Enrichment of Endoplasmic Reticulum with Cholesterol Inhibits Sarcoplasmic-Endoplasmic Reticulum Calcium ATPase-2b Activity in Parallel with Increased Order of Membrane Lipids

IMPLICATIONS FOR DEPLETION OF ENDOPLASMIC RETICULUM CALCIUM STORES AND APOPTOSIS IN CHOLESTEROL-LOADED MACROPHAGES

Macrophages in advanced atherosclerotic lesions accumulate large amounts of unesterified, or “free,” cholesterol (FC). FC accumulation induces macrophage apoptosis, which likely contributes to plaque destabilization. Apoptosis is triggered by the enrichment of the endoplasmic reticulum (ER) with FC, resulting in depletion of ER calcium stores, and induction of the unfolded protein response. To explain the mechanism of ER calcium depletion, we hypothesized that FC enrichment of the normally cholesterol-poor ER membrane inhibits the macropage ER calcium pump, sarcoplasmic-endoplasmic reticulum calcium ATPase-2b (SERCA2b). FC enrichment of ER membranes to a level similar to that occurring in vivo inhibited both the ATPase activity and calcium sequestration function of SERCA2b. Enrichment of ER with ent-cholesterol or 14:0–18:0 phosphatidylcholine, which possess the membrane-ordering properties of cholesterol, also inhibited SERCA2b. Moreover, at various levels of FC enrichment of ER membranes, there was a very close correlation between increasing membrane lipid order, as monitored by 16-doxyl-phosphatidylcholine electron spin resonance, and SERCA2b inhibition. In view of these data, we speculate that SERCA2b, a conformationally active protein with 11 membrane-spanning regions, loses function due to decreased conformational freedom in FC-ordered membranes. This biophysical model may underlie the critical connection between excess cholesterol, unfolded protein response induction, macrophage death, and plaque destabilization in advanced atherosclerosis.

The cholesterol-loaded macrophage, or foam cell, is a hallmark of atherosclerotic lesions (1, 2). Cholesterol accumulates in these cells as a result of the internalization of arterial-wall lipoproteins (3). In early atherosclerosis, most of the lipoprotein-derived cholesterol is esterified to fatty acids by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), which is localized in the endoplasmic reticulum (ER) (4). In late lesions, however, macrophages accumulate mostly unesterified, or “free,” cholesterol (FC) (5, 6). Although the mechanism of FC accumulation is not known, there is evidence in vivo to suggest that ACAT1 dysfunction, likely coupled with defects in cellular cholesterol efflux, contribute to this process (7). Most importantly, FC accumulation in late lesional macrophages induces apoptosis and secondary necrosis in these cells (7). These events are thought to contribute to lesional necrosis and to promote plaque disruption, which in turn leads to acute atherothrombotic cardiovascular events (7, 8). Thus, an important goal in this area of research is to understand the mechanisms of cholesterol-induced cytotoxicity in FC-loaded macrophages.

In this context, recent work in our laboratory has begun to elucidate the cellular and molecular events involved in FC-induced apoptosis. The key event appears to be induction of the ER stress pathway known as the unfolded protein response (UPR) (9). One of the effector molecules of the UPR, a transcription factor called CHOP (GADD153), has been implicated in a number of apoptotic pathways (10, 11), and we have shown that macrophages from Chop−/− mice are resistant to FC-in-
duced apoptosis (9). Moreover, genetic and pharmacologic manipulations that block FC trafficking to the ER block both CHOP induction and apoptosis. Thus, FC trafficking to the ER somehow induces the UPR, and the CHOP branch of the UPR then leads to the induction of apoptosis pathways in these cells. In the Apo-" mouse model of atherosclerosis, FC-rich macrophages in advanced atherosclerotic lesions express CHOP, and inhibition of FC trafficking to the ER blocks lesional macrophage apoptosis and lesional necrosis (9, 12).

