Effect of Chronic Renal Failure on Cardiac Contractile Function, Calcium Cycling, and Gene Expression of Proteins Important for Calcium Homeostasis in the Rat

DAVID KENNEDY, EIAD OMRAN, SANKARIDRUG M. PERIYASAMY, JALAA NADOOR, ANUMEET PRIYADARSHI, JAMES C. WILLEY DEEPAK MALHOTRA, ZIJIAN XIE, and JOSEPH I. SHAPIRO

Departments of Medicine and Pharmacology, Medical College of Ohio, Toledo, Ohio.

Abstract. Patients with chronic renal failure frequently develop cardiac hypertrophy and diastolic dysfunction; however, the mechanisms by which this occurs are still unclear. Male Sprague-Dawley rats were subjected to 5/6 nephrectomy and studied for their isolated myocyte function, calcium cycling, and gene expression of proteins important in calcium homeostasis after 4 wk. Comparable rats subjected to suprarenal aortic banding for the same duration were used for comparison. Rats subjected to 5/6 nephrectomy and aortic banding developed comparable hypertension; however, rats subjected to 5/6 nephrectomy experienced a greater degree of cardiac hypertrophy and downregulation of cardiac sodium potassium ATPase (Na⁺/K⁺-ATPase) activity than rats subjected to aortic banding. Moreover, cells isolated from the 5/6 nephrectomy rat hearts displayed impaired contractile function and altered calcium cycling compared with cells isolated from control or aortic constriction rat hearts. The 5/6 nephrectomy rat heart cells displayed a prolonged time constant for calcium recovery following stimulation, which corresponded to decreases in homogenate sarcoplasmic reticulum calcium ATPase-2a (SERCA2a) activity, protein density, and mRNA for SERCA2a. In conclusion, chronic renal failure leads to alterations in cardiac gene expression, which produces alterations in cardiac calcium cycling and contractile function. These changes cannot be explained only by the observed increases in BP.

Patients with renal failure usually develop cardiac complications. In end-stage renal disease (ESRD) patients treated with hemodialysis in the United States, annual mortality rates exceed 20%, with more than 50% attributed to cardiac mortality (1). Although the term “uremic cardiomyopathy” had previously been used to refer to a dilated cardiomyopathy complicating renal failure, more recent studies suggest that the most common form of heart disease in renal failure patients is one characterized by diastolic dysfunction and left ventricular hypertrophy (2). Although a number of known factors have been implicated in the pathogenesis of the left ventricular hypertrophy and diastolic dysfunction, our understanding of these processes is still incomplete (3).

For many years, it has been known that the sodium pump is abnormal in chronic renal failure and that circulating inhibitor(s) can be demonstrated in the serum of uremic patients (4–6). We have observed that sodium pump inhibition initiates a signal cascade that can cause alterations in gene transcription and ultimately produce hypertrophy in cardiomyocytes grown in culture (7–9). We have also observed that sodium pump inhibitors, including those circulating in the serum of uremic patients, can acutely cause altered calcium cycling and cardiomyocyte relaxation through sodium pump inhibition (10). The following studies were performed to further examine how cardiac growth and function are chronically modified by the uremic milieu.

Materials and Methods

Animals

Male Sprague-Dawley rats (200 to 250 g) were subjected to either 5/6 nephrectomy produced by removal of the right kidney and segmental infarction of two thirds of the remaining kidney with silk ligatures, suprarenal aortic constriction (produced by tying a silk ligature [4–10] around a 21-gauge needle and the suprarenal abdominal aorta and then removing the needle or sham surgery). The surgical approaches have been described in detail in previous publications from our laboratory (7,11). After surgery, the rats were allowed to recover for 4 wk having access to ad libitum food (Rodent Laboratory Chow 5001; PMI Nutrition International, Inc., Brentwood, MO) and water. The content of this chow mix is listed on the company web page, but the nutritional essentials are as follows: protein 23.4%, fat 4.5%, crude fiber 5.8%, and total digestible nutrients 76%. The calcium and phosphorus contents of the chow were 1.00% and 0.61%, respectively. At the end of 4 wk, some animals were anesthetized and BP was determined by placing a catheter in the carotid artery before removal of the heart for subsequent studies. In some experiments, cardiomyocytes were isolated for subsequent study. In other experiments, hearts were removed and immediately homogenized to allow...
for the determination of the enzymatic activities of the Na\(^+/\)K\(^+\)-ATPase and the SERCA2a as well as these protein densities using Western blots (7). In other experiments, the left ventricle was quickly excised and frozen in liquid nitrogen. This frozen tissue was then stored at −80°C until it was subsequently analyzed with quantitative PCR (StaRT-PCR) for determination of mRNA for several gene products.

