protein^{-1} for ischemic cardiomyopathy versus 4.2±1.24 for dilated cardiomyopathy). In ischemic cardiomyopathy, a higher tendency of iNOS activity (7.7±2.04 versus 3.3±0.47 pmol · min^{-1} · mg protein^{-1}) and significantly higher iNOS/cNOS ratio (10.5±1.87 versus 2.5±0.57 pmol · min^{-1} · mg protein^{-1}, P<0.05) were found in infarcted than noninfarcted regions. This could not be explained either by the difference in iNOS expression in cardiac myocytes or by the difference in myocyte population between infarcted and noninfarcted regions. The iNOS activity was significantly correlated with the density of infiltrating macrophages that expressed iNOS (Figure 6, r=0.992, P<0.0001). Macrophage accumulation was evident in 6 of 13 infarcted regions of ischemic cardiomyopathy, 2 of 2 myocarditides, 1 of 8 dilated cardiomyopathies, and all 3 septic hearts.

Discussion

The present study demonstrates increased expression of both eNOS and iNOS in human hearts with end-stage heart failure of various pathogeneses compared with nonfailing human hearts. In failing hearts, increased expression of eNOS was localized to cardiac myocytes of left ventricular subendocardial areas, whereas iNOS was consistently seen in most cardiac myocytes and infiltrating macrophages. The increased expression of eNOS in cardiac myocytes was more often seen in patients with β-blocker treatment compared with β-agonist treatment, but this was not associated with a significant decrease in cAMP contents in myocardial tissue. In contrast to consistent expression of iNOS in failing cardiac myocytes, iNOS activity was variable among individual failing hearts and among different regions of the same heart and was significantly correlated with the density of infiltrating macrophages.

Apparent iNOS mRNA and protein expression has already been shown in human heart failure of various pathogeneses.[11,12] Immunohistochemical studies have identified cardiac myocytes as the principal cell type that expresses iNOS in failing human hearts.[11,10] Conversely, other groups have reported that iNOS activity or immunoreactivity is increased in human failing hearts with inflammatory heart disease but not in failing hearts with other pathogeneses.[9,10] Using right ventricular subendocardial tissue, DeBelder et al[9] also showed that in contrast to iNOS, cNOS activity in the early phase of inflammatory heart disease was reduced compared with that in ischemic heart disease. The discrepancy among previous studies related to iNOS and eNOS expression in failing human hearts may be explained in part by our observation that NOS activity measured in heart homogenates did not necessarily reflect NOS immunoreactivity in cardiac myocytes. For example, iNOS activity and iNOS/cNOS activity ratios were higher in infarcted than noninfarcted regions of failing hearts with ischemic cardiomyopathy. This could not be explained by either the difference in iNOS immunoreactivity in cardiac myocytes or the difference in myocyte population between infarcted and noninfarcted regions. In the present study, the NOS activity was measured in the myocardial tissue just adjacent to the specimens used for histological study. Indeed, cNOS activity showed a tendency to be lower in infarcted than noninfarcted regions, as expected from a decrease in myocyte population in the infarcted region.

Nevertheless, apparent expression of both eNOS and iNOS was found in cardiac myocytes of failing human hearts but not in myoctes of nonfailing hearts by use of specific antisera against eNOS or iNOS whose specificity was confirmed by preabsorption tests and incubation with nonimmune serum and between which there was no cross-reaction. Our results are consistent with a recent report by Yamamoto et al,[13] who showed increased cNOS as well as iNOS activity in heart homogenates and isolated myocytes from dogs with rapid pacing–induced heart failure. They have also demonstrated a diminished inotropic responsiveness to the β-adrenergic agonist isoproterenol in isolated failing myocytes, which was reversed by L-NAME, an NOS inhibitor. Some evidence suggests that NO produced by either cNOS or iNOS within cardiac myocytes participates in diminished inotropic responsiveness to the isoproterenol in an autocrine and/or paracrine fashion.[17,18] Thus, increased expression of both eNOS and iNOS in cardiac myocytes themselves may be involved in the myocardial dysfunction in the failing human heart.

In the present study, variable eNOS immunoreactivity in cardiac myocytes of subendocardial areas was intimately related to the treatment with β-agonists (lower grade) or β-blockers (higher grade) before the heart transplant operation. Recently, Belhassen et al[14] showed that increases in intracellular cAMP levels by β-adrenergic or adenylate cyclase stimulation diminish eNOS expression in rat cardiac myocytes. In failing human hearts, it has been shown that myocyte β_1-adrenergic receptors, a major β-receptor subtype in the heart, are downregulated particularly in subendocardial layers.[19] Indeed, cAMP levels have been shown to be decreased in the failing human myocardium under basal and isoprenaline-stimulated conditions,[20] probably because of an impaired β_1-adrenergic receptor–G protein–adenylate cyclase pathway.[21] Thus, it is reasonable to speculate that decreased

cAMP levels may underlie increased eNOS expression in failing cardiac myocytes, which was evident in subendocardial areas of hearts of patients with β-blocker treatment.

