In Vivo Quantification of Myocardial Muscarinic Receptors in Heart Transplant Patients

Dominique Le Guludec, MD; Jacques Delforge, PhD; André Syrota, MD, PhD; Michelle Desruennes, MD; Heric Valette, MD; Iraj Gandjbakhch, MD; Pascal Merlet, MD

Background Decreased myocardial adenylate cyclase activity in response to guanine nucleotide stimulation has been recently demonstrated in denervated myocardium of transplant patients, suggesting that changes in left ventricular muscarinic receptors may occur.

Methods and Results The concentration and affinity constants of myocardial muscarinic receptors were determined by positron emission tomography with $^{11}$C-labeled methylquinuclidinyl benzilate (MQNB), a specific hydrophilic antagonist, in six transplant patients 4.7±2.3 months after surgery and in six normal subjects. Patients had no sign of cardiac rejection at endomyocardial biopsy. After intravenous injections of MQNB, time-activity curves were obtained over different regions of interest and were fitted to a nonlinear mathematical model. No difference in the concentration of muscarinic receptors was found in transplant patients compared with control subjects: 24±4 versus 26±7 pmol/mL tissue, respectively ($P=NS$). The association rate constant $k_{+1}$, the dissociation rate constant $k_{-1}$, and thus the equilibrium-dissociation constant $K_e$ were the same in transplant patients compared with control subjects.

Conclusions Despite known decreased GTP-stimulated adenylate cyclase activity in transplant patients, the density and affinity constants of myocardial muscarinic receptors are not altered. This suggests abnormalities of the signal-transduction function, such as a change in the guanine nucleotide binding proteins. (Circulation. 1994;90:172-178.)

Key Words • positron emission tomography • acetylcholine

Muscarinic cholinergic receptors (MR), in balance with $\beta$-adrenergic receptors, play a key role in the regulation of the rate and force of contraction of the heart (see Reference 1 for review). Their role in the inhibition of GTP-activated adenylate cyclase activity, mediated by a GTP binding protein, $G_i$, is now well established.2,3 The density and affinity of the receptors may be altered in physiopathological conditions, as well as their responsiveness in MR-coupled second messenger systems.4,5 Exposure to the specific agonist induces inactivation or desensitization of receptors.6,7 However, changes in myocardial MR in heart disease have not been extensively evaluated. This is due in part to the absence of noninvasive methods for quantification of MR receptors. In vitro binding studies require large amounts of tissue, difficult to obtain by endomyocardial biopsy. This technique is not free of complications and not suitable for normal subjects for comparative studies. Quantification of MR on peripheral lymphocytes has been used as an index of myocardial MR, but no correlation between lymphocyte and myocardial receptor density has been demonstrated thus far. Furthermore, discrepancies have been observed in quantification of $\beta$-adrenergic receptors and $G_i$ in peripheral lymphocytes and myocardium after denervation.4

Myocardial denervation in transplant patients leads to important clinical, physiological, and pharmacological alterations: elevated basal heart rate, delayed increase in heart rate during exercise with reduced exercise tolerance, silent ischemia despite severe coronary artery disease, arrhythmias, and differences in drug efficiency.8 Denervation generally induces a supersensitivity to agonists and an increase in receptor density. Changes in myocardial $\beta$-adrenergic receptors have been evidenced.10 Decrease in GTP-activated adenylate cyclase activity has been recently reported in animal models and transplant patients.8,11 This may be due to alteration in myocardial density or affinity of receptors coupling with the inhibitory guanine nucleotide binding protein, among which MR take place. Vatner et al10 reported a decrease in canine ventricular MR density after denervation. However, no data are available on MR in the denervated human heart.

The potential for noninvasive quantification of ventricular MR in vivo using positron emission tomography (PET) has recently been noted.12-14 This technique enables measurements of the concentration and affinity constants of left ventricular MR using $^{11}$C-labeled methylquinuclidinyl benzilate (MQNB), a highly specific antagonist, in combination with a multi-injection protocol and a mathematical model.15 The methodology was first applied in dogs and recently applied in normal subjects.15

The purpose of the present study was the in vivo quantification of myocardial MR in human denervated hearts as a result of orthotopic heart transplantation.