How might FC trafficking to the ER induce the UPR? We have shown that the earliest detectable event in the FC-UPR-apoptosis pathway is depletion of ER calcium stores (9). Low calcium in the ER lumen is a known inducer of the UPR, most likely by causing dysfunction of calcium-dependent protein chaperones such as calreticulin, calnexin, and BiP (13, 14).

For this connection in FC-loaded macrophages must await future experiments in which ER calcium pools could somehow be maintained in FC-loaded macrophages. However, pending such proof, we sought to explore how FC loading of the ER might lead to ER calcium depletion.

A critical molecule involved in the maintenance of ER calcium stores is sarcoplasmic reticulum calcium ATPase (SERCA), which pumps calcium from the cytosol into the ER lumen (15). The SERCA family is encoded by three distinct genes, numbered 1–3, each of which has multiple isoforms (16–18). Although these SERCA isoforms have similar overall structures and function, there are differences in certain specific structural features, functional characteristics, and regulatory properties (19). Most notably, certain isoforms are only expressed in muscle, which contains abundant sarcoplasmic reticulum and high SERCA expression. Specifically, SERCA1a and -1b are expressed exclusively in fast-twitch skeletal muscle, SERCA2a is expressed in cardiac and slow-twitch skeletal muscle, and SERCA2b, -3a, and -3b are expressed in nonmuscle cells (20).

We now report that FC loading of ER membranes containing SERCA2b, the form found in macrophages, profoundly inhibits SERCA2b ATPase activity and calcium sequestration. Moreover, this inhibition is directly correlated with an increase in SERCA2b, the form found in macrophages, profoundly inhibits SERCA2b ATPase activity and calcium sequestration. More-

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The homogenates were then made isotonic by the addition of 0.8 ml of low-ionic strength buffer containing 1.48 M sucrose and then centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant (4.8 ml) was divided into two equal portions of 2.4 ml each, loaded onto two sucrose density gradient tubes, and centrifuged at 100,000 × g for 2 h at 4 °C. This procedure resulted in visible bands at each of the four interfaces plus a pellet. Five fractions were collected as depicted in Fig. 1A. The pellet, which was enriched in endoplasmic reticulum (see “Results”), was washed twice and then resuspended in 250 μl of Buffer A. Equal amounts of protein from each fraction were analyzed by SDS-PAGE and immunoblotting for organelle markers. Lipids from each fraction were extracted using the method of Bligh and Dyer (28) and analyzed for free cholesterol content by gas chromatography and for phospholipid content by the Bartlett assay (31).

Enrichment of Microsomal/ER Membranes with Sterols or Phospholipid in Vitro—Various amounts of sterols in complex with MβCD, or vesicles containing phospholipids with saturated or unsaturated fatty acids, were added to 100 μg of microsomal or endoplasmic reticulum membranes from HEK293T cells, and the final volume adjusted to 500 μl with Buffer A. After incubation at 25 °C for 30 min with gentle agitation, the samples were centrifuged at 120,000 × g for 1 h at 4 °C. The pellet was washed twice and then resuspended in Buffer A to a final concentration of 1 μg/μl for the assays described below.

Measurements of Ca2+-dependent ATPase Activity—SERCA ATPase activity was measured using an enzyme-coupled spectrophotometric assay in which hydrolysis of ATP is coupled to the oxidation of NADH (32). The depletion of NADH was then detected by a decrease in absorbance at 340 nm using a Spectra Max 190 fluorospectrometer (Molecular Devices) maintained at 30 °C. The reaction was started by adding 5 μl of the calcium ionophore A23187. The pH was adjusted to 7.0 with KOH before addition of the enzymes, and the free Ca2+ concentration in the solution was determined to be ~3 μM. The reaction was started by adding 10 μg of the membranes to 200 μl of the assay buffer in wells of a 96-well UV-visible transparent bottom plate, followed by rapid mixing. The absorption at 340 nm was recorded at 30-s intervals for 30 min using the SOFTmax PRO 3.0 program.

