Mechanistic Insights into Sympathetic Neuronal Regeneration
Multitracer Molecular Imaging of Catecholamine Handling After Cardiac Transplantation

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Background—Post-transplant reinnervation is a unique model to study sympathetic neuronal regeneration in vivo. The differential role of subcellular mechanisms of catecholamine handling in nerve terminals has not been investigated.

Methods and Results—Three different carbon-11–labeled catecholamines were used for positron emission tomography of transport (C-11 m-hydroxyephedrine, HED), vesicular storage (C-11 epinephrine, EPI), and metabolic degradation (C-11 phenylephrine). A 2-day protocol was used, including quantification of myocardial blood flow by N-13 ammonia. Resting myocardial blood flow and EPI, HED and phenylephrine retention were homogeneous in healthy volunteers (n=7). Washout was only observed for phenylephrine (T_{1/2} 49±6 min). In nonrejecting, otherwise healthy heart transplant recipients (>1 year after surgery, n=10), resting myocardial blood flow was also homogenous. Regional catecholamine uptake of varying degrees was observed in the anterior left ventricular wall and septum. Overall, 24±19% of left ventricle showed HED uptake levels comparable with healthy volunteers, whereas it was only 8±7% for EPI (P=0.004 versus HED). Phenylephrine washout was not different from healthy volunteers in the area with restored EPI and HED retention (T_{1/2} 41±7 min; P>0.05), but was significantly enhanced in the EPI/HED mismatch area (T_{1/2} 36±8 min; P=0.008), consistent with inefficient vesicular storage and enhanced metabolic degradation.

Conclusions—Regeneration of subcellular components of sympathetic nerve terminal function does not occur simultaneously. In the reinnervating transplanted heart, a region with normal catecholamine transport and vesicular storage is surrounded by a borderzone, where transport is already restored but vesicular storage remains inefficient, suggesting that vesicular storage is a more delicate mechanism. This observation may have implications for other pathologies involving cardiac autonomic innervation. (Circ Cardiovasc Imaging. 2015;8:e003507. DOI: 10.1161/CIRCIMAGING.115.003507.)

Key Words: catecholamines ◼ heart transplantation ◼ positron-emission tomography ◼ regeneration ◼ sympathetic nervous system

The sympathetic nervous system plays a key role in maintaining the homeostasis and integrity of the cardiovascular system. It is impaired in the natural course of various cardiovascular diseases, where imbalance contributes to electromechanical instability and progression of heart failure.1–3 Regeneration of impaired innervation, however, may also occur.

See Clinical Perspective

The transplanted heart is a unique model in this regard. After transplantation, the heart becomes denervated because of surgical disruption of the extracardiac sympathetic nerve fibers at the base of the heart. However, this process is not complete as the intrinsic cardiac network or ganglia of the transplanted heart has the potential to function independently in the absence of central neuronal inputs.4 Various studies have shown that the intrinsic cardiac nervous system becomes partly reinnervated by extracardiac sympathetic efferent neurons with time after transplantation.5–8 This has been associated with improved left ventricular (LV) performance and exercise capacity after heart transplantation9 and with improved regulation of myocardial perfusion10 and substrate utilization.11 Studies evaluating the presence, extent, and functional consequences of restored extracardiac sympathetic innervation, however, have typically used only a single tracer to determine catecholamine uptake and washout. This provides only a snapshot of nerve terminal integrity and does
not give insights into subcellular components of catecholamine handling.

The availability of various radiolabelled catecholamines with different molecular properties may enable the integrated assessment of specific mechanisms involved in myocardial catecholamine handling by positron emission tomography (PET). Prior experimental and clinical work has shown that carbon-11 (C-11) m-hydroxyephedrine (HED), a lipophilic, metabolically resistant catecholamine analogue, mainly reflects activity of the sarcolemmal norepinephrine transporter (uptake-1), whereas the physiological neurotransmitter C-11 epinephrine (EPI) is avidly taken up by uptake-1, but then requires vesicular storage within presynaptic neurons for protection from metabolic degradation. C-11 phenylephrine (PHEN) is another synthetic catecholamine which, following uptake and storage, leaks from vesicles even under physiological conditions and is then subjected to intraneuronal enzymatic degradation by monoaminooxidase. These 3 tracers may thus be used to dissect the contribution of neuronal transport (HED), vesicular storage (EPI), and leakage/metabolic degradation (PHEN) to various functional and dysfunctional states of sympathetic nerve terminals. They have been successfully and safely applied to image myocardial sympathetic nerve terminals before, but they have never been combined in the same imaging protocol in humans.

