Dyscholesterolemia Protects Against Ischemia-Induced Ventricular Arrhythmias

Running title: Baartscheer et al.; Dyscholesterolemia and ventricular arrhythmias.

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Abstract:

**Background** - Hypercholesterolemia protects against ventricular fibrillation in patients with myocardial infarction. We hypothesize that hypercholesterolemia protects against ischemia induced re-entrant arrhythmias due to altered ion channel function.

**Methods and Results** - ECGs were measured in LDL-receptor (LDLr-/-), ApoA1 (ApoA1-/-) knockout and wild type mice (WT). Action potentials (AP), calcium handling and ion currents were recorded in ventricular myocytes. Gene expression was determined by qPCR and Western blot. In isolated perfused hearts regional ischemia was induced and arrhythmia inducibility was tested. Serum LDL cholesterol was higher in LDLr-/- mice than in WT (2.6 vs 0.4 mM) and HDL cholesterol was significantly lower in ApoA1-/- mice than in WT (0.3 vs 1.8 mM). LDLr-/- and ApoA1-/- myocytes contained more cholesterol than WT (34.4±2.8 and 36.5±2.4 versus 25.5±0.4 µmol/gr protein). The major potassium currents were not different in LDLr-/- and ApoA1-/- compared to WT mice. The L-type calcium current (I_{Ca}) however, was larger in LDLr-/- and ApoA1-/- than WT (12.1±0.7, 12.8±0.8 versus 9.4±1.1 pA/pF). Calcium transient amplitude and fractional SR calcium release were larger and AP and QTc duration longer in LDLr-/- and ApoA1-/- than WT mice (APD_{90}: 102±4, 106±3 vs 84±3.1 ms; QTc: 50.9±1.3, 52.8±0.8 vs 43.5±1.2 ms). During ischemia, VT/VF inducibility was larger in WT than LDLr-/- and ApoA1-/- hearts. Sodium channel and Ca-handling genes were not significantly different between groups.

**Conclusions** - Dyscholesterolemia is associated with AP prolongation due to increased I_{Ca} and reduces occurrence of re-entrant arrhythmias during ischemia.

**Key words:** arrhythmia; ischemia; cholesterol; ion channel; calcium
Introduction

Hypercholesterolemia is associated with high plasma levels of low-density lipoprotein (LDL) cholesterol and low levels of high-density (HDL) cholesterol. Hypercholesterolemia is a major risk factor for cardiovascular disease and can result in atherosclerosis and development of myocardial infarction (MI)\(^1\).

In a study by Dekker et al. it was demonstrated that in a patient group with acute MI followed by ventricular fibrillation (VF), hypercholesterolemia was significantly less present than in the patient group with acute MI without VF. This suggests that hypercholesterolemia protects against primary VF in patients with a myocardial infarction\(^2\).

The lipid composition of the myocyte membrane is an important modulator of ion channel function and thus of action potential configuration, and has important implications for the genesis of cardiac arrhythmias\(^3\),\(^4\). For example, a diet rich in fish oil shortens the ventricular action potential in the absence of circulating fish oils\(^5\). Also, LDL lowering therapy is associated with a shortening of the QT interval in patients with advanced chronic heart failure\(^6\), and infusion of rHDL causes a shortening of the QT-interval in patients with hypercholesterolemia\(^7\). On the other hand, in obese patients with high levels of cholesterol\(^8\), the electrocardiographic QT segment is prolonged, which may protect them from primary ventricular fibrillation.

Prolongation of the QT segment is a result of prolongation of the ventricular action potential caused by altered ion channel function and may provoke arrhythmias caused by triggered activity\(^9\), but may antagonize (ischemia-induced) re-entrant arrhythmias\(^10\). Indeed, in an animal model of heart failure\(^11\), a diet rich in fish oil protected against arrhythmias caused by triggered activity based on early after-depolarizations, but facilitated the occurrence of re-entrant arrhythmias\(^12\).
We surmise that cholesterol directly modulates cardiac electrophysiology and arrhythmogenesis in the absence of structural abnormalities resulting from atherosclerosis. We thus hypothesize that both increased levels of serum LDL and decreased levels of serum HDL cholesterol lead to increased membrane cholesterol, prolong the ventricular action potential duration and thereby protect from re-entrant arrhythmias (during acute myocardial ischemia).

Methods

This study was approved by the local institutional ethical committee. Animal care and handling conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Design of the study

We used two validated dyslipidemic mouse models without structural cardiac changes and atherosclerosis: 1) LDL receptor knock mouse (LDLr-/-) that recapitulates human patients with elevated serum LDL cholesterol\(^{13}\) and 2) the Apolipoprotein A1 knock out mouse (ApoA1-/-) mice) that recapitulates human patients with decreased serum HDL cholesterol levels\(^{14}\). Wild type mice (WT) were used as control. Male and female ApoA1-/- (B6;129P2-ApoA1lmm1umc/J), LDLr-/- (B6;129S7-LDLrtm1Her/J) deficient mice and WT mice (C57BL/6J) were purchased from Jackson Laboratories. The dyslipidemic mouse strains had been backcrossed to C57BL/6J mice for 10 generations. Only 10 week-old males were used for experiments.

The animals were anesthetized by isoflurane inhalation (0.8-1.0% volume) and a 6 lead surface ECG was measured. Then the mice were killed by cervical dislocation. The chest was opened and an incision was made in the aorta to collect blood for plasma cholesterol analysis. The heart was excised and used for either expression or functional studies.

Isolation of left ventricular myocytes was performed as described previously\(^{15}\). Myocytes

**ECG measurements**

Surface ECG’s were recorded using subcutaneous 23-gauge needle electrodes attached to each limb. Lead I was analyzed for heart rate, PQ, QRS, and QT duration. QT intervals were corrected for heart rate using the formula: $QTc = QT/((RR/100)^{1/2})$ with RR in msec. which is a mouse equivalent of Bazett’s formula.$^{16}$ Criteria for QRS duration were applied according to Boukens et al.$^{17}$

**Plasma cholesterol and sphingolipid content in left ventricular myocytes**

Plasma total and HDL-cholesterol were measured by enzymatic colorimetric spectrophotometry. LDL cholesterol was calculated with the Friedewald formula.$^{18}$

Tissue cholesterol and sphingolipids were measured in myocytes as described previously.$^{19}$ Myocytes from two hearts were pooled for reasons of sensitivity.