Alternatively, the use of β-agonists or β-blockers may have reflected the current state of heart failure. Intravenous β-agonists have been applied to patients with severe heart failure 3 to 10 days before operation, but within a few days after treatment, hemodynamic variables were improved in these same patients (cardiac index, from 2.2 to 3.6 L/min; pulmonary capillary wedge pressure, from 26 to 14 mm Hg). Thus, reduced eNOS expression in cardiac myocytes of patients with β-agonist treatment may have resulted from acute improvement of heart failure. Although elevation of cAMP levels in response to β-adrenergic stimulation is reduced in failing hearts,[20,21] it is known that the effects of intravenous β-agonists on heart failure are mediated in part by systemic vascular actions, including increased renal blood flow, resulting in increased urine volume. In addition, it has been shown that in failing dog and human hearts, stimulation of β_2-adrenergic receptors augments intracellular calcium transient amplitude without an increase in cAMP levels.[22] Because downregulation of β-adrenergic receptors has been shown to occur in the β_1- but not the β_2-subtype in the failing human heart,[19,23] activation of β_2-adrenergic receptors in cardiac myocytes may contribute to the improvement of heart failure by intravenous β-agonists.

Increased eNOS expression in cardiac myocytes was localized in subendocardial areas (5- to 10-cell layers) of some failing human hearts, whereas apparent iNOS expression was consistently seen in cardiac myocytes of most failing hearts. These observations suggest more regional and variable regulatory mechanisms for eNOS than iNOS expression in cardiac myocytes. A recent study showed that chronic hypoxia increases eNOS expression in the immature rabbit heart, relating to increased tolerance to ischemia.[24] However, a possible role for myocardial ischemia in subendocardial areas is unlikely, because eNOS expression in cardiac myocytes was similar in subendocardial areas of infarcted and noninfarcted regions (mean grade, 2.9 ± 0.30 versus 2.6 ± 0.53) and was not increased in surviving cardiac myocytes in the infarcted region (Figure 2E). Conversely, iNOS expression in cardiac myocytes has been shown to be induced by multiple factors, including lipopolysaccharide, cytokines (tumor necrosis factor-α, interferon-γ, interleukin-1β), angiotensin II, β-adrenergic receptor agonists,[3] and adrenomedullin.[25] Elevated systemic and/or intracardiac levels of these factors are all characteristic of sepsis or end-stage heart failure.[26–29] This may explain our observation of apparent iNOS expression in cardiac myocytes, which was consistently seen irrespective of any treatment before heart transplantation, including ACE inhibitors and β-agonists or β-blockers.

Another major finding of this study is that iNOS activity in failing human heart was variable despite consistently apparent iNOS expression in most cardiac myocytes, which was significantly correlated with the density of infiltrating macrophages. This suggests that increased iNOS activity in the heart homogenates may have mainly reflected iNOS activity within infiltrating macrophages rather than that in cardiac myocytes. Macrophages were found abundantly in infarcted regions of ischemic cardiomyopathy, myocarditides, and septic hearts but rarely in most hearts with end-stage dilated cardiomyopathy, noninfarcted regions of ischemic cardiomyopathy, and valvular heart disease. These findings are consistent with recent experimental studies using animal models of myocardial infarction (day 2 to 3),[5] cardiac allograft rejection,[7,8] and autoimmune myocarditis,[6] in which infiltrating inflammatory cells (mainly macrophages) have been shown to be the principal cell type responsible for increased iNOS activity or expression in myocardial lesions. Worrall et al[8] have shown contractile and electrophysiological dysfunction during early cardiac allograft rejection, which is in large part prevented by aminoguanidine, a selective iNOS inhibitor. Ishiyama et al[6] also reported that in rats with autoimmune myocarditis, treatment with aminoguanidine inhibited extensive myocardial destruction and massive infiltration of inflammatory cells. A possible role for NO in cytotoxic effects of infiltrating inflammatory cells on cardiac myocytes is supported by the study by Pinsky et al,[30] who demonstrated that iNOS expression by activated macrophages exerted cytotoxic effects on cocultured adjacent cardiac myocytes. Cytotoxic effects of excessive amounts of NO produced by activated macrophages is in part mediated by peroxynitrite formation resulting from the reaction of NO with superoxide anion.[31] Actually, peroxynitrite has been shown to induce cardiac myocyte injury through disturbance of Ca^{2+} transport systems in the plasma membrane and the contractile protein.[32] Thus, infiltrating inflammatory cells may contribute to the myocyte injury or death in addition to dysfunction found in inflammatory lesions of myocardial infarction, myocarditis, and sepsis. In the present study, there was no macrophage accumulation in most cases (7 of 8) of end-stage dilated cardiomyopathy, and iNOS activity in those tissues was not higher than that of ischemic cardiomyopathy. However, our results do not exclude abundant iNOS expression in infiltrating inflammatory cells and/or cardiac myocytes in the early stage of dilated cardiomyopathy.[9]