**Isolation and Culture of Cardiac Myocytes**

Details of the method of isolation and culture of calcium-tolerant adult myocytes may be found in several recent reports from our laboratory (9,10,12). This method of isolation produced a good yield of rod-shaped (70 to 80%) myocytes in each of the experimental groups presented here.

**Measurements of the Calcium Transient and Contractility**

The calcium transient was measured by using the calcium-selective fluorescent dye indo-1 and spectrofluorometer (Photon Technology International, Monmouth Junction, NJ). Myocyte contractility was measured simultaneously using an edge detector system (Crystal Biotech, Northboro, MA), which, along with the spectrofluorometer, was interfaced with an inverted microscope. The simultaneous observation of both indo-1 fluorescence and edge detection was accomplished by continuous illumination of the cells during field stimulation with a red light and splitting of the emission light based on wavelength to either a video imaging system or the spectrofluorometer. Again, details of this methodology may be found in recent reports from our laboratory (9,10,12). Calculations of cytosolic calcium concentration ([Ca\(^{2+}\)]\(_{i}\)) were made using the following formula:

\[
[\text{Ca}^{2+}]_i = \frac{K_d \times D_{\text{free}}/D_{\text{bound}} \times (R_{\text{Rmin}})/(R_{\text{Rmax}}-R)}
\]

where the Kd was assumed to be 250 nM, the Dfree and Dbound represent the intensity of the fluorescence at 485 nm after EGTA (4 mM) and Ionomycin (10 \(^{-6}\) M) treatment of the cell, respectively, and Rmin and Rmax were the ratios obtained under these conditions.

The time constant, \(\tau\), for recovery of length and calcium after electrical stimulation was performed by fitting a least square regression line to the log transformation of the edge detection and fluorescence data, respectively, as described by Bers et al. (13,14) and also reported previously by our group (10).

**StaRT PCR**

Analysis of gene expression for proteins important in calcium homeostasis as well as markers of cardiac hypertrophy was performed using standardized reverse transcription PCR (StaRT-PCR), which allows for quantitative measurement of gene expression on the basis of the ratio of native PCR products to specific competitive templates (CT) (15). Detailed description of the principles, reagents, and protocol for this methodology may be found in several recent reports (15–18). Briefly, left ventricles obtained from remnant and sham-treated rats were homogenized, and total RNA extraction was performed on the tissue homogenate as described by the TRI-REAGENT protocol (Molecular Research Center, Inc, Cincinnati, OH). Reverse transcription (RT) (5 min denaturing at 94°C, 1 h incubation at 37 (10), 5 min heat stopped at 94°C) and PCR was then performed.

Primers for all target and housekeeping genes that were evaluated in this study are listed in Table 1. Reaction volumes were 10 \(\mu\)l and each contained 0.05 \(\mu\)g of each primer, 0.5 U Taq polymerase, 1 \(\mu\)l PCR buffer, 0.2 mM dNTPs, 1 \(\mu\)l of a CT mixture containing the desired molarity of each CT, and 1 \(\mu\)l cDNA diluted such that native GAPDH competed equally with the GAPDH CT present in the chosen CT mixture. The PCR reaction mixtures were placed in capillary tubes and cycled 35 times in a Rapidicycler air thermocycler (Idaho Technology). Each cycle consisted of 5 s at 94°C, 10 s at 58°C, and 15 s at 72°C, with a slope of 9.9, for a total amplification time of approximately 25 min. The PCR products were electrophoresed on either DNA 7500 or 1000 assay LabChips (Agilent Technologies, Palo Alto, CA), and quantitative analysis was performed as described previously (15). Levels of expression are reported as units of messenger RNA (mRNA)/10\(^6\) copies of GAPDH.