We speculated that there may be subtle differences in the regenerative capacity of subcellular mechanisms of nerve terminal function and sought to study this by means of PET with multiple radiolabelled catecholamines using the reinnervating transplanted human heart as a model.

Methods

Subjects

Ten heart transplant recipients (9 men; 51±12 years; body mass index, 30±6) were included at >1 year post transplantation (mean, 33±11 years; range, 18–48 years) and 10 heart transplant recipients (9 men; 51±12 years; body mass index, 30±6) were included at >1 year post transplantation (mean, 33±11 years; range, 18–48 years). All but 1 were on cyclosporine, all but 1 were on mycophenolate (the other one was on azathiaprine), and 6 of 10 were on prednisone. Of note, on a previous visit from our group, the use of immunosuppressive regimens was not an important determinant of LV catecholamine retention after transplantation.

Exclusion criteria consisted of serum creatinine ≥2 mg/dl, history of diabetes mellitus, significant graft vasculopathy, ongoing transplant rejection, and use of medication that interferes with catecholamine uptake, storage, and metabolism, such as tricyclic antidepressants, clonidine, resepine, and monoaminooxidase inhibitors.

Seven healthy volunteers without any significant past medical history (6 men; 31±15 years; body mass index, 27±4) served as controls. They were excluded if they had any significant past medical history, including diabetes, hypertension, and heart disease. They also had blood testing to rule out chronic kidney disease and a resting ECG.

Volunteers were younger than transplant recipients (P=0.002) but matched to the age of donor hearts (P=non-significant). All subjects gave written informed consent before inclusion. The study protocol and consent form were approved by the Johns Hopkins Institutional Review Board (research protocol # NA_00006439).

PET Imaging

HED, EPI, and PHEN were synthesized as previously described. In all cases, specific activity at the end of synthesis was >3500 mCi/μmol, and radiochemical purity was >95%. N-13 ammonia was prepared as previously described. All radiotracers were prepared as sterile, nonpyrogenic solutions suitable for injection.

All subjects were imaged using a GE Discovery VCT PET/computed tomography (CT) scanner, equipped with an integrated lutetium yttrium orthosilicate crystal PET component and a 64-slice x-ray CT. The study protocol comprised 2 separate imaging sessions on subsequent days—on day 1, myocardial perfusion and EPI kinetics were measured as follows: before scanning, subjects had 2 peripheral intravenous catheters placed in each arm, one for radiotracer infusion and the second one for blood draws. Individuals were positioned with the help of CT topograms, and a low-dose CT scan (120 kV, 40 mA) was performed for attenuation correction of PET emission data. Then, myocardial perfusion at rest was assessed using 300 to 400 MBq of N-13 ammonia and dynamic imaging for 20 minutes. After 5 half-lives to allow for decay of N-13, EPI (300–400 MBq) was injected intravenously and acquired using a dynamic sequence of 16 frames over 60 minutes (6×30 s, 2×60 s, 2×150 s, 2×300 s, 4×600 s). A second PET session was performed within 1 to 3 days. Subjects again had 2 peripheral intravenous catheters in place and were positioned. Then, PHEN (300–400 MBq) was injected, followed by 60-minute dynamic acquisition with a protocol identical to EPI. After 1 hour to allow for C-11 decay, the same imaging procedure was repeated with HED (300–400 MBq).

Electrocardiography, heart rate, and blood pressure were monitored before, during, and after injection of each tracer. To determine the contribution of C-11–labeled metabolites to blood activity, venous blood samples were drawn at 2, 5, 10, 20, 40, and 60 minutes after injection and assayed using Sep-Pak cartridges as previously described.

Image Analysis

Reconstruction and Sampling

Attenuation-corrected transaxial images were reconstructed by filtered back projection. Using volumetric sampling of the last frame of the perfusion study, myocardial radioactivity was defined in 460 LV sectors and depicted in a polar map. For reproducible and comparable quantitative analysis, the so defined cardiac long axis and myocardial segments were automatically transferred to dynamic series of all other tracers, and time-activity curves were obtained. In addition, for each tracer, arterial input function was defined by a small region of interest in the LV cavity. In general, this was a semiautomated process, where manual software interaction was only required for the definition of the cardiac long axis view and arterial input function. Otherwise, all analyses were automated.

Myocardial Blood Flow

Absolute myocardial blood flow (MBF) was obtained at rest using an established 3 compartment model for N-13 ammonia. To correct for potential hemodynamic differences, resting MBF was corrected by the rate pressure product (RPP) using the following equation: corrected resting MBF=uncorrected resting MBF×10000/RPP.

