Regional In Vivo and In Vitro Characterization of Autonomic Innervation in Cardiomyopathic Human Heart

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Background—In contrast to healthy volunteers, regional differences of cardiac autonomic innervation have been described through the use of C11-hydroxyephedrine positron emission tomography (HED-PET) in the left ventricles of patients with dilated cardiomyopathy. The goal of the present study was to correlate HED-PET images with biochemical analysis of tissue samples.

Methods and Results—To assess the significance of altered HED uptake, we used HED-PET to examine eight patients with dilated cardiomyopathy before heart transplantation. After explantation, we measured the density and affinity of uptake1 (3H-mazindol binding) and tissue norepinephrine content as markers of presynaptic function, and we determined β-receptor density and affinity (3H-CGP 12177 binding) in the corresponding areas of the same patients. The density of uptake, and norepinephrine content showed marked regional variation, with highest values in the anterior septal wall and lowest in inferoapical and apical areas. Both parameters were closely correlated (r=.65, P=.05). Similarly, uptake, density or norepinephrine content and HED retention (PET) showed clear correlations (r=.63 and .60, respectively). Uptake1 affinities did not vary significantly and were not correlated to the other parameters. β-Adrenergic receptor density showed some, albeit less pronounced, regional variation and was weakly correlated to uptake, density and local HED retention (r=.38 and .31, respectively).

Conclusions—Uptake1 density and tissue norepinephrine content showed marked regional variation in cardiomyopathic left ventricles. HED-PET is significantly correlated to the density but not the affinity of uptake1 sites in the human heart, suggesting either loss of neurons or downregulation of uptake1 in dilated cardiomyopathy. HED-PET is a valuable marker for alterations of the presynaptic sympathoadrenergic system in humans. (Circulation. 1998;97:174-180.)

Key Words: heart failure ▪ cardiomyopathy ▪ tomography ▪ nervous system, autonomic

In the cardiac synaptic cleft, norepinephrine is eliminated via the presynaptic neuronal uptake1-carrier protein, whereas extraneuronal uptake, is of minor importance.1 Uptake, is known to play a pivotal role in several disturbances of cardiac sympathetic neurotransmission. A downregulation of cardiac uptake, density and function was documented in heart failure in both humans and animals.2-4 This impairment of presynaptic sympathetic function is a probable reason for the increased local release of norepinephrine and hence the increased exposure of the failing heart to catecholamines. Clinical studies of patients with heart failure have documented an increased cardiac norepinephrine spillover at rest5,6 and a failure to reach maximum spillover during exercise.7 Reduced and heterogeneous uptake, activity in the failing heart has also been documented in vivo through scintigraphic imaging with MIBG.8-11 A better regional resolution, and hence more detailed analysis of the left ventricular uptake, pattern, has been made possible by the introduction of positron emission tomography (PET) with use of the ligand C11-hydroxyephedrine (HED).12 In contrast to healthy subjects, patients with dilated cardiomyopathy demonstrated marked regional variation of left ventricular HED retention (F.H. et al, unpublished data, 1997). Decreased HED retention is related to functional changes of the sympathoadrenergic system, such as blood pressure variation.14 Left ventricular areas of reduced HED uptake show markedly reduced perfusion responses to sympathetic stimulation.15

To validate reductions in HED uptake, we sought to characterize the density and affinity of the uptake1-carrier protein in areas of reduced HED uptake. For this purpose, we compared the retention of the ligand HED, which is actively transported via uptake1, with the binding of an antagonist ligand to the carrier. The regional density and affinity of this protein were measured through radioligand binding in explanted human hearts of patients who had been investigated with the use of HED-PET before transplantation. Simulta-
neously, the regional tissue content of norepinephrine was determined because we wanted to characterize catecholamine stores as a marker for sympathetic neurons in the respective area. Finally, the density and the affinity of β-adrenergic receptors were measured because they are known to provide a representation of postsynaptic sympathetic function.16,17

Methods

Patients and Study Protocol
The study was performed with eight patients with heart failure (New York Heart Association functional class IV) due to dilated cardiomyopathy who were on the waiting list for heart transplantation (Table 1). All patients had nonischemic cardiomyopathy. The research protocol was approved by the institutional ethical committee, and each subject gave written informed consent before entering the study. Medical therapy consisted of cardiac glycosides, diuretics, and enalapril in all cases; none of the patients received catecholamines or β-receptor antagonists.