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Methods

Study Population

Patients

Six male patients (mean age, 45 ± 5 years) were studied 2 to 7 months (mean, 4.7 ± 2.3 months) after cardiac orthotopic transplantation. No patient was treated with β-blockers; all were on standard immunosuppressive therapy and calcium antagonists (nifedipine). An echocardiographic study was performed within the same week and did not show any abnormalities of systolic and diastolic parameters. The absence of significant rejection was confirmed at three consecutive endomyocardial biopsies: within the week of the PET study and 3 weeks before and 3 weeks after the PET study. All biopsy specimens were graded 0 or 1 according to the Billingham classification.16

Control Subjects

This group consisted of 6 normal healthy male volunteers (mean age, 32 ± 6 years [range, 24 to 44 years]). They were free of any cardiac disease on the basis of clinical, ECG, and echocardiographic examinations, and none was taking medication. Part of this control group was previously included in a first methodological study.15

Written informed consent was obtained from each subject. The study protocol was approved by the Ethics Committee of our institution (Commissariat à l’Energie Atomique, France).

PET Procedure

Preparation of [11C]MQNB

MQNB was labeled using [11C] by methylation of quinuclidinyl benzilate (QNB) with [11C]methyliodide. Labeled material had a specific radioactivity ranging from 11 to 45 GBq/μmol at the time of injection.

PET Measurements

PET studies were performed using a time-of-flight assisted positron camera (LETI TTV03, Commissariat à l’Energie Atomique). It allowed acquisition of seven cross-sectional images, 12 mm apart, with a 7-mm in-plane resolution on a reconstructed image using a modified Hanning window function. The axial resolution is 9 mm for a direct plane and 7 mm for a cross plane. Transmission scans were performed with a rotating 67 Ge source and used for subsequent attenuation correction. Emission data were recorded in list-mode starting with the first injection of [11C]MQNB until the end of the experiment. Sixty-two sequential images, using one of the seven cross sections, were reconstructed according to the specific experimental protocol used. Calibration was checked every week with a cylindrical phantom containing a uniform source of 67 Ge.

Experimental Protocol

The PET study included three injections of [11C]MQNB and/or MQNB. At the beginning of the experiment, approximately 370 MBq of [11C]MQNB was intravenously injected. Thirty minutes later, 0.3 mg of unlabeled ligand was intravenously injected (“displacement”). Sixty minutes later, a mixture of labeled (approximately 300 MBq) and unlabeled (0.3 mg) MQNB in the same syringe was administered (“coinjection”). The overall study lasted 90 minutes. The acquisition protocol was applied early in the morning on fasting subjects. The arterial blood pressure was measured before injection and every 2 minutes after each injection. The heart rate was continuously monitored and the ECG recorded every minute for 5 minutes after each injection, and then every 5 minutes.

PET Data Analysis

The image plane that best included the left ventricle was visually selected and used to generate a set of sequential images. The myocardial region of interest was automatically defined by a 70% isocountour plotting routine drawn on a 10-minute image (Fig. 1). In addition to the global left myocardial region of interest, three segmental regions were drawn: septal, apical, and lateral. The input function was obtained from a region of interest manually drawn in the left ventricular cavity. This method, which avoids arterial blood sampling, has previously been validated in normal volunteers.15 List-mode acquisition allowed the time-of-flight confidence weighted reconstruction of 10-second images during the first 2 minutes after labeled ligand injection and longer duration images (up to 5 minutes) when radioactivity decreased. [11C]MQNB concentration was measured in each region of interest after correction for 11C decay and expressed as picoatoms per milliliter after dividing by the specific radioactivity measured at time 0.

Myocardial wall thickness was measured by M-mode echocardiography and PET data corrected for loss in count recovery due to the small thickness of the heart wall compared with the spatial resolution of the PET system. The correction was performed using a recovery factor measured experimentally on a heart phantom with the same PET system. Spillover from blood cavity to myocardium was accounted for by using a vascular fraction (Fv) in the fitting procedure.

The Ligand-Receptor Model

The compartmental model used in this study and shown in Fig 2 was a nonequilibrium nonlinear model.13,15,17 It included two steps: (1) a transport of the ligand from the blood to a free ligand compartment and (2) a classic ligand-receptor interaction (Fig 2).