**[Ca$^{2+}$]$_i$ and SR calcium content**

[Ca$^{2+}$]$_i$ transients and SR calcium content were measured in left ventricular myocytes using Indo-1 as described previously.$^{20}$ [Ca$^{2+}$]$_i$ transients (6 Hz) were measured at $37^\circ$C using field stimulation and SR calcium content was estimated from the response of [Ca$^{2+}$]$_i$ to rapid cooling. Fractional SR calcium release was estimated from the ratio of [Ca$^{2+}$]$_i$ increase during a calcium transient and during rapid cooling.$^{21}$ (see supplemental material).

**Inducibility of arrhythmias during ischemia in Langendorff-perfused hearts**

Inducibility of arrhythmias was tested in WT, LDLr/- and ApoA1/- hearts (25, 20 and 19 hearts respectively). After cannulation of the aorta, hearts were allowed to recover for 15 minutes at
37°C with a solution containing (mM): NaCl 128, KCl 4.7, CaCl₂ 1.45, MgCl₂ 0.6, NaHCO₃ 27, NaH₂PO₄ 0.4, and glucose 11, which was gassed with 95% O₂ and 5% CO₂ (pH 7.4). A 30 minutes period of regional ischemia was induced by ligation of the left anterior descending (LAD) artery (1mm beneath its origin). From the last 5 minutes of the control period onward, the hearts were stimulated via a bipolar pacing electrode placed on the right ventricle with a basic cycle length of 120 msec. (1 msec pulse duration, twice diastolic stimulation threshold).

Electrograms were recorded via a unipolar electrode placed on the left ventricle, with a reference electrode placed on the cannula. The inducibility of arrhythmias during ischemia was tested every 30 sec (60 attempts/heart) by applying a train of 3 shortly coupled stimuli set 10 msec beyond the corresponding refractory period, followed by a pause of 500 msec (timing of the stimuli was determined prior to ischemia). The number and type of spontaneous arrhythmias following the three premature beats were documented. VT was defined as a spontaneous rhythm occurring with a cycle length below 90 ms containing regular monomorphic sequences. VF was defined as a spontaneous polymorphic irregular rhythm. When VF occurred, stimulation was stopped, and 20 seconds were allowed for the spontaneous termination of the arrhythmia. If the arrhythmia did not terminate spontaneously, the heart was defibrillated by an injection of ice-cold Tyrode’s solution. Then the heart was allowed to recover for 2 minutes and the stimulation protocol was resumed.

To determine the volume of the ischemic tissue the heart was perfused after the experiment with 0.2% Evans Blue and stored in 4% buffered formalin after which the hearts were embedded in 4% agarose and cut into 5 transversal slices for microscopic analysis. The relative ischemic volume (RIV%) was calculated by dividing the cumulated area of the ischemic tissue by the left ventricular cumulative area.
Cellular electrophysiology

Action potentials (AP) and sarcolemmal ion currents were recorded in left ventricular myocytes at 36±0.2°C with the amphotericin-B-perforated patch-clamp and ruptured patch-clamp technique respectively\(^5,22\).

APs were measured using solution A. Pipette solution contained (in mM): K-gluconate 125, KCl 20, NaCl 10, amphotericin-B 0.22, HEPES 10; pH 7.2 (KOH). APs were elicited at 6 Hz by 3-msec., 1.2x threshold current pulses. Data from 10 APs were averaged. The upstroke velocity of the AP was used to measure cardiac sodium current \((I_{Na})\)^\(^{23}\).

L-type Ca\(^{2+}\) current \((I_{Ca,L})\) and K\(^{+}\)-currents were measured by voltage-clamp. Current densities were calculated by dividing current amplitudes by cell membrane capacitance. Cycle length was 3 and 10 s for \(I_{Ca,L}\) and K\(^{+}\) currents, respectively. Extracellular solution for \(I_{Ca,L}\) measurements contained (mM): TEA-Cl 145, CsCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1.0, glucose 5.5, HEPES 5.0; pH 7.4 (NMDG-OH) and 0.2 mmol/L 4,4\textquoteleft diisothiocyanatostilbene-2,2\textquoteleft-disulfonic acid to block the Ca\(^{2+}\)-activated Cl\(^{-}\) current. Pipette solution for \(I_{Ca,L}\) measurements contained: CsCl 145, K\(_2\)-ATP 5.0, EGTA 10, HEPES 10; pH 7.2 ((NMDG)-OH). Extracellular solution for K\(^{+}\)-currents measurements was solution A with 0.3 mM CdCl\(_2\) to inhibit Ca\(^{2+}\)- and Na\(^{+}\)- currents; pipette solution for K\(^{+}\)-currents measurements contained (mM): K-gluconate 125, KCl 20, NaCl 10, MgATP 5.0, EGTA 10 HEPES 10; pH 7.2 (KOH). Outward K\(^{+}\) current has been shown to consist of different components\(^{22}\). Steady-state currents negative to −40 mV were defined as inward rectifier K\(^{+}\)-current \((I_{K1})\) and steady-state currents positive to −40 mV as the non-inactivating K\(_V\)-current \((I_{ss})\). The decay phases of the transient currents activated upon depolarization described the sum of two exponentials, reflecting the inactivating currents \(I_{so,f}\) and \(I_{K,slow}\)\(^{22}\) (see supplemental material).
Expression

Quantitative RT-PCR: 1 μg of RNA isolated from flash-frozen apex was reverse-transcribed using Oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed in 384-well plates using SYBR Green I master solution (Roche) and the LightCycler 480 (Roche). PCR amplification was performed in a total reaction volume of 10 μl, consisting of 1 μM forward and reverse primers, 2 μl cDNA, 5 μl 2x SYBR Green. The PCR was cycled between 95 °C/30 s and 60 °C/30 s for 50 cycles, following an initial denaturation step at 95 °C for 5 min. Primers were specific for mouse sequences and designed as described in detail before. Transcript quantities were normalized to the amount of endogenous control (Hprt and Gapdh) and analyzed using LinRegPCR software. (see supplemental material).