PET
All PET studies were performed with a Siemens/CTI 951 15-slice whole-body tomograph. A detailed description of the synthesis of HED has been documented previously.18

A perfusion scan using N13-ammonia was combined with HED dynamic imaging according to the following scheme: Imaging was initiated with an intravenous injection of 740 MBq (20 mCi) N13-ammonia to evaluate myocardial perfusion at rest. A 10-minute dynamic imaging according to the following scheme: Imaging was initiated with an intravenous injection of 740 MBq (20 mCi) N13-ammonia to evaluate myocardial perfusion at rest. A 10-minute dynamic imaging according to the following scheme: Imaging was initiated with an intravenous injection of 740 MBq (20 mCi) N13-ammonia to evaluate myocardial perfusion at rest (60 minutes). These retention fractions were then divided by the retention fraction of N13-ammonia to correct for flow variations.

Human Tissue
Tissue samples from the hearts of the same eight patients were taken after explantation. The mean time interval between PET study and transplantation was 3.4 ± 0.3 months. All patients gave written informed consent before surgery. General anesthesia was performed with fentanyl, fentanyl, and pancuronium bromide with enflurane. Cardiac surgery was performed with the patient on cardiopulmonary bypass. The entire explanted heart was then placed on ice immediately after removal from the body, allowing a standardized identification of apical and basal areas and of anterior, inferior, septal, and lateral walls. Equal amounts of tissue were cut from the central portion of each anatomically defined segment of the left ventricular wall. After excision, the tissue was immediately placed in ice-cold 0.5 to 30 nmol/L was carried out in 50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 5 mmol/L KCl with or without 10 μmol/L desipramine for 20 minutes at 22°C in a volume of 200 μL; an average of 207 μg of protein per tube was used. Mazindol is known to inhibit uptake of dopamine as well, but it binds to the dopamine carrier with an affinity ~10-fold lower than that for the uptake1 carrier.19

Radioligand Binding for Uptake
To define nonspecific binding, incubation of membranes with [3H]mazindol (DuPont–New England Nuclear) in concentrations ranging from 0.5 to 30 nmol/L was carried out in 50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 5 mmol/L KCl with or without 10 μmol/L desipramine for 20 minutes at 22°C in a volume of 200 μL; an average of 207 μg of protein per tube was used. Mazindol is known to inhibit uptake of dopamine as well, but it binds to the dopamine carrier with an affinity ~10-fold lower than that for the uptake1 carrier.19

In tissues rich in norepinephrine uptake, such as cerebral cortex, binding of [3H]-mazindol appears to correspond exclusively to norepinephrine uptake.21 Moreover, in a comparison of the

### TABLE 1. Clinical Data From Preoperative Cardiac Catheterization of Patients With Dilated Cardiomyopathy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age, y</th>
<th>LVEDP, mm Hg</th>
<th>EF, %</th>
<th>CI, L · min⁻¹ · m⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>57</td>
<td>20</td>
<td>19</td>
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<tr>
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<td>M</td>
<td>27</td>
<td>30</td>
<td>8</td>
<td>1.6</td>
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<tr>
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<td>M</td>
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<td>16</td>
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</tr>
<tr>
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<td>M</td>
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<td>2.1</td>
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<tr>
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<td>M</td>
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<td>21</td>
<td>2.3</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>50</td>
<td>25</td>
<td>10</td>
<td>1.4</td>
</tr>
<tr>
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<td>M</td>
<td>40</td>
<td>30</td>
<td>25</td>
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<td>8</td>
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<td>56</td>
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LVEDP indicates left ventricular end-diastolic pressure; EF, ejection fraction; and CI, cardiac index.
saturation binding of 3H-desipramine with that of 3H-mazindol, we found very similar $B_{\text{max}}$ values. The nonspecific binding of 3H-desipramine, however, accounted for 65% of the total binding in human cardiac tissue, so this radioligand could not be used for our experiments. The binding was fully saturable and showed a linear dependence on the amount of membrane protein used. Specific binding depended on the presence of sodium ions and was not detectable in the absence of sodium. Optimum binding was achieved at a concentration of 100 mmol/L NaCl.