The rate constant p characterized the transfer of ligand from blood to tissue (as milliliter of plasma per milliliter of tissue). It has been found to be linearly related to the heart rate.15 k characterized the transfer from tissue to blood (as min−1), and Vp (as milliliter of tissue per milliliter of tissue) is defined as the fraction of the region of interest delineated by PET in which the ligand can react with receptors. The product p · Vp is the clearance of the ligand (as milliliter of plasma per min per milliliter of tissue).

The model parameters introduced in the ligand-receptor interactions were similar to those used in in vitro studies: the concentration of available receptors (B∗max) and the association and dissociation rate constants (k1 and k−1, respectively).

By fitting the mathematical model to time-concentration curves, it was possible to obtain estimates of parameters p · Vp, k1, B∗max, k−1/Vp, k−1, and Fv. The volume of reaction Vp was deduced by assuming that the transport between blood and tissue was passive and the two parameters p and k had the same value. Thus, Vp could be estimated from the p · Vp/k ratio. This allowed the deduction of k−1 and thus the estimation of the equilibrium dissociation constant Kg.

Statistical Analysis

All values are expressed as mean ± SD. Data were compared using paired and unpaired Student’s t tests. Correlation coefficients, assuming linear regression, were calculated for paired variables. A value of P < .05 was considered statistically significant.

Results

Population Characteristics

Transplant patients were significantly older than control subjects (45 ± 5 versus 32 ± 6 years; P < .05), but donors were not (29 ± 10 years; P = NS). The end-diastolic left ventricular wall thickness, measured by echocardiography, ranged from 10.3 to 12.9 mm (mean, 11.0 ± 1 mm) in patients, not significantly different from that in control subjects (mean, 9.9 ± 0.6 mm).
Tolerance and Heart Rate Variations During the Experiment

The protocol experiment was well tolerated in all subjects; three transplant patients and four volunteers complained of a slight mouth dryness. The complete set of data was obtained in all patients.

The baseline heart rate in transplant patients was significantly higher than that of control subjects (87.6±8 versus 69.9±9 beats per minute [bpm], respectively; P<.01). The heart rate remained constant until the end of the procedure (maximal heart rate, 89.8±7 bpm) in all patients. In contrast, in normal volunteers the injection of unlabeled ligand at 30 minutes resulted in a slight heart rate increase (74±17 bpm). Moreover, within 5 minutes after the coinjection, heart rate began to increase, reaching a maximum 10 minutes after coinjection (98±13 bpm; P<.05 with basal heart rate), which persisted until the end of the procedure (Fig 3). There was no significant difference in the maximal heart rate between patients and control subjects.

PET Receptor Quantification

Fig 4 shows two examples of time-activity curves obtained in a normal volunteer and in a transplant patient. After the first tracer injection, the myocardial concentration increased rapidly, remaining constant until the displacement in normal volunteers, whereas it slowly increased in transplant patients. The displacement of [11C]MQNB by unlabeled MQNB resulted in a decrease in myocardial radioactivity concentration; the coinjection of both labeled and unlabeled MQNB produced a second increase in radioactivity concentration, immediately followed by its decrease.