Western blot analysis; Heart lysates were extracted from flash-frozen mouse hearts in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) as described previously. (see supplemental material).

Statistics

Statistical analysis was carried out with SigmaStat 3.5 software and data are presented as mean±S.E.M. Normality and equal variance assumptions were tested with the Kolmogorov-Smirnov and the Levene median test, respectively. Groups were compared using One-Way ANOVA, or Z-test when appropriate, and for I-V relationships by Two-Way Repeated Measures (RM) ANOVA followed by the Student-Newman-Keuls post hoc test. P<0.05 was considered statistically significant. The results obtained with the above procedure are consistent with a nonparametric analysis (Kruskall Wallis).

Results

Figure 1 shows plasma cholesterol (panel A) and the cholesterol and spingolipid content in left
ventricular myocytes (panel B). The plasma content of LDL cholesterol was increased in LDLr-/- mice and the HDL cholesterol was decreased in ApoA1-/- mice compared to WT mice. This was associated with an increase of the cholesterol and a decrease of the sphingolipid content in ventricular myocytes of both LDLr-/- and ApoA1-/- compared to WT mice (figure 1B). The alteration in cholesterol content did not change body weight, the heart weight/body weight ratio or the cellular capacitance(figure 1C).

Figure 2 shows typical examples of lead I surface ECG recordings (Figure 2A) and action potentials (6 Hz) and the corresponding dV/dt in left ventricular myocytes (Figure 2B). Overall, the QTc interval was significantly increased in both LDLr-/- and ApoA1-/- compared to WT mice (Figure 2C, left panel). We did not detect differences in RR interval, PQ interval and QRS duration. In line with the increased QTc interval action potential duration at 90 % of repolarization (APD$_{90}$) was prolonged in both LDLr-/- and ApoA1-/- compared to WT mice (Figure 2C, middle panel, pacing frequency 6 Hz). The upstroke velocity of the action potential in both LDLr-/- and ApoA1-/- was about 30% decreased compared to WT mice (Figure 2C, right panel) without a difference in resting membrane potential and the amplitude of the action potential.

Figure 3A shows typical examples of arrhythmias induced by 3 shortly coupled premature stimuli during regional ischemia (trace a: single spontaneous premature beat, trace b: ventricular tachycardia (VT), trace c: ventricular fibrillation (VF)). The prevalence of arrhythmias in hearts from the three groups of mice are shown in Figure 3B. In all 3 groups, practically all hearts displayed one or more episodes of spontaneous premature beats. However, the percentage of hearts displaying one or more episodes of VT or VF was significantly lower in dyscholesterolemic mice than in WT mice. The total occurrence of arrhythmias (1 or 2 premature
beats and VT/VF) was not statistically different between the groups: % of attempts
WT: 17.1±2.8, LDLr-/-: 18.2±3.1 and ApoA1-/-: 19.3±2.5). Figure 3C shows the incidence of one
or two premature beats after 3 shortly coupled stimuli during subsequent 10 minutes of ischemia,
as well as the incidence during the entire 30-minute ischemic period. The incidence of
extrasystoles was higher in the LDLr-/- and ApoA1-/- hearts compared to of WT hearts. In
contrast, the incidence of tachycardia or VF (Figure 3D) was significantly lower in both LDLr-/-
and ApoA1-/- hearts compared to WT hearts.

Table 1 shows characteristics of periods of VT and VF. The periods of VT in hearts of
WT mice have significant longer duration and higher frequency (shorter cycle length) compared
to LDLr-/- and ApoA1-/- hearts. VF duration was longer in WT compared to LDLr-/- and
ApoA1-/- hearts, but this did not reach significance because in hearts of the latter 2 groups only 1
period of VF was present. In the WT hearts 3 out 18 periods of VF were defibrillated after the
maximum allowed duration (20 sec).

The relative volume of the ischemic tissue after ligation of the LAD was not different
between groups (31.2±0.9, 28.9±0.7 and 29.7±0.9% for WT, the LDLr-/- and ApoA1-/- mice
respectively.

We next measured the major currents active during the plateau and repolarization phases
of the action potential to elucidate the mechanism underlying the longer action potentials in
LDLr-/- and ApoA1-/- mice. $I_{Ca,L}$ was investigated using a two-step protocol (Figure 4A)
consisting of series of depolarizing pulses (P1) to activate $I_{Ca,L}$ followed by a second
depolarization to 0 mV (P2) to establish the voltage dependency of inactivation. Figure 4B
shows representative $I_{Ca,L}$ recordings of WT, ApoA1-/- and LDLr-/- mice upon depolarizing
pulses to 0 mV. The average current-voltage (I-V) relationships of $I_{Ca,L}$ (Figure 4C) reveals that
ICa,L was larger in ApoA1-/- and LDLr-/ mice, than in WT mice, amounting to a difference of approximately 30%. Activation nor inactivation properties were different between ApoA1-/-, LDLr-/ and WT mice (Figure 4D). The decay of ICa,L (fitted with a double exponential function) was similar for the 3 groups (data not shown).

K+ currents were measured during 5 sec hyper- and depolarizing voltage clamp steps from a holding potential of –80 mV (voltage protocol Figure 5A). Figure 5B shows representative current recordings in response to hyperpolarizing pulses to –120 mV and depolarizing pulses to 50 mV in WT, ApoA1-/-, and LDLr-/ mice. Mean densities of steady-state currents, reflecting the inward rectifier current (IK1) and the non-inactivating Kv current (I_{SS}), were similar for the 3 groups (Figure 5C). The mean density of the total peak transient current (Figure 5D) was also not significantly different for ApoA1-/- and LDLr-/ mice versus WT myocytes.