**Western Blot Analysis SERCA2a and Sodium Calcium Exchanger (NCX-1)**

The hearts from sham and nephrectomized rats were excised, and the left ventricles were dissected out. Left ventricles were homogenized in 25 mM imidazole buffer pH 7.0 containing protease inhibitors. An aliquot of the homogenate was removed, and its protein content was determined (19). After solubilizing the homogenates in sample buffer (2% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue, and 50 mM Tris-HCl, pH 7.0), the proteins in the homogenates were resolved as described by Laemmli (20) on a SDS-PAGE using 10% gel. The proteins were transferred to nitrocellulose membrane following the method of Towbin et al. (21) and immunoblotted with anti-SERCA2 mAb or anti-NCX-1 mAb (Affinity Bioreagents, Inc., Golden, CO). The immunoreactive products were visualized with horseradish peroxide conjugated to donkey anti-mouse IgG (Affinity Bioreagents, Inc.) using an enhanced chemiluminescence substrate (Pierce, Rockford, IL). The images of the immunoreactive products were quantified with a Molecular Analyst software program (BioRad Laboratories, Hercules, CA).

**Measurement of Na\(^+\)/K\(^+\)-ATPase Activity**

Cardiac homogenates were prepared in the presence of protease inhibitors, and ouabain-sensitive Na\(^+\)/K\(^+\)-ATPase activity was measured as we have previously reported (7).

**Measurement of SERCA2a Activity**

To measure sarcoplasmic reticulum calcium ATPase activity (which we are assuming is predominantly SERCA2a activity [22,23]), the method of Simonides and Hardeveld (24) was used with modifications. Homogenates of left ventricles of rats were processed in a medium containing 25 mM imidazole (pH 7.0) and protease inhibitors. Homogenates were then subjected to freeze-thaw cycles five times to open up the vesicles formed during the homogenization. The assay medium consists of 1.0 ml containing 40 mM imidazole (pH 7.0), 100 mM KCl, 5 mM K\(^+\) oxalate, 5 mM Na\(^+\)_, 3 mM MgCl\(_2\), 2 mM ouabain, 200 to 220 μg of homogenerate, 3 mM (γ-32P)-ATP, and 10 μM Ca\(_{\text{Cl}}\) or 2 mM EGTA. After a 5 min preincubation at 37°C, enzymatic reaction was initiated by the addition of (γ-32P)-ATP and terminated 15 min later by the addition of 8% perchloric acid. Released inorganic (32P)-phosphate was measured as described by Askari et al. (25). The difference between ATPase activity in the presence and absence of Ca\(_{\text{Cl}}\) was considered as SERCA2a activity.

**Statistical Analyses**

Data obtained were first tested for normality. If the data did not pass the normality test, the Tukey test (for multiple groups) or the Mann-Whitney rank sum test were used to compare the data. If the data did pass the normality test, parametric comparisons were performed. If more than two groups were compared, one-way ANOVA was performed before comparison of individual groups with the
Results

Effect of 5/6 Nephrectomy on Heart Size and BP

The production of 5/6 nephrectomy and aortic constriction both resulted in considerable increases in both BP and heart size (Table 2). Although the increases in BP were comparable to that seen in rats subjected to suprarenal aortic constriction studied at a similar time point, the degree of cardiac hypertrophy was approximately 50% greater ($P < 0.05$, Table 2) in rats subjected to 5/6 nephrectomy. Rats subjected to aortic constriction maintained a normal hematocrit, whereas chronic renal failure rats had reduced hematocrit values (Table 2).