Measurements of 45Ca2+ Uptake into Membrane Vesicles—Ca2+ uptake activity was measured using an ATP-mediated oxalate-dependent assay (32). 45CaCl2 was added to the uptake buffer (120 mM KCl, 2 mM MgCl2, 1 mM ATP, 1.5 mM phosphoenolpyruvate, 1 mM dithiothreitol, 0.45 mM CaCl2, 0.5 mM EGTA, 25 mM MOPS/KOH, 0.32 mM NADH, 5 units/ml pyruvate kinase, 10 units/ml lactate dehydrogenase, and 2 μM of the calcium ionophore A23187. The pH was adjusted to 7.0 with KOH before addition of the enzymes, and the free Ca2+ concentration in the solution was determined to be ~3 μM. The reaction was started by adding 10 μg of the membranes to 200 μl of the assay buffer in wells of a 96-well UV-visible transparent bottom plate, followed by rapid mixing. The absorption at 340 nm was recorded at 30-s intervals for 30 min using the SOFTmax PRO 3.0 program.

The depletion of NADH was then detected by a decrease in absorbance at 340 nm using a Spectra Max 190 fluorospectrometer (Molecular Devices) maintained at 30 °C. The reaction was started by adding 5 μl of the calcium ionophore A23187. The pH was adjusted to 7.0 with KOH before addition of the enzymes, and the free Ca2+ concentration in the solution was determined to be ~3 μM. The reaction was started by adding 10 μg of the membranes to 200 μl of the assay buffer in wells of a 96-well UV-visible transparent bottom plate, followed by rapid mixing. The absorption at 340 nm was recorded at 30-s intervals for 30 min using the SOFTmax PRO 3.0 program.

Measurements of 45Ca2+ Uptake into Membrane Vesicles—Ca2+ uptake activity was measured using an ATP-mediated oxalate-dependent assay (32). 45CaCl2 was added to the uptake buffer (120 mM KCl, 25 mM MOPS/KOH, 3 mM MgCl2, 0.45 mM CaCl2, 0.5 mM EGTA, 5 mM potassium oxalate, 3 mM ATP, pH 7.0) to a final concentration of 1 μCi/ml. The reaction was started by adding 5 μg of membranes to 0.5 ml of Ca2+ uptake buffer in a clean glass tube at 25 °C with gentle mixing. At intervals of 5, 10, and 15 min, 150-μl aliquots were withdrawn from the reaction mixture and diluted into 3 ml of quench buffer (150 mM KCl, 1 mM LaCl3) in a glass tube on ice. The buffer was filtered through a Millipore filtration manifold with a pre-wetted 0.3-μm PHWP nitrocellulose membrane, followed immediately by washing with 5 ml of cold quench buffer. The membrane was removed from the manifold, and
radioactivity was measured by a liquid scintillation counter.

ESR Spectroscopy and Non-linear Least Squares Fitting of ESR Spectra—5 μl of 0.28% 16-doxyl-PC in methanol was added to 250 μl of PBS containing 250 μg of control or FC-enriched ER membranes. The labeled dispersion was vortexed for 30 s, then diluted with PBS to 4 ml and centrifuged at 120,000 g for 1 h at 4 °C. The pellet was transferred to a quartz capillary tube for ESR measurements. ESR spectra were recorded using a Bruker Instruments (Billerica, MA) ELEXTRON/EMX ESR spectrometer at a frequency of 9.34 GHz equipped with a Varian temperature controller. The non-linear least squares analyses of the spectra were performed using the latest version of the ESR fitting program (33). These analyses yield the following parameters: rotational diffusion rate $R_i$ and order parameters $S_0, S_2$. $R_i$ is the rotational rate of the nitroxide radical around an axis perpendicular to the mean symmetry axis for the rotation. This symmetry axis is also the direction of preferential orientation of the spin-labeled molecule (34). The order parameter $S_0$ is a measure of the angular extent of the rotational diffusion of the nitroxide moiety relative to the membrane director (the normal to the lipid bilayer). The larger the $S_0$, the more restricted is the motion, which usually means that laterally the lipid molecules surrounding the nitroxide radical are packed more tightly. $S_0$ is the non-symmetric order parameter, which represents the nonaxiality of the preferential orientation of the spin-labeled molecule relative to the membrane director. Thus, for $S_0$, the restriction of the wagging motion of the spin label is not symmetric about its main symmetry axis.