To establish the effects of hypercholesterolemia on intracellular calcium handling, we investigated [Ca2+]i transients and the response of [Ca2+]i upon rapid cooling (RC) in 6 Hz stimulated isolated ventricular myocytes (Figure 6A). In the LDLr-/- and ApoA1-/- mice the mean amplitude of the calcium transient was significantly increased compared to WT, without a significant change of diastolic calcium (figure 6B). Also, time to peak and the 80% recovery were similar in the 3 groups (data not shown). Moreover, the [Ca2+]i rise in response to RC was similar in LDLr-/- and ApoA1-/- mice as compared to WT mice, indicating no significant differences in SR calcium content (Figure 6B, middle panel). As a result, the fractional SR calcium release is significantly increased in the LDLr-/- and ApoA1-/- mice as compared to WT (Figure 6B, right panel). Of note, with the given the sample sizes, the minimum difference in SR content we could detect between the WT group and the LDLr-/- or the ApoA1-/- group, was 9%
and 14% resp. (power analysis).

In myocytes from LDLr-/- mice we applied 1 μM verapamil to decrease the L-type calcium current with 31.2%±1.2. Table 2 shows that the effect of such a decrease results in almost complete normalization of action potential duration and calcium transient amplitude, similar to values of WT mice, without significant effects on diastolic calcium and the amplitude of the calcium increase after rapid cooling.

To have an indication whether the increased calcium transient resulted from altered expression levels of membrane proteins, we measured mRNA levels. Figure 7A shows the averaged data (expressed as ratio to Gapdh and normalized to WT) in ApoA1-/-, and LDLr-/- and WT mice. No significant difference of relative mRNA levels of Caveolin-3, L-type Ca2+-channel, the cardiac Na+-channel or of the calcium handling proteins (SERCA2A, NCX and RyR channels) were observed between the transgenic and WT mice. Also, no difference was found when the household gene Hprt was used.

Because we did not find a difference in mRNA expression levels of the L-type calcium channels but an increased current in ApoA1-/-, and LDLr-/- mice compared to WT mice, we performed Western blot analysis of the L-type calcium channels. Figure 7B shows a typical example of a Western blot (top panel) and the average data: protein-signal densities were normalized to the corresponding Gapdh-signal densities (bottom panel). We did not find significant differences in protein level of the L-type calcium channels in ApoA1-/- and LDLr-/- mice compared to WT.

Discussion

This study shows that in two different lines of dyscholesterolemic mice, cellular cholesterol levels are increased and sphingolipid content is decreased. In both types of dyscholesterolemic
mice the ventricular action potential duration and the electrocardiographic QTc-interval were increased. Besides, there was a 25% reduction in the upstroke velocity of the action potential indicating a reduction of $I_{Na}$. Thus, the abnormal cholesterol and sphingolipid metabolism resulted in an inherent class III antiarrhythmic effect. This conclusion was supported by our observation that hearts from dyscholesterolemic mice were relatively protected against ischemia-induced ventricular tachy-arrhythmias during acute regional myocardial ischemia. We established that the mechanism underlying the increased action potential duration was an increase of $I_{Ca,L}$. The increment in calcium current was associated with increased calcium transient amplitudes in the LDLr-/- and ApoA1-/- mice, in the presence of an unchanged loading of the SR. Notably, the cellular and organismal electrophysiologic effects were observed in the absence of circulating cholesterol. The observed changes were not associated with changes in mRNA of the L-type Ca-channel, the cardiac sodium channel and calcium handling proteins (SERCA2A, NCX and RyR channels). Neither was the protein expression of the L-type calcium channel changed in LDL-/- and ApoA1-/- mice compared to WT.

Major risk factors for cardiovascular disease include saturated fats and low-density lipoprotein (LDL) cholesterol\(^1\) (serum levels). Conversely, polyunsaturated fatty acids and high-density lipoprotein (HDL) are thought to prevent or attenuate cardiac disease processes\(^{27,28}\). High levels of cholesterol may protect from primary ventricular fibrillation in patients with a MI, as suggested by the observation that in patients with an increased risk for VF following MI, hypercholesteremia was less common than in control MI patients\(^2\). The action potential prolongation that we have documented in dyscholesterolemic mice offers an explanation for this antifibrillatory effect. Several other studies also suggest that dyscholesterolemia affects action potential duration. Obese patients with high levels of cholesterol display prolongation of the QT
interval. LDL lowering therapy with atorvastatin shortens QT segment in patients with advanced chronic heart failure and infusion of reconstituted HDL in patients with familial hypercholesterolemia induces QT-shorting.

The LDL-/- mouse line is characterized by an increased serum LDL-cholesterol, the ApoA1-/- mouse line by a decreased serum HDL-cholesterol. Although cholesterol levels are differentially affected in these mice, the electrophysiological effects of these changes were virtually the same. This suggests that the effects are the result of changes in cholesterol and sphingolipid content of the plasma membrane. The distribution of the membrane content of cholesterol and sphingolipids follows a similar pattern. The plasma membrane contains between 50 and 90% of the total cellular cholesterol and more than 50% of the total sphingolipid content. Mitochondria and the sarcoplasmic reticulum have low cholesterol and sphingolipid content, whereas the Golgi compartment contains intermediate amounts of cholesterol. In addition, it has been suggested that the cholesterol and sphingolipid content of the plasma membrane are inversely related, which is in agreement with our results and the delivering of cholesterol by LDL to membranes and the efflux of deposited cholesterol by HDL cholesterol.