<table>
<thead>
<tr>
<th>Table 1. Primers used for StaRT PCR$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>$\alpha$Na$^+/K^+$ ATPase</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>$\alpha$2Na$^+/K^+$ ATPase</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>skACT</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>ANP</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>SERCA2a</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>NCX-1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

$^a$ $\alpha$1Na$^+/K^+$ ATPase, sodium potassium pump, $\alpha$ subunit, isoform 1; $\alpha$2Na$^+/K^+$ ATPase, sodium potassium pump, $\alpha$ subunit, isoform 2; skACT, skeletal actin; ANP, atrial natriuretic factor; NCX-1, sodium calcium exchanger; SERCA2a, sarcoplasmic reticulum calcium ATPase, isoform 2a; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Effect of partial nephrectomy (PNx) and aortic constriction (AC) on body weight, BP, and heart weight$^a$

<table>
<thead>
<tr>
<th></th>
<th>Sham ($n = 12$)</th>
<th>AC ($n = 14$)</th>
<th>PNx ($n = 14$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>467 ± 12</td>
<td>464 ± 9</td>
<td>458 ± 12</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>109 ± 2</td>
<td>175 ± 8$^c$</td>
<td>165 ± 7$^c$</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>51 ± 1</td>
<td>50 ± 2</td>
<td>38 ± 2$^{cd}$</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.24 ± 0.03</td>
<td>1.45 ± 0.04$^c$</td>
<td>1.56 ± 0.03$^{cd}$</td>
</tr>
</tbody>
</table>

$^a$ Results expressed as mean ± SEM.

$^b$ $P < 0.05$ versus Sham.

$^c$ $P < 0.01$ versus Sham.

$^d$ $P < 0.05$ versus aortic clip.

$^e$ $P < 0.01$ versus aortic clip.
crease was more modest than that seen in the 5/6 nephrectomy rats (Figure 1). Examining the Na\(^+\)/K\(^+\) ATPase isoforms, the 5/6 nephrectomy rat hearts demonstrated a considerable decrease in the expression of both \(\alpha_1\) and \(\alpha_2\) isoforms, whereas the decrease in Na\(^+\)/K\(^+\) ATPase appeared to be confined to the \(\alpha_2\) isofrom in the aortic constriction rat hearts (Figure 1).

**Effect of 5/6 Nephrectomy on Isolated Cardiac Myocyte Calcium Cycling and Contractile Function**

Cardiomyocytes isolated from rats subjected to aortic constriction demonstrated no significant alterations in contractile function and calcium cycling when compared with sham-operated rats after 4 to 6 wk. However, cardiomyocytes isolated from 5/6 nephrectomy rats demonstrated decreases in fractional shortening as well as substantial increases in both diastolic and systolic calcium concentrations. Moreover, the rats subjected to 5/6 nephrectomy showed substantially greater \(\tau\) values for both calcium and length recovery after stimulation (Table 3).

**Effect of 5/6 Nephrectomy on SERCA2a**

To further examine the mechanisms underlying the alterations in calcium cycling seen in the hearts of rats subjected to 5/6 nephrectomy, SERCA2a activity, protein density, and gene expression were examined. Rats subjected to 5/6 nephrectomy showed substantially decreased SERCA2a activity compared with sham-treated rats and rats subjected to aortic constriction (Figure 2). Quantification of protein density with Western blotting confirmed a decrease in SERCA2a in the 5/6 nephrectomy as compared with the sham-treated rat hearts (Figure 2). Interestingly, Western blotting to determine the protein density of the NCX-1 revealed nearly a 100% increase in the hearts from the 5/6 nephrectomy compared with sham-treated rats (1.94 ± 0.22 versus 1.00 ± 0.10; both \(n = 5\); \(P < 0.01\)).

**Effect of 5/6 Nephrectomy on Cardiac Gene Expression**

To further examine the mechanisms underlying the biochemical and physiologic changes described above, StaRT-PCR was used to quantify cardiac gene expression for the Na\(^+\)/K\(^+\)-ATPase isoforms, SERCA2a and NCX-1, as well as skeletal muscle actin (skACT) and atrial natriuretic peptide (ANP). Hearts isolated from 5/6 nephrectomy rats demonstrated significant decreases in message expression for the \(\alpha_2\) isofrom of Na\(^+\)/K\(^+\)-ATPase as well as increases in skACT and ANP (Table 4). In particular, SERCA2a message was reduced by 50% (\(P < 0.01\)) in these 5/6 nephrectomy hearts.