RESULTS

Cholesterol Accumulation in ER-enriched Membranes from FC-loaded Macrophages—FC trafficking to the ER is a necessary step in ER calcium depletion in FC-loaded macrophages (9). As a prelude to our study on the effects of increased ER cholesterol on SERCA activity, we determined the level of cholesterol accumulation in the ER in FC-loaded macrophages. For this purpose, we developed a sucrose density step-gradient fractionation procedure for the isolation of an ER-enriched fraction from RAW and mouse peritoneal macrophages (Fig. 1A). As shown in Fig. 1B for RAW macrophages and Fig. 1D for mouse peritoneal macrophages, the fifth fraction (pellet) was enriched in the ER-specific protein ribophorin II (26). The blot in Fig. 1B shows no detectable LAMP2 (lysosomes), Gas 28 (Golgi), or $\beta_1$-integrin (plasma membrane) in fraction 5. As expected for an ER-enriched fraction (35), the FC:phospholipid ratio of fraction 5 was relatively low (black bars in Fig. 1, C and D). With FC loading of the macrophages, the FC:phospholipid ratio of the ER-enriched fraction increased ~2-2.5-fold (gray bars in Fig. 1, C and D).

SERCA Expression in Control and FC-loaded Macrophages—In theory, ER calcium depletion in FC-loaded macrophages could be caused by activation of calcium release channels and/or inhibition of SERCA. Initial experiments with inositol 1,4,5-trisphosphate receptor and the ryanodine receptor intracellular calcium release channels reconstituted in planar lipid bilayer membranes with different FC:phospholipid ratios revealed no evidence of consistent cholesterol-induced activation of these channels (data not shown). We therefore focused on the hypothesis that cholesterol inhibits SERCA. In this context, we first determined which isoform(s) of SERCA were expressed in macrophages. As shown by the immunoblot in Fig. 2A (first two lanes), RAW macrophages and mouse peritoneal macrophages express SERCA2b; SERCA1 and -3 could not be detected (not displayed). Previous studies by Caspersen et al. (36) showed that SERCA2b expression in PC12 cells was increased by inducers of the UPR. Because FC loading induces the UPR in macrophages (9), we assayed SERCA2b protein levels in control and FC-loaded macrophages. As shown in Fig. 2B, FC loading led to an ~2-fold increase in SERCA2b expression that preceded maximum induction of the UPR effector CHOP.

As expected, SERCA2b activity in microsomes isolated from macrophages was too low to be measured in vitro. Because macrophages are very difficult to transfect, we used SERCA2b-transfected HEK293 cells as the source of SERCA2b-containing membranes for our study. As shown in Fig. 2A (third lane), endogenous SERCA2b was undetectable in HEK293 cells, whereas 24 h after transient transfection, the cells expressed abundant SERCA2b (fourth lane in Fig. 2A; note that 10 μg of protein was loaded in the fourth lane versus 50 μg for the first three lanes). Immunostaining of the transfected cells showed that SERCA2b was expressed in a distinct ER pattern (not shown).

Cholesterol Enrichment Inhibits SERCA2b Activity—To determine the effects of membrane cholesterol enrichment on SERCA2b activity in vitro, we incubated SERCA2b-containing microsomes with various amounts of cholesterol in complex with MβCD. The microsomes were then recovered by centrifugation, and SERCA2b activity was measured. Cholesterol enrichment of the microsomes inhibited both SERCA2b calcium ATPase activity (Fig. 3A) and SERCA2b-mediated 45Ca2+ uptake (Fig. 3B) in the same dose-dependent manner, with a 50% inhibition of both when incubated with 1.5 μg of cholesterol. Incubation with MβCD alone did not affect SERCA2b activity (inset in Fig. 3A). The inhibition of these processes by the specific SERCA inhibitor thapsigargin confirmed that these measurements represented SERCA activity.