In summary, we report that increased eNOS expression in cardiac myocytes was localized only in subendocardial areas of some failing human hearts, whereas apparent iNOS expression was consistently seen in cardiac myocytes of most failing hearts. This observation suggests a different regulatory mechanism for increased eNOS and iNOS expression in failing cardiac myocytes. However, increased eNOS and iNOS expression in cardiac myocytes did not seem to be explained simply by cAMP levels. Conversely, iNOS activity in failing myocardial tissue was more variable than cNOS activity and was significantly correlated with the density of infiltrating macrophages found in infarcted regions of ischemic cardiomyopathy, myocarditides, and septic hearts. In conclusion, whereas increased eNOS and/or iNOS expression in failing cardiac myocytes may contribute to the whole myocardial dysfunction, myocyte injury or death found in infarcted or inflammatory lesions may be mediated in part by abundant iNOS expression within infiltrating macrophages rather than cardiac myocytes.

Acknowledgment

This work was supported by the Heart and Stroke Foundations of Canada and Quebec.

References

1. Hare JM, Colucci WS. Role of nitric oxide in the regulation of myocardial function. *Prog Cardiovasc Dis*. 1995;38:155–166.
2. Förstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, Kleinert H. Nitric oxide synthase enzymes: characterization, purification, molecular cloning, and functions. *Hypertension*. 1994;23:1121–1131.
3. Kelly RA, Balligand J-L, Smith TW. Nitric oxide and cardiac function. *Circ Res*. 1996;79:363–380.
4. Brady AJB, Poole-Wilson PA, Harding SE, Warren JB. Nitric oxide production within cardiac myocytes reduces their contractility in endotoxemia. *Am J Physiol*. 1992;263(*Heart Circ Physiol* 32):H1963–H1966.
5. Wildhirt SM, Dudek RR, Suzuki H, Pinto V, Narayan KS, Bing RJ. Immunohistochemistry in the identification of nitric oxide synthase isoenzymes in myocardial infarction. *Cardiovasc Res*. 1995;29:526–531.
6. Ishiyama S, Hiroe M, Nishikawa T, Abe S, Shimojo T, Ito H, Ozasa S, Yamakawa K, Matsuzaki M, Mohammed MU, Nakazawa H, Kasajima T, Marumo F. Nitric oxide contributes to the progression of myocardial damage in experimental autoimmune myocarditis in rats. *Circulation*. 1997;95:489–496.
7. Yang X, Chowdhury N, Cai B, Brett J, Marboe C, Sciacca RR, Michler RE, Cannon PJ. Induction of myocardial nitric oxide synthase by cardiac allograft rejection. *J Clin Invest*. 1994;94:714–721.
8. Worrall NK, Pyo RT, Botney MD, Misko TP, Sullivan PM, Alexander DG, Lazenby WD, Ferguson TB. Inflammatory cell-derived NO modulates cardiac allograft contractile and electrophysiological function. *Am J Physiol*. 1997;273(*Heart Circ Physiol* 42):H28–H37.
9. de Belder AJ, Radomski MW, Why HJ, Richardson PJ, Martin JF. Myocardial calcium-independent nitric oxide synthase activity is present in dilated cardiomyopathy, myocarditis, and postpartum cardiomyopathy but not in ischemic or valvular heart disease. *Br Heart J*. 1995;74:426–430.
10. Habib FM, Springall DR, Davies GJ, Oakley CM, Yacoub MH, Polak JM. Tumour necrosis factor and inducible nitric oxide synthase in dilated cardiomyopathy. *Lancet*. 1996;347:1151–1155.
11. Haywood GA, Tsao PS, von der Leyen HE, Mann MJ, Keeling PJ, Trindate PT, Lewis NP, Byrne CD, Rickenbacher PR, Bishopric NH, Cooke JP, McKenna WJ, Fowler MB. Expression of inducible nitric oxide synthase in human heart failure. *Circulation*. 1996;93:1087–1094.
12. Thoenes M, Förstermann U, Tracey WR, Bleese NM, Nüssler AK, Scholz H, Stein B. Expression of inducible nitric oxide synthase in failing and non-failing human heart. *J Mol Cell Cardiol*. 1996;28:165–169.
13. Yamamoto S, Tsutsui H, Tagawa H, Saito K, Takahashi M, Tada H, Yamamoto M, Katoh M, Egashira K, Takeshita A. Role of myocyte nitric oxide in β-adrenergic hyporesponsiveness in heart failure. *Circulation*. 1997;95:1111–1114.
