Cardiac Systolic and Diastolic Dysfunction After a Cholesterol-Rich Diet

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Background—Although hypercholesterolemia is a well-established risk factor for coronary artery disease, little is known regarding its direct effects on cardiac function.

Methods and Results—We examined the effects of cholesterol feeding (0.5%) on cardiac function in rabbits. After 10 weeks, both systolic shortening and diastolic relaxation rates were impaired without any change in aortic pressure or ventricular hypertrophy. However, sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA)-2 mRNA levels were reduced within 4 days after initiation of cholesterol feeding. After this effect, SERCA-2 protein and SERCA-mediated Ca uptake into sarcoplasmic reticulum vesicles were impaired, and the ratio of MHC-β to MHC-α mRNA increased 5-fold. Suppression of the SERCA-2 message correlated temporally with enrichment of the cardiac sarcolemma with cholesterol.

Conclusions—These data demonstrate that dietary hypercholesterolemia induces a “cholesterol cardiomyopathy” characterized by systolic and diastolic dysfunction. These alterations were independent of vascular disease and demonstrate a dietary link to cardiac dysfunction. (Circulation. 2004;109:97-102.)

Key Words: heart failure ■ cardiomyopathy ■ myosin ■ sarcoplasmic reticulum ■ hypercholesterolemia

Although hypercholesterolemia is widely appreciated as a principal risk factor for coronary artery disease (CAD), little is known regarding the effects of hypercholesterolemia on cardiac function apart from CAD. However, observations in various clinical trials and animal models suggest an effect of cholesterol on myocardial function. For example, several trials have shown that statins, which dramatically reduce serum cholesterol levels, improve congestive heart failure (CHF). In the Scandinavian Simvastatin Survival Study (4S), mortality rates in patients developing CHF decreased 20% with simvastatin treatment compared with placebo.1 In the Evaluation of Losartan In The Elderly (ELITE), there was a significantly lower mortality (10.6%) in elderly CHF patients receiving statins compared with those who were not (17.6%).2 In another study enrolling post–myocardial infarction patients, those with left ventricular (LV) ejection fractions <40% had a 6% improvement in LV ejection fractions after 12 weeks on simvastatin therapy.3 Although these observations in humans suggest a noncoronary role in improving cardiac performance, they do not directly link dyslipidemia to impaired ventricular function. In animal and cell culture models, statins ameliorate CHF. In a murine model, fluvastatin decreased mortality and heart failure along with decreasing LV dilation, hypertrophy, and interstitial fibrosis.4 Cerivastatin produced a similar effect in a rat model of CHF after myocardial infarction.5 Thus, HMG-CoA reductase inhibitors seem to have ameliorating actions on cardiac dysfunction, but the degree to which this occurs by reducing cholesterol exposure to myocytes has not been addressed. Direct effects of cholesterol exposure on cardiac myocyte function have been demonstrated by Bastiaanse et al6 in a study that showed that decreased cytosolic calcium levels and impaired cardiac myocyte contractility occurred after elevation of membrane cholesterol content in LV myocytes in cell culture.

The present study was designed to determine whether diet-induced hypercholesterolemia alters cardiac function independently of CAD. Rabbits were fed cholesterol for up to 70 days, and LV myocytes were freshly isolated and immediately studied at various intervals. At day 70, we found (1) a significant reduction in systolic and diastolic function that was accompanied by (2) a marked increase in membrane cholesterol content, (3) reduced sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA)-2 mRNA and protein levels and SERCA-2 mediated Ca-uptake activity, and (4) an increased ratio of myosin heavy chain (MHC)-β to MHC-α. Lastly (5), these alterations occurred in the absence of ventricular hypertrophy or coronary lesions and were pre-

Received May 27, 2003; revision received August 25, 2003; accepted September 2, 2003.