To determine the increase in the FC:phospholipid molar ratio that is associated with inhibition of SERCA2b ATPase activity, we repeated the experiment described above using ER-enriched membranes from the SERCA2b-transfected HEK293 cells. Using a concentration of MβCD-cholesterol that inhibited SERCA2b ATPase activity by ~50% (Fig. 4A), the FC:phospholipid molar ratio of the membranes was increased by ~2-fold (Fig. 4B). As demonstrated by the data for fraction 5 in Fig. 1C, this is very similar to the increase in the FC:phospholipid molar ratio that occurs in the ER in FC-loaded macrophages.

Comparison of the Effect of Nat-Cholesterol, Ent-Cholesterol, and Epi-Cholesterol on SERCA Activity—There are two major mechanisms that could explain FC-induced SERCA2b inhibition, namely, alteration of ER membrane structure or direct
binding of cholesterol to SERCA2b or a SERCA2b-regulatory protein. To distinguish between these two mechanisms, we first compared the effect of nat-cholesterol and ent-cholesterol on SERCA2b activity. Nat-Cholesterol and its enantiomer, ent-cholesterol, have identical effects on membrane properties such as enhancement of membrane order, interaction with phospholipids, and promotion of membrane rafts (22). In contrast, cholesterol and ent-cholesterol interact differently with cholesterol-binding proteins such as cholesterol oxidase or Vibrio cytolsin (37). We therefore incubated SERCA2b-containing ER membranes with MβCD complexed with either nat- or ent-cholesterol and then assayed the membranes for the sterol:phospholipid ratio and SERCA2b ATPase activity. Similar amounts of nat- and ent-cholesterol were incorporated into the membranes, and SERCA2b ATPase activity was inhibited to the same extent (~60%) by the two sterols (Fig. 5A). On the other hand, when a similar amount of 3-epi-cholesterol was incorporated into the membranes, the SERCA2b ATPase activity was not affected (Fig. 5B), which is consistent with previous studies showing that the 3-epimer of cholesterol orders membrane lipids to a much lesser extent than cholesterol (21, 38). These data are most consistent with a model in which FC-induced changes in ER membrane structure is the cause of SERCA2b inhibition.

Manipulation of Phospholipid Composition—Cholesterol enrichment of biological membranes increases the order, or “stiffness,” of those membranes (39, 40). If this were the mechanism behind FC-induced inhibition of SERCA2b activity, we should be able to inhibit SERCA2b by other perturbations that increase membrane order. Other investigators have shown that the order of biological membranes containing unsaturated fatty acid-containing PC can be increased in vitro by replacing endogenous PC with PC containing saturated fatty acids (41). We therefore incubated SERCA2b-containing membranes with small unilamellar vesicles made from 14:0–18:0 PC or 18:1–18:1 PC, re-isolated the membranes, and then assayed SERCA2b ATPase activity. As shown in Fig. 6, membranes that had been incubated with 14:0–18:0 PC had much less SERCA2b activity than those incubated with 18:1–18:1 PC. These data further support the concept that SERCA2b activity is compromised when the membrane order is increased.

Direct Evaluation of Membrane Fluidity by Electron Spin Resonance—To further evaluate the hypothesis that FC-induced SERCA2b inhibition was related to the membrane or-
Cholesterol and related sterols are an essential component of membrane lipid bilayers in eukaryotic cells (43). Sterols have important effects on the physical properties of biological membranes, and these effects, in turn, can influence the structure and function of integral membrane proteins (44). Most notably, cholesterol increases phospholipid ordering and widens the bilayer by reducing the number of phospholipid molecules in the gauche conformation (45). In normal physiology, cholesterol-induced lipid ordering in membrane microdomains ("rafts") regulates a number of essential protein-protein interactions, such as those involved in signal transduction (46). However, studies in vitro have shown that excessive ordering of membrane lipids by the introduction of superphysiologic amounts of cholesterol can perturb protein function. Several mechanisms that might account for loss of membrane protein function under these conditions are a decrease in the conformational "freedom" of proteins, induction of dysfunctional protein "aggregation," an increase in bilayer thickness, and separation of lipid phases (40, 45, 47, 48).