Radioligand Binding for $\beta$-Adrenergic Receptors
The binding assay for $\beta$-adrenergic receptors was carried out similarly. The radioligand 3H-CGP 12177 was used in concentrations ranging from 0.05 to 5 nmol/L, and incubation lasted for 60 minutes. Propranolol was used as an antagonist to determine nonspecific binding. In contrast to uptake, binding, the incubation buffer did not contain NaCl or KCl.

Termination of the Binding Assay
The reaction was terminated with filtration through GF/B filters and washing with ice-cold incubation buffer. Filter radioactivity was determined by liquid scintillation counting. Uptake, carrier or $\beta$-adrenergic receptor density and affinity were determined from Scatchard plots of the counting data. Fig 1 shows the typical binding graph of $^3$H-mazindol to a membrane preparation from human ventricular tissue. The binding isotherm of $^3$H-CGP 12177 to cardiac membranes was similar, although it was characterized by a somewhat lower nonspecific binding.

Statistical Analysis
Data are expressed as mean±SEM values. For correlation of different parameters, a linear regression between two parameters was calculated, including 95% confidence intervals. A value of $P<.05$ was considered significant.

Results
HED-PET
Fig 2 shows a typical perfusion and neuronal PET image of the heart of a patient with terminal dilated cardiomyopathy. In the left ventricle of this patient, HED retention was markedly reduced in the lateral and inferior apical walls. In contrast, ammonia-PET documented nearly normal perfusion of all areas. Similar images were obtained in all patients. A reduced HED uptake in the inferoapical and apical wall areas was a common finding, as shown previously in a larger study population of patients with heart failure due to dilated cardiomyopathy (F.H. et al, unpublished data, 1997). The average ratio of HED retention relative to ammonia retention was significantly lower in the apical (0.84±0.04) and inferoapical (0.88±0.04) areas than in the basal septal wall (1.03±0.04, $P<.05$).

Uptake$_1$-Carrier Density
Soon after PET imaging, all patients had to undergo heart transplantation because of terminal heart failure. The different left ventricular areas of the explanted hearts were analyzed in vitro.

By studying radioligand binding to membrane preparations from the same nine areas, we found a marked regional variation in the density of uptake, in all investigated left ventricular areas. The different areas of the explanted hearts were analyzed in vitro.

Figure 2. Typical perfusion and neuronal PET images of the heart of a patient with terminal dilated cardiomyopathy. Top, Retention of $N^{13}$-ammonia. Bottom, Retention of HED. From left to right, the short axis (SA) and the horizontal long axis (HLA) and vertical long axis (VLA) are shown. In the left ventricle of this patient, HED retention was markedly reduced in the lateral and inferior apical walls. In contrast, ammonia-PET documented nearly normal perfusion in all areas.
ventricles, with lowest values in the apical and inferoapical left ventricular walls. These differences were even more pronounced in individual patients. To illustrate this finding, we demonstrate the correlation of the different regional parameters of all patients. Fig 3 shows the distribution of uptake, density (ordinate) and tissue norepinephrine content (abscissa) throughout the nine different left ventricular regions. In all patients, we observed a large range of regional variation, with mean densities between 80 and 280 fmol uptake1 sites/mg of protein. The affinity of uptake1 (determined as the k_d value of 3H-mazindol) showed some, although markedly less, regional variation. The k_d values ranged from 3 to 9 nmol/L, without any similarity or regional trend common to all investigated patients. There was no detectable correlation between uptake1 affinity and HED retention or regional tissue norepinephrine content (all tests not significant).