The mean receptor concentration (B′ unr) measured in patients was 24±4 pmol/mL tissue and was not different from that of control subjects: 26±7 pmol/mL tissue (P=NS). Individual data are given in Tables 1 and 2. In patients, the $K_d \cdot V_p$ product, directly identified from PET data, was significantly higher than control values: 0.55±0.14 versus 0.30±0.08 pmol/mL tissue (P<.05), and $k_{s1}/V_p$ was significantly lower: 0.48±0.08 versus 1.33±0.22 mL tissue/(pmol·min) (P<.001). However, these changes were due to a significant increase in the volume of reaction $V_p$; 0.26±0.08 mL tissue/mL tissue in patients versus 0.16±0.06 mL tissue/mL tissue in control subjects (P<.05). The values of $k_{s1}$, $k_{s1}$, and $K_d$ were not significantly different between patients and control subjects. The equilibrium dissociation constant $K_d$ was 2.2±1.2 pmol/mL tissue in patients and 2.1±0.7 pmol/mL tissue in control subjects (P=NS). No corre-
Fig 2. Drawing of compartmental ligand-receptor model used in analysis of myocardial tissue data obtained after intravenous injection of \(^{11}C\)-labeled methylquinuclidinyl benzilate. It includes three compartments corresponding to labeled ligand concentrations: the ligand in the arterial blood \(C^*(t)\), the free ligand in the tissue \(M^*(t)\), and the ligand specifically bound to the receptors \(M^*_b(t)\). All transfer probabilities of drug between compartments are linear except for the binding probability, which depends on the bimolecular association rate constant \(k_1\), and on the local concentration of free receptors, which is equal to \((B'_\text{max} - M^*_b - M_b)\). \(B'_\text{max}\) is the estimate of muscarinic receptor concentration, and \(M^*_b\) and \(M_b\) is the quantity of labeled and unlabeled ligand bound to receptor. \(V_R\) is the volume of reaction in which the free ligand can react with the receptor sites. The parameter \(k_1\), is the dissociation rate constant and \(k_1\), the association rate constant. Because the experimental protocol includes injections of unlabeled ligand, it is necessary to simulate the kinetics of this unlabeled ligand with the same model. The unlabeled ligand concentrations are not directly observable from positron emission tomography (PET) data, but the concentration of the unlabeled specifically bound ligand \(M^*_b(t)\) has an effect on the local concentration of free receptors and consequently on the binding probability of free labeled ligand. \(F_r\) indicates vascular fraction.

Fig 4. Line graph shows example of a time-activity curve obtained in a normal volunteer (•) and in a transplant patient (○) using a three-injection experiment. The protocol included a tracer injection of labeled ligand at time 0 (5.2 and 10.7 \(\mu\)g, respectively), an injection at 30 minutes of unlabeled ligand (0.30 mg), and a coinjection at 60 minutes of labeled (22.7 and 33.1 \(\mu\)g, respectively) and unlabeled (0.3 mg) ligand. The level of the plateau is dependent on the injected dose of radioactive tracer, which is included in the model. The two solid lines correspond to the fitting procedures obtained from the model. MQNB indicates methylquinuclidinyl benzilate.

Parasympathetic Innervation of the Human Ventricular Myocardium

The parasympathetic innervation of the human ventricular myocardium, although spare compared with the atria, was confirmed approximately 10 years ago.\(^{18,19}\) It was first demonstrated by conventional histochemical techniques, using three biochemical markers of parasympathetic innervation: acetylcholine content, choline acetyltransferase activity, and acetylcholinesterase activity. However, the first studies using acetylcholinesterase staining underestimated cholinergic innervation of the ventricular myocardium. More recently, sensitive immunohistochemical methods and specific antisera to neural markers protein, allowing the quantification of immunoreactive nerve fibers and acetylcholinesterase activity, confirmed the distribution of parasympathetic innervation throughout the human atrial and ventricular tissues, with a gradient from the former to the latter. The enzyme activity seems to persist, although at a lower degree, in cardiac allograft tissues.\(^{20}\) Finally, the presence of ventricular cholinergic neurons was recently confirmed using PET and tracers that bind to the vesicular acetylcholine transporter of cholinergic neurons, such as \([\text{F}^{18}]\)fluoroethoxybenzovesamicol\(^{21}\) or \([\text{C}]\)physostigmine (H.V., unpublished data, 1993).

Denervation Consequences

Myocardial denervation after transplantation induces dramatic changes in the physiology and physiopathology of the heart. The general phenomenon occurring after surgical interruption of autonomic innervation is
an increased response to the administration of the neurotransmitter that has been lost. This “denervation supersensitivity” may be the consequence of both presynaptic nerve loss and postsynaptic changes involving the receptor-effector system. This phenomenon was first described for the nicotinic receptor in the skeletal muscle. This was confirmed for both sympathetic and parasympathetic systems in other organs. In the heart, such changes have been extensively studied for the β-adrenergic system. An increased total number of β-adrenoreceptors was reported by Vatner et al for humans. However, a similar upregulation was not confirmed by Brodde et al in transplant patients, suggesting species differences. Brodde et al only noted a change in the ratio of β1/β2 adrenoreceptors, with a correlation between this ratio and the time after transplant, suggesting changes between acute and chronic stages of cardiac denervation. The supersensitivity of the myocardium in response to β-agonists observed in animal models has not been found in humans.