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Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000109213.10461.F6
ceded by significant reductions in SERCA-2 mRNA levels after only 4 days of cholesterol feeding. These findings demonstrate, for the first time, a dietary link to the development of an intrinsic “cholesterol cardiomyopathy.”

Methods

Animals

Control and cholesterol-fed (“diet”) New Zealand White rabbits were used in this investigation. Control rabbits were maintained on standard calibrated chow, and diet rabbits were fed batch-matched standard chow supplemented with cholesterol (0.5%) for up to 10 weeks. All animals were handled in accordance with Accreditation of Animal Laboratory Animal Care guidelines.

Isolation of Myocytes

Calcium-tolerant myocytes were isolated and maintained in Henseleit buffer containing 1 mmol/L CaCl₂ and 1% BSA at 35 °C and gassed with 95% O₂/5% CO₂.

Contractile Measurements

The freshly isolated myocytes were perfused (3 to 5 mL/min) in a chamber and stimulated (0.5 Hz, 5-msec duration, and 40 to 80 V). Video-based edge-detection software was used to measure changes in cell length during contraction and relaxation.

Electrophysiological Techniques

Freshly isolated myocytes were superfused with various Tyrode’s-based solutions at 1 to 2 mL/min. All action potentials were recorded at a stimulation rate of 0.5 Hz at 35 °C. Voltage-clamp experiments were performed in the whole-cell recording mode as described previously with low-resistance (2- to 5-MΩ) suction-type pipettes containing cesium filling solution. For calcium currents, a holding potential of −40 mV was used to inactivate Iᵥ, Iᵣ, and T-type calcium currents. Membrane potential was stepped from −45 to +60 mV in 5-mV increments. Iᵥ was measured from peak to steady state at each voltage step.

SERCA-2 Probe Synthesis and Northern Blot Analysis

An 840-bp fragment of SERCA-2 (GenBank accession No. X02814.1) containing the downstream end of the coding region and part of the 3’ untranslated region of rabbit SERCA-2 cDNA was subcloned into the pCR-TM II vector (Invitrogen). Total RNA was extracted from fresh LV tissue, separated by electrophoresis, and transferred onto nylon membranes. Blots were hybridized in the presence of the α-³²P–labeled SERCA-2 cDNA probe. For loading and transfer control, the blots were rehybridized with a α-³²P–labeled 1.2-kb fragment of mouse 18S rRNA gene (Ambion, catalog No. 7328).

SERCA-2 Western Blot Analysis

Homogenates of frozen LV tissue were subjected to 7.5% SDS-PAGE for SERCA-2 immunoblotting, transferred to PVDF membranes (catalog No. IPVH09120, Millipore), blocked for 1 hour with 5% nonfat dry milk, and incubated for 2 hours with an anti–SERCA-2 monoclonal antibody (catalog No. MA3-910, Affinity BioReagents, Inc) at 1:2500 dilution. After washing, filters were incubated with 1:10 000 dilution of the secondary antibody for 60 minutes.

SERCA-2 Activity

Thapsigargin (TSG)-sensitive ⁴⁰Ca uptake was measured in microsomes prepared from LV myocardium. Fresh LV tissue was homogenized and centrifuged at 4000g for 5 minutes, and the postnuclear supernatant was collected for assay. ⁴⁰Ca⁺ (1 μCi) was added to Ca⁺⁺ uptake incubation medium (500 μL) containing ATP (2 mmol/L) and oxalate (10 mmol/L) with and without TSG (1 μmol/L) at 37°C and 200 μg protein and incubated at 37°C for 0, 10, 30, and 60 minutes followed by scintillation counting.

MHC Isoform Expression

After reverse transcription of mRNA isolated from fresh LV tissue, forward (5’-GCCAAGGGTGAAAGGAGTGA3’) and reverse (5’-CTCTCTCTCAGGTCACTCAG-3’) primers were used to amplify MHC-α and MHC-β cdNA (Accession No. S62056 and Z34886, respectively). After complete digestion of the polymerase chain reaction product with HindIII, fragments of the amplification product (lengths, 660 bp for α-MHC and 460+200 bp for β-MHC) were separated on 2% agarose gel, and the ratio of β- to α-MHC fragments was quantified by densitometry.