Kinetics of C-11–Labeled Tracers

Myocardial retention (%/min) of all C-11–labeled tracers was defined as the concentration in myocardial tissue between 40 and 60 minutes, normalized to the integral of the metabolite-corrected arterial input function, and expressed in a parametric polar map. Tracer clearance was determined by monoexponential fitting of myocardial time activity curves between 10 and 60 minutes to obtain a washout rate constant.

Definition of Normal Catecholamine Uptake and Washout

Global and regional myocardial retention index and washout rates for all C-11–labeled tracers in healthy volunteers were gathered and used to create reference databases for polar map analysis. Polar maps of transplant recipients were then compared with the normal reference maps. Normal catecholamine uptake was defined as the fraction of polar map segments with myocardial retention within 2 SD of the mean reference.
**Statistical Analysis**

Statistical analysis was performed using SPSS (version 21). Data normality was determined using the Shapiro–Wilk test and normal Q–Q plots. Continuous variables are presented as mean±SD. Two-tailed, paired *t* test was used to assess differences between 2 variables intra-individually, and 1-way, factorial ANOVA combined with Scheffe’s test for post hoc analysis and correction for multiple comparisons was performed to compare mean of >2 variables intra-individually. The 2-tailed, unpaired *t* test was used to assess differences between subgroups of individuals. Spearman correlation was used to evaluate associations between time from transplantation and catecholamine uptake. *P*<0.05 was considered statistically significant.

**Results**

**Global and Regional MBF**

Regional MBF was homogenous in all LV walls, both in healthy individuals and transplant recipients. Global resting MBF was elevated in transplant recipients (1.02±0.26 versus 0.70±0.10 mL/min per g; *P*=0.01) because of higher baseline heart rate and RPP. After correction for RPP, resting MBF was not significantly different between both groups (0.89±0.20 versus 0.73±0.08 mL/min per g; *P*=0.6).

**Myocardial Catecholamine Kinetics in Healthy Volunteers**

Global myocardial retention of EPI was highest, and that of PHEN was lowest among the 3 tracers in healthy volunteers (Table). Only PHEN showed significant washout from healthy myocardium, contributing to its lower retention. There was no washout of EPI or HED from normal myocardium. Regional retention was homogeneous throughout the LV for all 3 catecholamines (Figures 1 and 2A).

Plasma metabolism of HED was lower compared with EPI and PHEN because of its resistance to degrading enzymes (Figure 3A).

**Myocardial Catecholamine Kinetics in Heart Transplant Recipients**

In transplant recipients, mean plasma metabolism of all 3 tracers was comparable with normals, although, more variance in
EPI metabolites in plasma was observed in transplant recipients (Figure 3B). Taken together with the absence of perfusion differences, this rules out systemic effects on myocardial catecholamine tracer kinetics.

Global LV retention of HED, EPI, and PHEN was significantly reduced, whereas washout of PHEN was enhanced compared with healthy volunteers (Table).

Despite globally reduced retention, an LV region with tracer retention within the normal range was observed, mostly in the anteroseptal wall (Figures 2B and 4A). The extent of myocardium with normal HED uptake, consistent with regionally restored catecholamine uptake-1, was $24\pm19\%$ of LV. Of note, the extent of myocardium with normal EPI retention, consistent with regionally restored vesicular storage, was significantly lower at $8\pm7\%$ of LV ($P=0.004$; Figure 4B). The region with restored vesicular storage according to EPI was typically located in the center of the larger region with restored catecholamine uptake according to HED (Figure 5A). Consistent with prior work, there was a moderate correlation (although not statistically significant) between time from cardiac transplantation and normal catecholamine uptake according to HED ($r=0.65$; $P=0.058$) and EPI ($r=0.54$; $P=0.1$).