Thus, unbalanced lipoprotein levels may cause cholesterol to accumulate in the plasma membrane, which also influences sphingolipid content. We cannot rule out that cholesterol and sphingolipid content is also altered in intracellular membranes, e.g. the sarcoplasmic reticulum. However, the observation that pharmacological inhibition of $I_{Ca,L}$ by about 30% in myocytes from LDLr-/- mice resulted in almost complete normalization of the action potential and calcium handling (Table 2), suggests that SR function has not changed. Moreover, the absence of alterations in body and heart weight and cell capacitance (figure 1C), indicates that cardiac remodeling has not occurred. This is in line with previous studies that report no development of
cardiac structural changes or atherosclerosis at this age in these mouse lines\textsuperscript{13,14}. Overall, these data suggest that the increased plasma membrane cholesterol levels have mediated the electrophysiological effects.

We cannot exclude the presence of microstructural changes, such as alterations in the structure of caveolae, the specialized cholesterol- and sphingolipid-enriched membrane microdomains that contain the structural protein caveolin-3. Cardiac ion channels, including L-type calcium- and Na\textsuperscript{+}-channels\textsuperscript{34}, have been associated with caveolae, which are involved in regulation of ion channel function. Nevertheless, we did not find differences in expression levels of caveolin-3.

Modulation of membrane lipid composition due to a fish oil diet has been shown to shorten the cardiac action potential and to protect from triggered activity, but to cause re-entrant arrhythmias\textsuperscript{12}, suggesting that lipid modulation of the diet has important implications for cardiac arrhythmias depending on the mechanism of the arrhythmia. We here report that when all the trigger-induced arrhythmias (i.e. the total number of VES+VT+VF) are included in the analysis of arrhythmia indelibility, no differences between groups were evident. However, although the number of spontaneous premature beats (triggers) were the same in the different groups, the incidence of VT/VF was lower, with periods of shorter duration and longer cycle length in the dyscholesterolemic mice. This implies a difference in arrhythmogenic substrate, but not in its triggers.

The 30\% reduction of the AP upstroke velocity occurred in the absence of depolarization of the resting membrane. It has been shown that AP upstroke velocity is an adequate measure of $I_{Na}$\textsuperscript{23} this finding indicates that sodium current density is decreased with about 30\% and lies at the basis of this phenomenon. This is in agreement with previously published results in a rabbit.
model of hypercholesterolemia where a 40% increase of plasma membrane cholesterol content was associated with a 38% decrease in $I_{Na}$ in ventricular myocytes, without a change of inactivation of $I_{Na}$\textsuperscript{35}. Together these data suggest that alterations of $I_{Na}$ are not the cause of the action potential and QTc prolongation.

A modest reduction of sodium current does not lead to conduction slowing in itself\textsuperscript{36} and may therefore not have influenced the class III antiarrhythmic effect of dyscholesterolemia in the conditions of our study. On the other hand, in the setting of e.g. the Brugada Syndrome, a small reduction in sodium current may provoke the typical electrocardiographic signs (right precordial ST-segment elevation) and arrhythmias in the presence of preexisting structural abnormalities\textsuperscript{37}. It has been reported that the Brugada electrocardiographic sign can be provoked by a heavy meal (full stomach test)\textsuperscript{38}. Our experimental observation may underlie the mechanism of this provocation test, although it can also be explained by increased vagal tone.

**Action potential duration**

Changes in the level of membrane cholesterol, have been shown to regulate multiple types of K\textsuperscript{+}-channels\textsuperscript{3}. We found a significant increase in $I_{Ca,L}$ to be responsible for the action potential prolongation. The increase of $I_{Ca,L}$ is consistent with augmentation of $I_{Ca,L}$ in myocytes from portal vein of rabbits with dietary atherosclerosis\textsuperscript{39} and the reduction of $I_{Ca,L}$ in skeletal muscle of mice with cholesterol depletion\textsuperscript{40}. On the other hand it has been described that cholesterol enrichment inhibits $I_{Ca,L}$ channels in gall bladder cells of guinea pigs treated with cholesterol-saturated cyclodextrin complex\textsuperscript{41} and in myocytes from conduit coronary arteries of hypercholesterolemic miniature swine\textsuperscript{42}.

**Calcium handling**

We show that the altered myocyte function was not due to altered protein expression levels,
indicating that alteration of the lipid composition of the plasma membrane directly modulates cardiac electrophysiology and arrhythmogenesis in the absence of structural abnormalities of the vessels. A variety of ion channels have been shown to be sensitive to changes in membrane cholesterol, among which ion channels that influence action potential characteristics. In our two models of dyscholesterolemia, the amplitude of the calcium transient was increased without a significant increase in SR calcium content, alteration of calcium transient characteristics or diastolic calcium. This indicates that the trigger to release calcium from the SR, i.e., the calcium influx through the L-type calcium channels, was increased. Indeed, fractional SR calcium release and the $I_{Ca,L}$ were larger in the two dyscholesterolemic mouse models. As the minimum detectable difference in SR calcium content between the WT and the LDLr-/- or ApoA1-/- groups was 9 and 14% respectively, we cannot rule out that the increase of the calcium transient was not in part due to a small increase of the SR calcium content. However, pharmacological normalization of $I_{Ca,L}$ in LDLr-/- mice (table 2) resulted in an almost complete normalization of the calcium transient amplitude, fractional SR release and action potential duration, indicating that increased $I_{Ca,L}$ is the main contributor. The observed altered calcium homeostasis is in agreement with other studies. We did not observe an increased diastolic calcium in dyscholesterolemic mice, which can be explained by a secondary increase in NCX activity following the increased amplitude of the calcium transient.

Two general mechanisms have been proposed for alteration of the lipid content of the sarcolemma of regulation of ion channels. One possibility is that cholesterol may interact directly with the transmembrane domains of the ion channels. We observed antiarrhythmic electrophysiological changes both in the presence and absence of circulating cholesterol, making direct binding a less likely mechanistic option. Another explanation is that the increased...
cholesterol content may regulate ion channels by hydrophobic mismatch between the transmembrane of domains and the lipid bilayer. In this case, a stiffer membrane will increase the energy that is required for the transition from closed to open states of the channel and thus influence the ion channel kinetics\textsuperscript{46}. These mechanisms are not mutually exclusive and our methods do not allow discrimination between these possibilities, although we were not able to detect changes in ion channel kinetics (figure 4D).