Tissue Norepinephrine Content
Similarly, tissue norepinephrine content showed a pronounced regional variation that was apparent in all patients. The abscissa of Fig 3 shows the mean regional norepinephrine contents. Regional norepinephrine content was clearly correlated to the respective uptake1 density, reaching statistical significance in all investigated patients (mean values are given in Table 2).

β-Adrenergic Receptors
The β-adrenergic receptor density also showed some regional variation, which was clearly less pronounced than the local differences in uptake1 and norepinephrine content. Although there was a trend for higher β-adrenergic receptor densities in the septal wall, this trend did not reach statistical significance in the investigated subjects. Fig 4 demonstrates the regional distribution of mean regional β-adrenergic receptor densities (ordinate) in comparison with the uptake1 densities of the same areas (abscissa). The affinity of β-receptors, determined as the k_d value of 3H-CGP12177, was fairly homogeneous, ranging from 0.4 to 0.7 nmol/L. Neither the density nor the affinity of β-receptors showed a correlation to other in vivo or in vitro parameters (all tests not significant), although the average correlation coefficient between β-adrenergic receptor density and uptake1 density was .38 (range, .1 to .7).

Correlation of PET Data and Biochemical Measurements
In all investigated patients, we detected a significant correlation of flow-corrected HED retention and uptake1 density. Fig 5 illustrates the correlation of mean uptake1 densities (abscissa) and mean HED-to-flow ratios (ordinate). Mean correlation coefficients with 95% confidence intervals and P values based on the analysis of 72 different areas in eight patients are given in Table 2.

Tissue norepinephrine content and flow-corrected HED retention were correlated (Fig 6). This association did reach significance for the correlation of regional mean values but failed to reach significance for the calculation of mean correlation coefficients (Table 2). In contrast, uptake1 affinity showed no such correlation with HED-to-flow ratios (all tests not significant). Mean absolute HED retention varied among patients (range, 2.1 ± 0.06%/min in patient 2 to 8.7 ± 0.6%/min in patient 5) and was correlated to the average uptake1 density (r = .5, P = .05) and average norepinephrine content of the respective patients (r = .53, P = .04).

Discussion
The present study documents a marked regional variation of HED retention in the left ventricles of patients with...
cardiomyopathy, with lowest values in inferior and apical areas. We identified a marked reduction in uptake1-carrier density, the underlying pathophysiological substrate, in these areas. Simultaneously, local norepinephrine content was clearly reduced in these regions. β-Adrenergic receptor density and affinity, however, showed less regional variation and were not significantly related to alterations in HED uptake.