Finally, for a regional analysis of catecholamine turnover, polar maps of transplant recipients were subdivided into 3 regions: (1) denervated myocardium (with abnormally low retention of HED and EPI), (2) normal myocardium (with normal retention of HED and EPI), and (3) myocardium with normal catecholamine uptake but abnormal vesicular storage (Figure 5B). Washout of PHEN in region 2 was comparable with healthy volunteers. Whereas, region 3 demonstrated significantly higher PHEN washout compared with healthy volunteers. PHEN washout was significantly higher in the denervated region 1 compared with the other regions of the transplanted and healthy heart.
In this study, we used for the first time 3 different positron-labeled catecholamine analogues for intraindividual comparison in humans. Global and regional kinetics of the agents in healthy subjects and in reinnervating cardiac transplant recipients provide unique insights into subcellular mechanisms of sympathetic nerve regeneration. Expectedly, and consistent with prior studies using single tracers or 2 of the 3 agents, regional distribution of the catecholamines HED, EPI, and PHEN was homogeneous in healthy subjects. Global myocardial retention of EPI was highest among the 3 agents. EPI is a physiological neurotransmitter, which is avidly taken up by the uptake-1 into sympathetic nerve terminals and requires efficient storage in intraneuronal vesicles for protection from metabolic degradation by cytosolic monoaminooxidase. This has been confirmed by prior experimental work showing absent myocardial retention after desipramine-induced blockade of uptake-1, but also after reserpine-induced blockade of vesicular storage.13 Global retention of HED was mildly lower than EPI, consistent with prior studies.19 HED is a catecholamine analogue, which has a high affinity for uptake-1 but does not require vesicular storage for efficient retention in nerve terminals because of its resistance to monoaminooxidase metabolism. Its retention is closely correlated to uptake-1 density.23 HED is thought to undergo a continuous uptake and release by nerve terminals via uptake-1, as suggested by prior experimental work, where blockade of uptake-1 after administration of HED resulted in washout of previously retained tracer—a characteristic that was not observed for EPI in the same setting.13 Finally, PHEN showed lowest myocardial retention in healthy subjects among the 3 agents. This is a result of its relatively low affinity to uptake-1, but also of its steady washout because of metabolic degradation within nerve terminals.24 Prior work in isolated perfused hearts has suggested that PHEN is a marker of leakage from neuronal vesicles and of subsequent metabolic degradation by monoaminooxidase.14 Consistently, PHEN was the only agent that showed significant washout from normal myocardium.

Analysis of plasma metabolites was used for the correction of arterial input function to determine myocardial tracer retention. Of note, the degree of plasma metabolism was highest for EPI versus PHEN and HED, consistent with the above-described differences in tracer characteristics. Also, plasma metabolism was comparable between healthy subjects and cardiac transplant recipients, suggesting a lack of contribution of differences in systemic catecholamine handling to myocardial kinetics. Furthermore, differences between normal and transplant recipients cannot be explained by differences in myocardial perfusion. Blood flow was regionally homogeneous in all individuals. Mildly elevated global resting flow in transplant recipients is attributed to a higher RPP which is mostly because of higher heart rate as a consequence of lack of vagal tone from atrial parasympathetic denervation.25 This finding is consistent with

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**Discussion**

In this study, we used for the first time 3 different positron-labeled catecholamine analogues for intraindividual comparison in humans. Global and regional kinetics of the agents in healthy subjects and in reinnervating cardiac transplant recipients provide unique insights into subcellular mechanisms of sympathetic nerve regeneration. Expectedly, and consistent with prior studies using single tracers or 2 of the 3 agents, regional distribution of the catecholamines HED, EPI, and PHEN was homogeneous in healthy subjects. Global myocardial retention of EPI was highest among the 3 agents. EPI is a physiological neurotransmitter, which is avidly taken up by the uptake-1 into sympathetic nerve terminals and requires efficient storage in intraneuronal vesicles for protection from metabolic degradation by cytosolic monoaminooxidase. This has been confirmed by prior experimental work showing absent myocardial retention after desipramine-induced blockade of uptake-1, but also after reserpine-induced blockade of vesicular storage.13 Global retention of HED was mildly lower than EPI, consistent with prior studies.19 HED is a catecholamine analogue, which has a high affinity for uptake-1 but does not require vesicular storage for efficient retention in nerve terminals because of its resistance to monoaminooxidase metabolism. Its retention is closely correlated to uptake-1 density.23 HED is thought to undergo a continuous uptake and release by nerve terminals via uptake-1, as suggested by prior experimental work, where blockade of uptake-1 after administration of HED resulted in washout of previously retained tracer—a characteristic that was not observed for EPI in the same setting.13 Finally, PHEN showed lowest myocardial retention in healthy subjects among the 3 agents. This is a result of its relatively low affinity to uptake-1, but also of its steady washout because of metabolic degradation within nerve terminals.24 Prior work in isolated perfused hearts has suggested that PHEN is a marker of leakage from neuronal vesicles and of subsequent metabolic degradation by monoaminooxidase.14 Consistently, PHEN was the only agent that showed significant washout from normal myocardium.

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prior work.\textsuperscript{10,26} and cannot explain regionally heterogeneous innervation nor globally reduced retention of all 3 catecholamines in transplant recipients.