Also, we cannot entirely rule out that the subunit isoform type of ion channels and/or phosphorylation state of the myocytes are influenced by alteration of the lipid composition of the sarcolemma. We did not find alterations in decay and/or activation and inactivation characteristic of the L-type calcium channel, nor changes in the calcium transient decay and SR calcium content, two characteristics found in myocytes with an increased phosphorylation state. Therefore, changes in subunit type and/or altered phosphorylation state are not likely to be responsible for the observed changes.

**Conclusions**

Our study shows that dyscholesterolemia alters the lipid content of cardiac myocytes. This affects ion channel properties underlying $I_{Na}$ and $I_{Ca,L}$ resulting in a decrease of upstroke velocity and increased duration of the action potential and QT duration of the ECG. These electrophysiological changes resulted in reduced inducibility of lethal arrhythmias induced by acute MI. Thus, altered cholesterol homeostasis is an important modifier of cardiac electrophysiology. Change of sarcolemmal cholesterol levels (depending on the underlying pathophysiology of altered electrophysiological characteristics) without increasing plasma cholesterol levels may provide the basis of therapeutic interventions to alter cardiac electrophysiology and to reduce arrhythmogenesis especially in dyslipidemic patients.
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Conflict of Interest Disclosure: None.

References:


Table 1: Characterization of the periods of ventricular tachycardia and fibrillation

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<th>WT</th>
<th>LDLr-/- (n=16)*</th>
<th>ApoA1 (n=13)*</th>
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<tr>
<td>Duration VT (msec)</td>
<td>2590±319 (n=82)</td>
<td>647±120</td>
<td>759±147</td>
</tr>
<tr>
<td>Cycle length VT (msec)</td>
<td>50±0.8 (n=82)</td>
<td>60±0.19</td>
<td>60±3.1</td>
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<tr>
<td>Duration VF (msec)</td>
<td>6224±1389 (n=18)</td>
<td>1318 (n=1)</td>
<td>2145 (n=1)</td>
</tr>
</tbody>
</table>

*p<0.05 versus WT

Table 2: The effect of pharmacological inhibition of $I_{Ca,L}$ on action potential duration and calcium handling characteristics in LDLr-/- myocytes.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>LDLr-/- (n=10)</th>
<th>LDLr-/- 1 μM Verapamil (n=10)</th>
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<tbody>
<tr>
<td>APD$_{90}$ (ms)</td>
<td>84±3.2</td>
<td>102±4.4†</td>
<td>85±4.6*</td>
</tr>
<tr>
<td>Diastolic calcium (nM)</td>
<td>93±2.8</td>
<td>99±3.9</td>
<td>92±5.5</td>
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<tr>
<td>Amplitude Calcium transient (nM)</td>
<td>42±2.9</td>
<td>82±6.5†</td>
<td>45±2.6*</td>
</tr>
<tr>
<td>Amplitude RC (nM)</td>
<td>169±8.2</td>
<td>183±11.7</td>
<td>177±13.2</td>
</tr>
<tr>
<td>Fractional release (%)</td>
<td>27±2.3</td>
<td>50±2.8†</td>
<td>29±2.1*</td>
</tr>
</tbody>
</table>

*p<0.05 versus LDLr-/-
†p<0.05 versus WT (see also figure 6)
Figure Legends:

**Figure 1:** Lipid content in wild-type and dyscholesterolemic mice. Panel A: Plasma levels of total, LDL and HDL cholesterol in WT (n=8), LDLr-/- (n=8) and ApoA1-/- (n=8) mice. Panel B: Levels of total cholesterol and sphingolipids in left ventricular myocytes from WT (n=12), LDLr-/- (n=12) and ApoA1-/- (n=12) mice. Panel C: Body weight, heart weight/body weight ratio and myocyte capacitance WT (n=21), LDLr-/- (n=19) and ApoA1-/- (n=22). Data expressed as mean±SEM. *p<0.05 versus WT.

**Figure 2:** Electrocardiograms and action potentials in wild-type and dyscholesterolemic mice. Panel A,B: examples of surface ECG’s (lead I) and action potentials in left ventricular myocytes with the corresponding dV/dt (6 Hz). Panel C: Average data of QTc from WT (n=24), LDLr-/- (n=25) and ApoA1-/- (n=19) mice and AP characteristics: duration at 90% repolarization (APD90) and upstroke velocity; WT (n=16), LDLr-/- (n=18) and ApoA1-/- (n=12). Data expressed as mean±SEM. *p<0.05 versus WT.

**Figure 3:** Trigger-induced arrhythmias during 30 minutes of ischemia. Panel A: examples of arrhythmias after applying a train of 3 shortly coupled stimuli set followed by a pause of 500ms. Panel B: number of hearts (%) in which the attempts to induce arrhythmias resulted in at least one arrhythmia. Panel C,D: average of attempts/heart to induce arrhythmias(%) that resulted in premature beats (C) or VT/VF (D). WT (n=25), LDLr-/- (n=19) and ApoA1-/- (n=20). Data expressed as mean±SEM. *p<0.05 versus WT.
**Figure 4:** L-type calcium current in wild-type and dyscholesterolemic mice. Panel A: voltage protocol. Panel B: examples of current traces following hyperpolarizing pulses to –60 mV and depolarizing pulses to 40 mV. Panel C: Average current-voltage relation. Panel D: Activation and inactivation relationships. Data expressed as mean±SEM. *p<0.05 versus WT. WT (n=14), LDLr-/- (n=11) and ApoA1-/- (n=10).

**Figure 5:** K⁺ currents in wild-type and dyscholesterolemic mice. Panel A: voltage protocol. Panel B: examples of current traces following hyperpolarizing pulses to –120 mV and depolarizing pulses to 50 mV. Panel C. Mean current density-voltage relation of steady state current. D. Mean density-voltage relation of transient peak current. Data expressed as mean±SEM. *p<0.05 versus WT. WT (n=10), LDLr-/- (n=9) and ApoA1-/- (n=8).