Membrane and Blood Cholesterol Measurements

Whole-cell homogenates from both control and diet animals were subjected to lipid extraction by standard methods. Free cholesterol was quantified by gas-liquid chromatography, and phospholipid mass was assessed with a phospholipid phosphorus assay. In addition, purified myocyte sarcolemmal membranes were isolated at the beginning and end (day 70) by cell disruption and differential ultracentrifugation. The membranes were assayed for cholesterol and phospholipid content and expressed as the free cholesterol/phospholipid (FC/PL) mole ratio. Lastly, blood cholesterol levels were measured by gas-liquid chromatography.

Data Analysis

A total of 48 animals were used in this study (20 diet and 28 controls). For contractile data, n=6 consecutive contractions were analyzed from a single cell, and 3 to 6 cells were averaged to provide an n=1 for each animal. Data are shown as the mean±SEM, and the data between groups were tested for significance with a nonpaired Student t test or ANOVA followed by a Bonferroni correction when appropriate. The null hypothesis was rejected at a value of P<0.05.

Results

Contractile Dysfunction Is Associated With Dietary Hypercholesterolemia (n=22 and 32 Cells From 8 Control [C] Versus 9 Diet [D] Animals)

Significant contractile alterations were found in the diet myocytes compared with the control group. Figure 1A illustrates typical tracings obtained from cells isolated from control and test animals. Although the magnitude of contraction was similar between the 2 groups (9.1±0.3% versus 9.3±0.3% resting cell length, C versus D), there was a 27% decrease (P<0.01) in the maximum rate of shortening (+dL/dt) and a 25% decrease (P<0.05) in the rate of relaxation (−dL/dt) (Figure 1B). The decrease in the rate of shortening was accompanied by a 60% increase in the time to peak (0.276±0.020 versus 0.453±0.18 seconds, C versus D; P<0.001). The decrease in the rate of relaxation was accompanied by a 34.8% increase in the time to relaxation (T₉₀) in the diet group (0.290±0.012 versus 0.391±0.091 seconds, C versus D; P<0.01).

Only minor differences occur in action potentials (n=22 and 32 cells from 8 C versus 9 D animals) and calcium currents (n=22 and 32 cells from 8 C versus 9 D animals). Action potential durations were slightly shorter (3% to 8%) in the cells from the diet animals at the 25%, 50%, and 75% repolarization points but were not statistically different between the 2 groups (data not shown). There was, however, a small (2.6-mV) but significant difference in the resting membrane potential (−76.1±0.4 versus −73.5±0.3 mV; P<0.05, C versus D) and action potential amplitude (5.0 mV).
(124.9±1.0 versus 119.9±0.8 mV; P<0.05, C versus D). For calcium currents, the peak current density (normalized for capacitive surface area) was smaller in the diet group, but these differences were not statistically significant at any voltage except -10 mV (P<0.05) (data not shown).

SERCA-2 mRNA, Protein, and Activity Are Suppressed by Dietary Hypercholesterolemia

Because the reduction in systolic and diastolic function in the diet group was not accompanied by remarkable alterations in membrane calcium currents, we measured SERCA-2 mRNA and protein levels in the control and diet cells. A decrease in SERCA-2 expression of 17% approached significance (P=0.054) 4 days after the initiation of cholesterol feeding when the blood cholesterol levels were ~295±31 mg/dL and became significant at 8 days (21.5%), decreasing further to 31% by 10 weeks (P<0.05) (Figure 2A). Unlike mRNA levels, SERCA-2 protein levels remained unchanged through 16 days but were significantly reduced (40%; P<0.05) by day 70 (Figure 2B). In addition, at day 70, SERCA-2 activity (TSG-sensitive 45Ca2+ uptake) was reduced on average by 50% (P<0.05 to 0.01) (Figure 2C).