14. Belhassen L, Kelly RA, Smith TW, Balligand J-L. Nitric oxide synthase (NOS3) and contractile responsiveness to adrenergic and cholinergic agonists in the heart: regulation of NOS3 transcription in vitro and in vivo by cyclic adenosine monophosphate in rat cardiac myocytes. *J Clin Invest*. 1996;97:1908–1915.
15. Giaid A, Saleh D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med*. 1995;333:214–221.
16. Hussain SNA, Giaid A, Dawiri QEI, Sakkal D, Hattori R, Guo Y. Expression of nitric oxide synthase and GTP cyclohydrolase I in the ventilatory and limb muscles during endotoxemia. *Am J Respir Cell Mol Biol*. 1997;17:173–180.
17. Balligand J-L, Kelly RA, Marsden PA, Smith TW, Michel T. Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. *Proc Natl Acad Sci U S A*. 1993;90:347–351.
18. Balligand J-L, Ungureanu-Longrois D, Simmons WW, Pimental D, Malinski TA, Kapturczak M, Taha Z, Lowenstein CJ, Davidoff AJ, Kelly RA, Smith TW, Michel T. Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. *J Biol Chem*. 1994;269:27580–27588.
19. Beau SL, Tolley TK, Saffitz JE. Heterogeneous transmural distribution of β-adrenergic receptor subtypes in failing human hearts. *Circulation*. 1993;88:2501–2509.
20. Danielsen W, v. der Leyen H, Meyer W, Neumann J, Schmitz W, Scholz H, Starbatty J, Stein B, Döring V, Kalmár P. Basal and isoprenaline-stimulated cAMP content in failing versus nonfailing human cardiac preparations. *J Cardiovasc Pharmacol*. 1989;14:171–173.
21. Böhm M, Reiger B, Schwinger RHG, Erdmann E. cAMP concentrations, cAMP dependent protein kinase activity, and phospholamban in non-failing and failing myocardium. *Cardiovasc Res*. 1994;28:1713–1719.
22. Altschuld RA, Starling RC, Hamlin RL, Billman GE, Hensley J, Castillo L, Fertel RH, Hohl CM, Robitaille P-ML, Jones LR, Xiao R-P, Lakatta EG. Response of failing canine and human heart cells to β_2-adrenergic stimulation. *Circulation*. 1995;92:1612–1618.
23. Bristow MR, Ginsburg R, Umans V, Fowler M, Minobe W, Rasmussen R, Zera P, Menlove R, Shah P, Jamieson S, Stinson EB. β_1- and β_2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective β_1-receptor down-regulation in heart failure. *Circ Res*. 1986;59:297–309.
24. Baker JE, Holman P, Kalyanaraman B, Pritchard KA. Chronic myocardial hypoxia increases eNOS expression to produce elevated levels of nitric oxide for cardioprotection. *Circulation*. 1996;94(suppl I):I-468. Abstract.
25. Ikeda U, Yamamoto K, Kanbe T, Kawahara Y, Shimada K. Adrenomedullin augments inducible nitric oxide synthase expression in cytokine-stimulated cardiac myocytes. *Circulation*. 1996;94(suppl I):I-700. Abstract.
26. Matsumori A, Yamada T, Suzuki H, Matoba Y, Sasayama S. Increased circulating cytokines in patients with myocarditis and cardiomyopathy. *Br Heart J*. 1994;72:561–566.
27. Torre-Amione G, Kapadia S, Lee J, Durand J-B, Bies RD, Young JB, Mann DL. Tumor necrosis factor-α and tumor necrosis factor receptors in the failing human heart. *Circulation*. 1996;93:704–711.
28. Hokimoto S, Yasue H, Fujimoto K, Sakata R, Miyamoto E. Increased angiotensin converting enzyme activity in left ventricular aneurysm of patients after myocardial infarction. *Cardiovasc Res*. 1995;29:664–669.
29. Jougasaki M, Rodeheffer RJ, Redfield MM, Yamamoto K, Wei C-M, McKinley LJ, Burnett JC Jr. Cardiac secretion of adrenomedullin in human heart failure. *J Clin Invest*. 1996;97:2370–2376.
30. Pinsky DJ, Cai B, Yang X, Rodriguez C, Sciacca RR, Cannon PJ. The lethal effects of cytokine-induced nitric oxide on cardiac myocytes are blocked by nitric oxide synthase antagonism or transforming growth factor. *J Clin Invest*. 1995;95:677–685.
31. Szabó C, Day BJ, Salzman AL. Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger. *FEBS Lett*. 1996;381:82–86.
32. Ishida H, Ichimori K, Hirota Y, Fukahori M, Nakazawa H. Peroxynitrite-induced cardiac myocyte injury. *Free Radic Biol Med*. 1996;20:343–350.