Although an abnormal response to acetylcholine has not yet been demonstrated in the human transplanted heart, the evaluation of potential changes in MR seems of great interest with regard to the modifications recently found in the adenylate cyclase system both in humans and animals. Muscarinic agonists inhibit cardiac adenylate cyclase activity, with reduced cyclic AMP formation in humans. The coupling of MR agonist binding to inhibition of adenylate cyclase activity is measured by guanine nucleotide binding proteins (G proteins). Recently Denniss et al demonstrated significantly depressed adenylate cyclase activity in response to guanine nucleotide stimulation in myocardial biopsy samples from transplant patients. Such a change in the GTP-stimulated adenylate cyclase activity has also been reported by Horn et al in transplant patients. This might be due to changes in G proteins, as suggested by Horn et al, who recently found significantly greater G1 levels in the myocardium of autotransplanted baboons. In other models of denervation, Hodges et al also demonstrated that selective chronic parasympathectomy of the canine heart was associated with an increase in G1. On the other hand, the decrease in the GTP-stimulated adenylate cyclase activity induced by denervation may be related to a decreased stimulatory G protein, Gs. Nevertheless, such changes in the level of Gs were not found by Horn et al. These findings suggest a reduction in GTP-stimulated adenylate cyclase activity and an increase in Gi, with no changes in β-receptor total density and Gi content, as a result of cardiac denervation. From these results, an enhanced number or affinity to agonists of receptors coupling to the inhibitory guanine nucleotide binding protein may be hypothesized. This may concern essentially the MR and the A1-adenosine receptors.

In the only available animal study, Vatner et al reported a slight but significant (20%; P<0.01) decrease in MR number without change in affinity in surgically denervated dog myocardium. However, the changes in adenylate cyclase activity found in patients have not been found in denervated dogs, suggesting differences between MR in animals and patients.

The absence of changes in patients’ myocardial MR is in agreement with the unchanged sensitivity to muscarinic agonists found in the denervated heart. An absence of postsynaptic supersensitivity to muscarinic agonists, with enhanced sensitivity to nicotine, has been reported in animal models. A supersensitivity to acetylcholine, initially suggested by Jacobs et al, has not been observed by others. Priola and Spurgeon found no exaggerated negative inotropic response to acetylcholine in left denervated ventricle in dogs and found even a lower depression in contractility of denervated hearts compared with intact hearts when using the same dose of acetylcholine. Oda et al have also demonstrated that the denervated heart had a subnormal content of acetylcholine. This may be due to the fact that surgical parasympathetic denervation is

### TABLE 1. Parameter Estimates for Subjects

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<th>Subject 2</th>
<th>Subject 3</th>
<th>Subject 4</th>
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<td>0.11</td>
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### TABLE 2. Parameter Estimates for Patients

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preganglionic, in contrast with sympathetic denervation. This is concordant with the persistence of some parasympathetic innervation in cardiac allograft tissues. Therefore, the estimation of the MR $B'_\text{max}$ should not be hampered by discrepancies in endogenous ligand.

Few data are available on sensitivity to parasympathetic neurotransmitters in transplant patients. Results obtained in animal models cannot be extrapolated to patients, as demonstrated by the differences between experimental and clinical situations reported for the sympathetic system. In transplant patients, Landzberg et al. studied the effect of an intracoronary infusion of atropine and acetylcholine on the left ventricular $dP/dt$ peak under basal conditions and during $\beta$-adrenergic stimulation with intracoronary dobutamine infusion. They showed that MR stimulation attenuated the $\beta$-adrenergic response to dobutamine similarly in transplant patients and control subjects. This absence of human supersensitivity after transplantation suggested the functional integrity of ventricular MR, in agreement with the present PET data.

**Limitations of the Study**

In the present study no comparison between PET measurements and in vitro quantification of the left ventricular MR was available. This would have required a left catheterization, which is not performed at this period of the follow-up in our institution. However, the PET values of $B'_\text{max}$ obtained in normal patients in our laboratory were very close to the measurements of MR density reported by Böh m et al. in normal human myocardium using $[^1\text{H}]\text{QNB}$: 275 pmol/g protein, which corresponds to 22.5 pmol/g tissue when taking in account the ratio of protein to tissue.