MHC-α and MHC-β mRNA Expression Levels Are Altered by Dietary Hypercholesterolemia

Because of the close similarity of the MHC-α and MHC-β isoforms (molecular mass difference <0.2%), separation of these proteins by Western blot has been problematic, especially in rabbits. Accordingly, we used a novel strategy in which primers for the MHC mRNA were designed that flanked a sequence difference between the α and β isoforms that contained a recognition site for HincII on MHC-β. After

Figure 1. A, Typical tracings from myocytes isolated from left ventricle of control and cholesterol-fed rabbits. B, Myocytes isolated from cholesterol-fed rabbits display impaired systolic shortening (open bars) measured as rate of decrease in resting cell length on stimulation. Similar impairment is seen in diastolic shortening (hatched bars) measured as rate of increase in cell length on relaxation. Data are expressed as mean±SEM; P<0.05.

Figure 2. A, SERCA-2 mRNA levels assessed from Northern blots decreased within 4 days after initiation of cholesterol feeding and fell to ~30% from days 8 through 70. B, SERCA-2 protein levels assessed from Western blots remaining unchanged over first 16 days on cholesterol diet and fell ~55% by day 70. n=3 animals per time point. C, Time-dependent SERCA-2 activity assessed with a TSG-sensitive 45Ca uptake technique into LV SR was decreased ~50% after 70 days on high-cholesterol diet. n=3 control and 3 diet animals Differences between time points were tested with ANOVA with a post hoc Bonferroni correction. Data are mean±SEM; P<0.05 to 0.01.
Animals, C Versus D)

Combining the 2 MHC/H9252 extracts accurately reflects plasma membrane cholesterol levels (Figure 4). The FC/PL molar ratio in tissue in the diet animals that paralleled the increase in blood rise in membrane cholesterol content between days 0 and 16 days 0, 4, 8, 12, 16, and 70 days demonstrated a progressive addition, membrane lipid analyses of LV homogenates at restrictions digest with HincII, transcripts of 660 bp (MHC-α) and 460 and 200 bp (MHC-β) were obtained (Figure 3A). Combining the 2 MHC-β fragments, we found a 5-fold increase in the ratio of LV MHC-β to MHC-α mRNA (Figure 3B) after 70 days of cholesterol feeding.

Cardiac Sarcolemma Enriches With Cholesterol as Serum Cholesterol Levels Increase

Analysis of purified cardiac sarcolemmal membranes isolated from fresh LV tissue obtained from control and cholesterol-fed (70 days) animals revealed a significant increase (1.74-fold; P<0.05) in unesterified (free) cholesterol content, expressed as the FC/PL mole ratio (0.270±0.037 FC/PL, C versus D; n=3 to 6). The total membrane phospholipid content did not differ between the 2 groups (333.1±62.0 versus 319.6±67.9 μg, C versus D). In addition, membrane lipid analyses of LV homogenates at days 0, 4, 8, 12, 16, and 70 days demonstrated a progressive rise in membrane cholesterol content between days 0 and 16 in the diet animals that paralleled the increase in blood cholesterol levels (Figure 4). The FC/PL molar ratio in tissue extracts accurately reflects plasma membrane cholesterol content.12,13,15

Myocardial Hypertrophy Does Not Occur With Dietary Hypercholesterolemia (n=17 and 13 Animals, C Versus D)

The heart weight (8.7±0.2 versus 9.1±0.4 g, C versus D), body weight (3.3±0.05 versus 3.5±0.14 kg, C versus D), and heart weight-to-body weight ratios (2.62±0.07 versus 2.57±0.08 mg/kg, C versus D) were similar in the 2 groups. At the cellular level, the capacitive surface area, an index of cell size, was also similar in the 2 groups (81.9±7.5 versus 81.2±7.7 pF, C versus D). These data are consistent with the conclusion that myocardial hypertrophy does not occur in this model.