**Figure 6:** Intracellular calcium handling in wild-type and dyscholesterolemic mice. Panel A: examples of [Ca²⁺]ᵢ transients and the response of [Ca²⁺]ᵢ on rapid cooling in left ventricular myocytes (6 Hz) from WT, LDLr-/- and ApoA1-/- mice. Panel B: average of diastolic calcium, calcium transient amplitude, response to RC and fractional SR calcium release (calcium transient amplitude expressed as the percentage of the responses to RC). WT (n=32), LDLr-/- (n=48) and ApoA1-/- (n=25). mean ± SEM. *p<0.05 versus WT.

**Figure 7:** mRNA and protein expression in wild-type and dyscholesterolemic mice. Panel A: mRNA mean expression: mean+/−SEM (expressed as ratio of the household gene Gapdh and normalized to WT) for WT, LDLr-/- and ApoA1-/- mice. Caveolin-3, Na-channel, NCX: Sodium Calcium Exchanger. RyR-channels: Ryanodine Receptor channels. SERCA-2A: Sarcoplasmatic
calcium pump. LTTC: L-type calcium channel. WT (n=6), LDLr-/- (n=6) and ApoA1 (n=6).
Panel B: western blot analysis of L-type calcium channel. Protein-signal densities were normalized to the corresponding Gapdh-signal densities and normalized to WT. WT (n=6), LDLr-/- (n=6) and ApoA1-/- (n=6).
Figure A shows the comparison of total cholesterol (Tot chol), low-density lipoprotein cholesterol (LDL chol), and high-density lipoprotein cholesterol (HDL chol) levels among WT, LDLr/-, and ApoA1/- mice.

Figure B illustrates the analysis of cholesterol and sphingolipids expressed as μg/gram protein.

Figure C presents data for body weight in grams (gr), heart weight/body weight in mg/gram, and myocyte capacitance in pF.
Dyscholesterolemia Protects Against Ischemia-Induced Ventricular Arrhythmias
Antonius Baartscheer, Cees A. Schumacher, Vincent Wekker, Arie O. Verkerk, Marieke W. Veldkamp, Ralph J. van Oort, Ies Elzenaar, Roelof Ottenhoff, Cindy van Roomen, Hans Aerts and Ruben Coronel

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SUPPLEMENTAL MATERIAL
**Myocyte isolation.**

Left ventricular myocytes were isolated as described previously\(^1\). Hearts were isolated and perfused via the aorta for a control period of 15 min, followed by a perfusion with low calcium Hepes solution containing: (mmol/L) [Na\(^+\)] 156, [K\(^-\)] 4.7, Ca\(^{2+}\) 0.01, [Mg\(^{2+}\)] 2.0, [Cl\(^-\)] 150.6, [HCO\(_3^-\)] 4.3, [HPO\(_4^{2-}\)] 1.4, [Hepes] 17, [Glucose] 11, Creatine 10, pH 7.3. After 10 min collagenase B (7 mg/10ml) was added to the perfusion fluid. After 30 minutes the heart was removed and cut in small pieces and put into 10 ml enzyme solution and shaken in Gyrotory waterbath shaker at 37\(^\circ\)C under continuous oxygen superfusion for 10 minutes. After the supernatant was removed 10 ml of the same solution was added now containing 1% bovine albumin (Sigma A6003 essentially acid free) and the tissue was shaken for another 10 min. Myocytes were harvested and allowed to sedimented. The supernatant was discarded and the sedimented cells were resuspended in the same solution as described above containing 1% albumin and 1.3 mM Ca\(^{2+}\) without creatine and allowed to sedimented again. Supernatant was removed and cells were stored until us in separate vials each containing 3 ml solution of the same content and about 10\(^4\) myocytes. This final suspension contained 60 to 80% rod shaped myocytes.

**Calcium measurement.**

Before each individual experiment, cells were loaded during 30 minutes with 5 \(\mu\)mol/L indo-1/AM, washed twice with fresh Hepes solution (Ca\(^{2+}\)=2.6 mM without albumin), and kept for another 15 minutes to ensure complete de-esterification. Hardware for data recording consisted of two homemade differential amplifiers for photomultiplier signals and a combined A/D and D/A board (DAS1802AO, Keithley Metrabyte) controlled by custom made software (Test-point). Loaded myocytes were attached to a poly-D-lysine (0.1 g/l) treated cover slip placed on a temperature controlled (37\(^\circ\)C) microscope stage of an inverted fluorescence microscope (Nikon Diaphot) with quartz optics. A temperature controlled perfusion chamber (height 0.4mm, diameter 10 mm, volume 30 lL, with two needles at opposite sides for perfusion purposes, was tightly positioned over the
cover slip. The content of the chamber could be replaced within 100 ms. Bipolar square pulses for field stimulation (40 V/cm) were applied through two thin parallel platinum electrodes at a distance of 8 mm. A quiescent single myocyte was selected and the measuring area was adjusted to the rod-shaped surface of the myocyte with a rectangular diaphragm. Dual wavelength emission fluorescence of Indo-1 was recorded with 1 kHz (410/516 nm, excitation at 340 nm) and corrected for fluorescent of unloaded myocytes. Free cellular calcium ([Ca^{2+}]) was calculated as described previously. The increase of [Ca^{2+}] upon rapid cooling (RC) was used to estimate SR calcium content. RC causes complete depletion of calcium from SR and calcium released remains confined to the cytoplasm. RC was performed by rapid superfusion with ice-cold Hepes solution of the same composition; low temperature (0–1°C) was reached within 200 ms.

**Cellular electrophysiology**

Quiescent rod-shaped cross-striated cells with a smooth surface were selected for measurements. APs and sarcolemmal ion currents were recorded at 36±0.2°C with the amphotericin-B-perforated patch-clamp and ruptured patch-clamp technique, respectively, using an Axopatch 200B Clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA). Voltage control, data acquisition, and analysis were accomplished using custom software. Potentials were corrected for the estimated change in liquid junction potential. Adequate voltage control was achieved with low-resistance pipettes (1.5-2.5 MΩ), and series resistance was compensated by ≥80%. Signals were filtered (low-pass, 1-kHz) and digitized (2-kHz), except for AP measurements, where filtering and digitizing frequencies were 5-kHz and 20-kHz, respectively. Cell membrane capacitance was determined as described previously.