**Discussion**

Although the term “uremic cardiomyopathy” has been used for many years, our concept of the clinical features has changed dramatically (27). Foley et al. (28) have demonstrated that, although systolic dysfunction is demonstrable in the minority of chronic renal failure patients, hospitalization for fluid overload or congestive heart failure occurs very commonly (29). Recent work suggests that echocardiographically demonstrable diastolic dysfunction is extremely common in ESRD patients treated with hemodialysis (30). As discussed briefly in the introduction, we have observed that sodium pump inhibition, which appears to accompany the chronic renal failure state, may acutely cause or contribute to both diastolic dysfunction and cardiac hypertrophy (7,10). To gain further insights into this issue we conducted the current study.

We observed that chronic renal failure induced by partial (5/6) nephrectomy was associated with marked increases in cardiac size, a phenomenon that could not be accounted for only by the hypertension associated with this model. We also noted characteristic changes in message expression quite similar to that seen in ouabain-induced cardiac hypertrophy in vitro. Specifically, we saw increases in the transcription of ANP and skACT, which appears to accompany all pressure overload type cardiac hypertrophy (31) as well as decreases in the \(\alpha_2\) isoform of Na\(^+\)/K\(^+\)-ATPase. The fall in \(\alpha_2\) expression is analogous to the decrease in \(\alpha_3\) expression in neonatal cardiac myocyte hypertrophy induced with ouabain (32,33) or observed with the coincident exposure to both pressure overload and potassium depletion together (7) or pressure overload alone (34). We have postulated that the decrease in \(\alpha_2\) (or \(\alpha_3\)) expression may constitute a negative feedback attenuating hypertrophy induced by ouabain or other digitalis-like substances (9,32,33). In the remnant kidney cardiac cells, we noted that in addition to the decreases in \(\alpha_2\) Na\(^+\)/K\(^+\)-ATPase, the \(\alpha_1\) isoform protein expression was also significantly decreased. We did not examine \(\alpha_3\) isoform expression in the current study.

We must point out that the hematocrit was diminished in the chronic renal failure rats, raising the possibility that the accelerated hypertrophy in these animals was due to an additive or synergistic effect between pressure and volume overload as suggested to occur in patients with chronic renal failure (35). Although our gene expression data appear more consistent with a purely “pressure overload” phenotype (34), it is not possible to exclude this possibility from our data.

Our findings regarding sodium pump gene expression are...
submitted on a literature background, which is plagued by inconsistency, at least in the case of chronic renal failure. Greiber et al. (36) reported essentially no difference in α1 and α2 isoform mRNA or protein expression in a similar model of chronic renal failure studied at approximately the same time point. Da Silva et al. (37) found significant decreases in cardiac mRNA for α2 without any changes in α2 protein observed. Bonilla et al. (38) reported decreases in both Na⁺/K⁺ ATPase activity as well as mRNA for the α1 isoform in skeletal muscle; however, these workers found an increase in α2 mRNA in this tissue. At present, the reason(s) for the discrepancy between our findings and these other studies is/are not clear.

Regarding the myocyte function, we found that the cells isolated from the hearts of rats bearing remnant kidney demonstrated both systolic and diastolic dysfunction in vitro. Our findings were quite similar to that reported by McMahon et al. in 1996 (39). This was in contrast to the heart cells isolated from the aortic clip rats, which demonstrated grossly normal function and calcium cycling at the time of isolation, which was between 4 to 6 wk after surgery; this observation was also consistent with previous studies performed early after induction of aortic banding (40). The systolic dysfunction seen in the heart cells from the remnant kidney rats was strikingly similar to that observed when heart cells are isolated from rats with congestive heart failure from a variety of causes, including aortic constriction (41). We particularly noted marked calcium insensitivity in these heart cells with the diastolic calcium value substantially elevated compared with sham-treated rats. It appears that it takes considerably longer periods of aortic constriction to produce abnormal systolic function and calcium insensitivity in these isolated myocytes than the induction of uremia by the 5/6 nephrectomy model that is associated with very similar BP elevation in the species and strain that we employed (42). Although most patients with chronic renal failure display normal (or even supranormal) systolic function (2), we suggest that the digitalis-like substances that circulate...
in such patients might mask underlying contractile problems (10).