It has not been known whether changes in cardiac uptake1-carrier protein density or affinity contribute to reduced HED uptake into the myocardium or whether HED uptake depends on other variables. In our study, we document for the first time a marked regional variation of the density of uptake1 carriers in the left ventricles of patients with dilated cardiomyopathy, which was clearly correlated to local differences in HED uptake. In membranes prepared from different left ventricular areas, the density and affinity of uptake1-carrier proteins were investigated by studying the binding of tritiated antagonist radioligands, which are not transported by the carrier or metabolized in the neuron. Using this assay, we identified a binding site in human heart membranes to which both desipramine and mazindol bound saturably and with high affinity. Radioligand binding depended strongly on the presence of sodium ions. A detailed characterization of this binding site had been carried out in a previous study using rat myocardium.23 Therefore, we obtained a more static view of the presence and density of uptake1 that complements the information about functional catecholamine uptake as assessed with HED-PET. Previous studies have documented reduced uptake1-carrier densities in failing hearts of both humans4 and dogs.5 This finding was accompanied by a decreased norepinephrine uptake, as assessed by in vitro tissue uptake of 3H-norepinephrine,2,3 or as measured by the amount of cardiac extraction of norepinephrine.24 These reports did not differentiate regional alterations of uptake1, in the left ventricle. Although we were not able to include a control group of healthy subjects in vitro measurement, we assume that the low average levels of uptake1-carrier sites in our study reflect a general reduction of these proteins in failing human hearts, corroborating the earlier reports. This reduction might be due to loss of neurons or downregulation of uptake1 per neuron, which could not be distinguished in the present study. A study of experimental heart failure in dogs, however, suggested that decreases of cardiac norepinephrine uptake are related to a loss of noradrenergic nerve terminals.25 Also, histological analysis of the right atria of patients with dilated cardiomyopathy revealed a decreased number of autonomic neurons.26 Therefore, it is tempting to speculate that a regionally heterogeneous loss of neuronal tissue is the basis for reduced uptake1 density, and hence HED uptake in these areas. Unfortunately, we could not conclusively investigate this issue because we were not able to obtain whole explanted hearts for neurohistological examination.

Similarly, up to threefold differences of regional tissue norepinephrine content were found, and they clearly correlated with uptake1 density and HED retention. Due to the terminal state of the patients included in this study (New York Heart Association functional class IV), average tissue levels of norepinephrine were low, corresponding to the lower range of
the largest study published to date. Previous reports have documented a correlation between the overall cardiac retention of $^{123}$I-MIBG with norepinephrine content in endomyocardial biopsies from patients with dilated cardiomyopathy or in dogs with pacing-induced heart failure. The proportional variation of HED retention is probably lower than the variation of tissue norepinephrine because many biological factors, such as pharmacokinetics or tissue availability, influence the in vivo distribution of an agent and therefore might mask the extent of variation, whereas the in vitro measurement of another parameter is more direct.

The observed regional variation of presynaptic sympathetic function might have important implications for the development and complications of heart failure. Several recent investigations of norepinephrine extraction in vivo have identified reduced uptake, as the most important causal factor for increased norepinephrine spillover in heart failure. Reduction of cardiac MIBG uptake in dilated cardiomyopathy has been linked to the prognosis of the patients. Regional variation of uptake might be the basis for some forms of ventricular arrhythmia observed in failing hearts. Assessment of myocardial refractoriness has revealed an increased refractory period in areas of reduced HED retention, which might thereby promote reentry arrhythmias. Decreased MIBG uptake has been linked to arrhythmogenic right ventricular cardiomyopathy. Reductions in cardiac HED uptake were also related to differences in blood pressure variation, a functional marker for the autonomic innervation of the heart. A recent report showed that variations in local HED uptake are also reflected by differences in maximum blood flow due to vasodilation after sympathetic stimulation. In general, different conditions of myocardial damage appear to be accompanied by similar reductions in inferior and lateral HED retention, such as diabetic neuropathy. This finding implies even more far-reaching consequences of local uptake regulation.

The fact that myocardial $\beta$-adrenergic receptor density was not significantly linked to the distribution of uptake, implies that postsynaptic regulation might depend on factors other than the presynaptic components. A clear statement in this regard will, however, require the investigation of a larger group of patients because we did detect a trend for local variation that did not reach statistical significance. A somewhat divergent regulation of presynaptic and postsynaptic components of the sympathoadrenergic system might, however, contribute to the marked arrhythmogenicity observed in the failing cardiomyopathic heart.

In conclusion, marked regional variations of both density of uptake, and tissue norepinephrine stores were observed in failing human hearts. These differences were comparable to those measured with HED-PET and did not correspond to differences in perfusion, as assessed by $^{11}$-ammonia-PET. HED-PET appears to be a reliable marker for biochemical alterations of the presynaptic sympathetic function.

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