Hypercholesterolemia Does Not Alter Aortic Pressure (n=17 and 13 Animals, C Versus D)

The mean arterial pressure, systolic and diastolic pressures, pulse pressure, and heart rate were also not altered in the diet group.

Discussion

In this report, we describe observations demonstrating that diet-induced hypercholesterolemia impairs systolic and diastolic function. Although the absolute magnitude of cell shortening was not affected, the rate of contraction and rate of relaxation were significantly suppressed. The contractile changes associated with cholesterol feeding are similar to those seen in models of myocardial hypertrophy16 but without the accompanying hypertrophy or hemodynamic overloading. In previous studies, we found that dietary hypercholesterolemia altered contractile function in arterial smooth muscle cells (SMCs), which was mediated by cholesterol enrichment of the SMC plasma membrane and alterations in membrane calcium permeability.13,17 Accordingly, we examined membrane lipid composition and calcium currents in LV myocytes. Like the SMCs, we found that the cardiac sarcolemmal membranes became enriched with cholesterol during the development of serum hypercholesterolemia. Specifically, the membrane FC/PL mole ratio increased 74% over the course of 10 weeks on the high-fat diet. Estimates of membrane cholesterol content at shorter time points indicated that membrane cholesterol content virtually paralleled blood cholesterol levels (Figure 4), elevating after only 4 days of cholesterol feeding when blood cholesterol levels were ~300 mg/dL. In SMCs12,13 in vivo and macrophages in culture,18 as well as in synthetic membranes, similar degrees of cholesterol enrichment result in a marked increase (up to 20%) in

Figure 3. A, Reverse transcription–polymerase chain reaction of MHC transcripts from normal and diet LV tissue before (top) and after (middle) restriction digestion with HincII. Decreased expression of MHC-α and increased expression of MHC-β transcripts are seen in diet tissue compared with control (N) tissue for each of 3 animals. B, Summarizing expression levels for MHC-α and MHC-β reveals a 5-fold increase in ratio of MHC-β to MHC-α.

Figure 4. Temporal relationship between blood and LV myocyte sarcolemmal cholesterol levels and LV SERCA-2 expression over course of study period reveals parallel relationship between blood and membrane cholesterol content and their reciprocal relationship with LV SERCA-2 levels. Data are expressed as mean±SEM; n=3 control and ≥3 diet animals.

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membrane bilayer width.\textsuperscript{12} We have suggested that this swelling effect of excess cholesterol on the cell membrane is linked to a variety of alterations in membrane protein activity, including reduced adenylate cyclase,\textsuperscript{19} Na/K-ATPase,\textsuperscript{13} and alkaline phosphatase\textsuperscript{20} activities, and may underlie the changes we observed in the present study. In addition, cholesterol enrichment may alter membrane caveolae and/or caveolin activity, as shown in other cell systems.\textsuperscript{21} Because caveolae are cholesterol-rich membrane microplatforms heavily involved in cell signaling,\textsuperscript{22} this possibility deserves further attention. The alterations in membrane lipids were accompanied by only minor electrophysiological alterations that were not altered sufficiently by cholesterol feeding to explain the functional alterations observed. This is in contrast to SMCs, and the basis for the differences between cardiac and SMC responses to cholesterol enrichment are not clear.