Other investigators have also noted increases in cytosolic calcium during experimental uremia, but this has been ascribed to the associated secondary hyperparathyroidism (43). Although we did not attempt to dissect out the role of parathyroid hormone in this current study, we have previously reported that acute sodium pump inhibition by deproteinated serum extract as well as administration of cardiac glycosides elevates cardiac cytosolic calcium (7,9,10). We speculate that chronic exposure to such sodium pump inhibition could alter cardiac calcium cycling, contractile function, and gene expression on a chronic basis. However, this speculation as well as the role that parathyroid hormone plays in the regulation of circulating inhibitors of the sodium pump requires additional study.

In addition to this systolic dysfunction, we also observed substantial diastolic dysfunction characterized by delayed recovery of length in parallel with a longer time constant (τ) describing calcium recovery after stimulation (44). This has also been observed in a variety of cardiomyopathies, including that induced by prolonged (> 12 wk) aortic banding (45). Although the decrease in cytosolic calcium after stimulation depends on several processes, the most quantitatively important are through reuptake of calcium into the sarcoplasmic reticulum and extrusion out of the cell via the sodium calcium exchanger. Bassani et al. (46) reported that sarcoplasmic reticulum calcium reuptake is the major determinant of the rate of decay of the calcium transient in rat cells with the sodium calcium exchanger accounting for substantially less of this calcium recovery in the rat. Although the sodium calcium exchanger is responsible for the majority of calcium efflux in myocytes, it can also serve as a calcium influx mechanism (47).

Experimental and human heart failure are typically characterized by a decrease in SERCA2a (48,49) and an increase in NCX-1 (50), although it should be noted that some studies have observed NCX-1 to be unchanged or even decreased (51,52). Thus, some authors suggest that the [Ca2+]i is determined by the interactions between these two calcium handling proteins taken together (i.e., the ratio of NCX-1 to SERCA) rather than either one separately (53). Thus, to elucidate the mechanisms responsible for deranged calcium metabolism seen with 5/6 nephrectomy, we focused our investigation on SERCA2a and NCX-1, as these proteins represent the predominant cardiac isoforms of both the sarcoplasmic reticulum calcium ATPase and the sodium calcium exchanger, respectively. In our setting, we have decreased levels of SERCA2a and increased levels of NCX-1. It has been suggested that in pathophysiologic conditions where there is a reduction in SERCA2a function, overexpression of NCX-1 may participate in a compensatory attempt to maintain normal cardiac homeostasis (54). On the basis of our data, it appears that transcriptional downregulation of SERCA2a leads to decreases in sarcoplasmic reticulum calcium reuptake and impaired myocyte relaxation in this model. As discussed above, it is unclear what role parathyroid hormone and circulating digoxins-like substances play in the regulation of these transport proteins in the setting of chronic renal failure, and further examination of this important area is certainly warranted.

In summary, we observed that uremia induced by 5/6 nephrectomy caused marked cardiac hypertrophy and changes in cardiac gene expression that could not be explained by only the observed increases in BP. We also noted that calcium cycling and contractile function were deranged in the myocytes isolated from these hearts and that transcriptional downregulation of the SERCA2a may account for the impaired diastolic function seen in these myocytes.

Acknowledgments

Some of these data were presented in abstract form at the 2000 American Society of Nephrology Meetings. The authors would like to thank Ms. Carol Woods for her excellent secretarial assistance as well as Jie Chen and Dr. Wei Han for their technical efforts. Portions of this study were supported by the American Heart Association (National and Northwest Ohio Affiliate) and the National Institutes of Health (HL57144 and HL63238 and HL67963).

References