The observation of regionally heterogeneous restoration of sympathetic innervation, which occurs predominantly in the basal anteroseptal wall (Figure 2B) and increases with time after transplantation, is consistent with several previous studies.\textsuperscript{5,6,8,9} The novelty of this study is that intraindividual comparison of the kinetics of EPI, HED, and PHEN in transplant recipients suggests molecular differences in the recovery of subcellular mechanisms (catecholamine uptake, vesicular storage, and enzymatic degradation) required for normal catecholamine handling in cardiac presynaptic sympathetic nerve terminals. Uptake-1 density (as evidenced by HED retention) is more extensively restored compared with vesicular storage recovery (as observed by EPI retention). Prior work by Münch et al.\textsuperscript{20} obtained similar results by observing lower global transplant/normal retention ratios of EPI compared with HED, but regional analysis was not performed in similar detail and a polar map–based definition of regional SD from the normal mean was not conducted.

A unique feature of our study is the addition of PHEN as a third catecholamine for imaging. Despite its comparably low uptake-1 affinity, PHEN is uniquely suited to characterize vesicular leakage and enzymatic degradation by washout analysis. We used PHEN to identify differences between the myocardial region demonstrating matched recovery of vesicular storage (EPI) and uptake-1 (HED), and the surrounding area showing recovery of uptake-1 by HED only. In the former, PHEN washout was similar to healthy volunteers, confirming normal sympathetic nerve integrity of this segment in the transplanted hearts. In contrast, the surrounding region with restored uptake-1 but abnormally low EPI retention revealed significantly enhanced PHEN clearance rates, consistent with inefficient or absent vesicular storage and, therefore, enhanced metabolic degradation. This gives further support to the notion that vesicular storage is restored to a lesser extent compared with uptake-1 activity in the reinnervating transplanted heart.

Limitations
The main study limitation is the lack of serial PET studies to demonstrate reinnervation at the subcellular level and on a patient basis. Yet, we have previously demonstrated that regional reinnervation does occur after transplantation.\textsuperscript{6} In our experience, myocardial retention of catecholamines is completely absent in subjects early after heart transplantation but returns, although partially, at later stages.

We also acknowledge that we do not have a clinical end point similar to prior studies that have focused on the clinical consequences of reinnervation/denervation, such as regulation of MBF, metabolism, and regional contractile function, after transplantation.\textsuperscript{5,10,11} Instead, and because of the known intra-individual heterogeneity of the cardiac autonomic system, we used the transplanted heart as a model to obtain basic insights into the biology of the myocardial adrenergic nervous system. We think that such insights into its biology and into the differential kinetic behavior of various catecholaminergic agents add important basic knowledge to the field.

Conclusions
In summary, our study using molecular imaging with multiple radiolabelled catecholamines that underlie different kinetic properties suggests that regeneration of subcellular components of sympathetic nerve terminal function does not occur simultaneously. In the reinnervating transplanted heart, a region with normal catecholamine transport and vesicular storage is surrounded by a borderzone, where transport is already restored but vesicular storage remains inefficient. This suggests that vesicular storage is a more delicate mechanism which requires more time for restoration in the process of neuronal regeneration. This observation may have implications for other pathologies involving cardiac autonomic innervation, such as myocardial ischemia, infarction, heart failure, metabolic, and neurodegenerative diseases, where impaired innervation has been identified and where the presence and contribution of nerve regeneration is less well defined.\textsuperscript{1,3-5,7} Of note, vesicular storage may not only require more time for restoration but it may also be damaged at an earlier stage in disease, as suggested by preclinical work in myocardial infarction.\textsuperscript{22} Whether this has implications for adverse outcome, or whether it may emerge as a target for regenerative therapies, should be a subject of future studies.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

In this study, using molecular positron emission tomography imaging, we investigated the differential role of distinct subcellular mechanisms of catecholamine handling after cardiac transplantation. Our findings suggest that regeneration of subcellular components of sympathetic nerve terminal function does not occur simultaneously. A region with normal left ventricular catecholamine transport and vesicular storage is surrounded by a borderzone where transport is already restored but vesicular storage remains inefficient. This suggests that vesicular storage is a more delicate mechanism which requires more time for restoration in the process of neuronal regeneration. This observation may have implications for other pathologies involving cardiac autonomic innervation, such as myocardial ischemia, infarction, heart failure, metabolic, and neurodegenerative diseases, where impaired innervation has been identified and where the presence and contribution of nerve regeneration is less well defined.
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