In the absence of altered membrane calcium currents, we hypothesized that the decreased rate of contraction and relaxation in cardiac myocytes of the diet group resulted from an alteration in intracellular calcium handling. We found that SERCA-2 mRNA levels were reduced by 17\% after only 4 days of cholesterol feeding and continued to decline to 31\% by day 70 (Figure 2A). Hence, the effect of hypercholesterolemia on SERCA-2 expression was surprisingly rapid. Interestingly, the SERCA-2 mRNA level was reciprocally related to the increase in membrane and blood cholesterol levels over the course of the cholesterol feeding period (Figure 4), suggesting a role for increased membrane cholesterol content in mediating the decrease in SERCA-2 message. SERCA-2 protein levels, however, were unchanged through 16 days on diet but fell by day 70, consistent with the explanation that SERCA-2 protein half-life is longer than that of SERCA-2 message. The reductions in SERCA-2 mRNA and protein content were accompanied by a 50\% decrease in SERCA-2 activity. Thus, these alterations could account for the altered contractile function observed in the diet cells. In this scenario, sarcoplasmic reticulum (SR) calcium uptake would be reduced, leading to reduced relaxation rate and reduced SR calcium content and therefore reduced SR calcium release and decreased shortening velocity.\textsuperscript{23,24} There is a growing body of evidence implicating alterations in SERCA-2 expression in heart failure.\textsuperscript{25} In addition, transgenic mice overexpressing SERCA-2 show decreased mortality and preserved myocyte function even in the presence of hemodynamic overload.\textsuperscript{26} It has been suggested that down-regulation of SERCA-2 activity is accompanied by a switch from the \( \alpha \) to the \( \beta \) isofrom of MHC, a switch that would be expected to impair contractile mechanics.\textsuperscript{27,28} Consistent with this notion, we demonstrate an isoform shift away from the \( \alpha \-) and toward the \( \beta \-) MHC isofrom that accompanied suppressed SERCA-2 mRNA, protein, and activity, and the appearance of myocyte dysfunction induced by the cholesterol-rich diet. Accordingly, the alterations in contractile function observed may have resulted from the combined effects of altered calcium handling and altered MHC expression. In this regard, it is noteworthy that these alterations in cardiac function associated with changes in SERCA-2 expression, activity and MHC isoforms are similar to myocyte alterations associated with aging.\textsuperscript{29}

Impaired cardiac function associated with serum hypercholesterolemia may have important implications. For example, Wu et al\textsuperscript{30} recently reported that elevated aortic afterload accelerated ventricular dysfunction in hypercholesterolemic apolipoprotein E–knockout mice compared with their wild-type controls. In their study, a decline in ventricular function over a period of 8 weeks in apolipoprotein E–knockout mice without elevated afterload failed to achieve statistical significance, but when coupled to elevated afterload, failure was much more rapid than in mice with normal cholesterol levels.

In light of their observations, we suggest that hypercholesterolemia may sensitize the myocardium to the additional insults (eg, hemodynamic overloading, myocardial ischemia, diabetes) by altering the membrane lipid bilayer and disturbing intracellular calcium handling and MHC isoform expression patterns.

Whether these changes occur in humans during serum hypercholesterolemia is not clear at this time. However, Wang et al\textsuperscript{31} reported a positive correlation between serum HDL levels and LV ejection fractions in human subjects with serum hypercholesterolemia (225 mg/dl) even in the absence of angiographic evidence of CAD. Because HDL functions in reverse cholesterol transport, low HDL levels would be expected to be associated with elevated tissue cholesterol levels. In addition, in postmenopausal women with moderate hypertension, a significant positive correlation between total and LDL cholesterol levels and impaired diastolic function and a negative correlation between HDL levels and diastolic dysfunction have been observed.\textsuperscript{32} Considering that >40\% of hypercholesterolemic patients have hypertension,\textsuperscript{33} the occurrence of this diet-induced cardiomyopathy in the human population could have dramatic consequences. These findings also may help to explain the rapid rise in the incidence of heart failure.

Acknowledgments

This project was supported in part by National Institutes of Health grants P01-HL-07443, R01-HL-66273, and R01-HD-40284 (Dr Tulenko), R01-HL-068657 (Dr Narula), and R01-HL033921 and R01-HL-061495 (Dr Houser).

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*Circulation.* 2004;109:97-102; originally published online December 15, 2003;
doi: 10.1161/01.CIR.0000109213.10461.F6
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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