The heart rate of transplant patients was higher than that of control subjects at baseline and did not increase after injection of the muscarinic antagonist, an equivalent of atropine. This discrepancy between posttransplant patients and control subjects was expected because of the absence of tonic vagal input. The heart rate does not respond to innervation-dependent pharmacological stimuli such as atropine. This different pattern of heart rate changes implies a different pattern of the input function of the tracer during the procedure. The differences in heart rate variations after MQNB injection are included in the model. The differences in the volume of reaction between patients and control subjects can be interpreted as an increase in the interstitial water volume, since MQNB is a very hydrophilic antagonist. This increase in $V_h$ probably reflects edema, which has been reported to exist in transplanted hearts even in the absence of characterized rejection. Edema indeed existed in our patients. On the other side, the right ventricular biopsy performed within the same week as the PET study, edema was visually evaluated with a semiquantitative score from 0 to 4; all specimens were graded 1 or 2. However, a precise quantification of edema is not easy, and samples were not obtained in the left ventricle. The edema induces a decrease in the number of cells in the region of interest of approximately 10% ($V_h$ from 0.16 to 0.26 mL tissue per milliliter tissue); this could account for a similar decrease in $B'_\text{max}$ which is expressed per milliliter of tissue. Indeed, the $B'_\text{max}$ of patients was 8% lower than that of control subjects.

No differences in affinity constants between patients and control subjects were found in the present study. PET receptor quantification uses labeled antagonist of the receptor because of its higher affinity compared with that of the agonist. Burgisser et al. previously reported that not only agonist affinity but also antagonist affinity are regulated by guanine nucleotide. MR in frog heart display high-agonist/low-antagonist and low-agonist/high-antagonist receptor affinity states, with interconversion from the former to the latter induced by guanine nucleotides in vitro. Berrie et al. showed that the binding properties of MR for agonist and antagonist are inversely altered in the presence of guanine nucleotides in homogenates of rat atria and ventricular myocardium. GTP produced no change in the concentration of antagonist binding sites but an increase in the affinity of the antagonist ($N$-methylscopolamine) and a larger decrease in the agonist (carbachol) affinity. According to this hypothesis, Syrota et al. previously suggested that vagal stimulation would be characterized by a conversion to the low-agonist/high-antagonist affinity form of the MR. The absence of vagal stimulation in transplanted patients would be presumed to result in a high-agonist/low-antagonist affinity form of MR and would be evidenced using an agonist.

In the present study no correlation was found between MR density or affinity and the time after transplant. However, no conclusions could be drawn on this point because of the narrow range of time from surgery to PET study in this limited series of patients. Further studies performed later after denervation are required to confirm our results in patients without rejection and without coronary artery disease. This time was chosen to avoid the acute period after transplantation as well as any potential reinervation and coronary artery disease, which is most likely to occur after the first year after transplantation. This is of importance because regional changes in coronary blood flow could influence the PET results. Partial sympathetic reinervation has been reported by Wilson et al. 1 year after surgery and by Schweiger et al. using $[^1\text{H}]\text{hydroxyephedrine}$ and PET. Smith et al. reported the absence of increased heart rate period variability in denervated donor atria of patients as long as 8 months after transplantation. In the present study the absence of increased heart rate after injection of pharmacological doses of MQNB in transplant patients suggests the lack of significant parasympathetic reinervation.

Finally, other factors may alter myocardial MR in patients. Interference with pharmacological therapy may occur. Denniss et al. did not find any differences in adenylyl cyclase activity between transplant patients whether or not they received azathioprine therapy. The effect of corticosteroids on the binding affinity of cardiac autonomic receptors has been studied for $\beta$-adrenergic receptors but not for MR. The mean age of our transplant patients was significantly greater than that of control subjects, and an inverse correlation between the $K_d$ of lymphocytal MR for MQNB and age has been found by Usami and Kaku; however, the mean age of donors was not different than that of control subjects.

In conclusion, we report here the absence of change in the myocardial MR density and affinity for antagonist after orthotopic transplantation in patients. Factors other than MR should be studied to understand the previously found decrease in GTP-stimulated adenylyl cyclase activity.
Acknowledgments
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