*Current-clamp experiments.*

APs were measured using a modified Tyrode’s solution containing (mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1.0, glucose 5.5, HEPES 5.0; pH 7.4 (NaOH). Pipette solution contained: K-glucuronate 125, KCl 20, NaCl 10, amphotericin-B 0.22, HEPES 10; pH 7.2 (KOH). APs were elicited at 6-Hz by 3-ms, 1.2x threshold current pulses through the patch pipette. We analyzed resting
membrane potential (RMP), maximal upstroke velocity (dV/dt_{max}), and AP duration 90% repolarization (APD_{90}). Data from 10 consecutive APs were averaged.

**Voltage-clamp experiments.**

L-type Ca\(^{2+}\) current (I_{Ca,L}) and K\(^+\) currents were measured by voltage-clamp protocols shown in the appropriate figures. Cycle length was 3 and 10 s for I_{Ca,L} and K\(^+\) currents, respectively. Extracellular solution for I_{Ca,L} measurements contained (mM): TEA-Cl 145, CsCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1.0, glucose 5.5, HEPES 5.0; pH 7.4 (NMDG-OH). Pipette solution for I_{Ca,L} measurements contained: CsCl 145, K\(_2\)-ATP 5.0, EGTA 10, HEPES 10; pH 7.2 (NMDG)-OH). Extracellular solution for K\(^+\) measurements was Tyrode’s solution; pipette solution for K\(^+\) measurements contained (mM): K-gluconate 125, KCl 20, NaCl 10, MgATP 5.0, EGTA 10 HEPES 10; pH 7.2 (KOH). I_{Ca,L} was measured in the presence of 0.2 mmol/L 4,4’diisothiocyanatostilbene-2,2’-disulfonic acid (DIDS; Sigma-Aldrich (MO, USA) to block the Ca\(^{2+}\)-activated Cl\(^-\) current. K\(^+\) currents were measured in the presence of 0.3 mM CdCl\(_2\), which strongly inhibits Na\(^+\) currents and blocks Ca\(^{2+}\) currents, and thereby also prevents activation of the transient outward Ca\(^{2+}\)-activated Cl\(^-\) current\(^5\).

Current densities were calculated by dividing current amplitudes by C_m. Voltage-dependence of (in)activation of I_{Ca,L} was determined by fitting a Boltzmann function \((y=[1+\exp((V-V_{1/2})/k)])^{-1}\) to the individual curves, yielding half-maximal voltage \(V_{1/2}\) and slope factor \(k\). Current decay of I_{Ca,L} were fitted with a double-exponential function to obtain the time constants of the fast and the slow components of current decay: \(y=A_{f}\times\exp(-t/\tau_f) + A_s\times\exp(-t/\tau_s)\), where \(\tau_f\) and \(\tau_s\) are the time constants of fast and slow components, and \(A_f\) and \(A_s\) the fractions of the fast and slow component.

In adult mouse ventricular myocytes, total outward K\(^+\) current has been shown to consist of various different components\(^6\). We defined steady-state currents negative to \(-40\) mV as the inward rectifier K\(^+\) current (I_{K1}). Steady-state currents positive to \(-40\) mV were defined as the noninactivating K_V current (I_{ss}). The decay phases of the transient currents activated upon depolarization are best described by the sum of two exponentials, reflecting the inactivating currents I_{no,f} and I_{K,slow} \(^6\). The amplitudes of I_{no,f} and I_{K,slow} were analyzed using \(A_f\) and \(A_s\) of the double-exponential function described above.
Quantitative RT-PCR

1 μg of RNA isolated from flash-frozen apex was reverse-transcribed using Oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed in 384-well plates using SYBR Green I master solution (Roche) and the LightCycler 480 (Roche). PCR amplification was performed in a total reaction volume of 10 μl, consisting of 1 μM forward and reverse primers, 2 μl cDNA, 5 μl 2x SYBR Green. The PCR was cycled between 95 °C/30 s and 60 °C/30 s for 50 cycles, following an initial denaturation step at 95 °C for 5 min. Primers were specific for mouse sequences (Supplemental Table 1) and designed as described in detail before7. Transcript quantities were normalized to the amount of endogenous control (Hprt and Gapdh) and analyzed using LinRegPCR software8.

Western Blot

Western blot analysis; Heart lysates were extracted from flash-frozen mouse hearts in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) as described previously9. Heart lysate aliquots were size-fractionated on 7.5% (for Cav1.2) or 12% (for GAPDH) SDS-polyacrylamide gels. The resolved gels were electro-transferred on PVDF membranes. The membranes were probed with rabbit anti-Cav1.2 (1:200; Alomone Labs, Jerusalem) or mouse anti-GAPDH (1:10,000; Fitzgerald, Acton, MA) antibody at 4°C overnight, followed by incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000; GE-Healthcare, Buckinghamshire, UK). Images were acquired by enhanced chemiluminescent detection by the ImageQuant LAS-4000 (GE-Healthcare, Buckinghamshire, UK). Integrated densities of protein bands were measured using ImageJ Data Acquisition Software (National Institute of Health, Bethesda, MD). Protein-signal densities were normalized to the corresponding GAPDH-signal densities.
<table>
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<tr>
<th>Gene</th>
<th>sense</th>
<th>anti-sense</th>
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<td>Scn5a (Na-channel)</td>
<td>TTGTCATCCTCTCCATCGTG</td>
<td>AGACGGATGACACGGAAGAG</td>
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<tr>
<td>Slc8a1 (NCX)</td>
<td>GGACCAACAGCTGGAGAGAG</td>
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<td>TACTGACCCCTGCCCCCGAC</td>
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*Table 1. Primer sequences.*
References.