Whereas these in vitro studies examining effects of excess cholesterol on protein function have established important theoretical principles, they have generally not been connected with specific pathophysiological processes. The present study was conceived in the context of a critical cell biological event occurring in advanced atherosclerosis, namely accumulation of excess FC by macrophages. In vitro data suggest that a portion of this FC overloads the ER membrane (9, 12), and cell culture data have shown that depletion of ER calcium stores is an important consequence that can be directly attributable to ER cholesterol overload (9). Because excess membrane cholesterol does not appear to activate ER calcium release channels, we hypothesized that inhibition of SERCA2b, the form of SERCA that regulates a number of essential protein-protein interactions, was the primary cause of loss of ER calcium. In the context of these in vivo findings, we show here that enrichment of ER in vitro with a level of cholesterol that occurs in vivo in FC-loaded macrophages does indeed inhibit the activity of SERCA2b.

The inhibition of SERCA2b activity by ent-cholesterol and by phospholipids containing saturated fatty acids suggested that the mechanism was because of increased ordering of the ER membrane rather than a direct interaction with cholesterol per se. To directly examine changes in the biophysical characteristics of ER membranes as cholesterol loading is increased, we used ESR spectral analysis of 16-doxyl-PC incorporated into ER membranes. As shown in Table I, the $R_1$, $S_o$, and $S_d$ of the ER membrane with no added cholesterol (FC:phospholipid molar ratio $= 0.12$) are similar to those of liquid-disordered membranes, which is consistent with previous studies of the properties of ER membranes (21). As the cholesterol content is increased to a FC:phospholipid ratio of 0.33, the order parameter $S_o$ increases significantly. The $S_d$ of 16-doxyl-PC in ER membranes loaded with these high levels of cholesterol is close to the $S_d$ of 16-doxyl-PC in the liquid-ordered phase of lipid bilayers consist-

![Image](https://example.com/image.png)
of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol at a molar ratio of 1:1, which is 0.22 at 25–35 °C (49). The \( S_0 \) values are also similar to those obtained in detergent-resistant membranes from rat basophilic leukemia (RBL) cells, which is 0.20 at 37 °C (49). These changes in the order parameter indicate that the ER membrane undergoes a significant extent of ordering with FC enrichment, with its properties becoming similar to liquid-ordered membranes. The \( R/H \) values change less dramatically as cholesterol is increased, with values that are also consistent with a liquid-ordered membrane organization.

In considering these data, it is important to note that the ER membrane normally has a relatively low FC:phospholipid ratio and saturated fatty acid content and is therefore one of the most fluid membranes in the cell (21, 35). Indeed, a recent report showed that the ER membrane, unlike the plasma membrane, is actually permeable to small charged molecules, which is a property of low-order membranes (50). Thus, one would expect that a number of integral membrane proteins in the ER are adapted to function optimally in this fluid membrane environment and would therefore be adversely affected by an increase in membrane order. In this regard, SERCA contains multiple transmembrane domains, and its calcium pumping cycle requires several changes in protein conformation (51, 52).

**Fig. 5.** Effect of ent-cholesterol and epi-cholesterol enrichment of SERCA2b-containing microsomes on SERCA2b activity. 500 μg of ER-enriched membranes from SERCA2b-transfected HEK293 cells were incubated with 7.5 μg of nat-cholesterol (Chol) (A and B), ent-cholesterol (A), or epi-cholesterol (B) in complex with MJCD and then re-isolated by centrifugation. Sterol and phospholipid (PL) mass and Ca\(^{2+}\)-ATPase activity of these samples were measured. For both experiments, \( n = 3 \) measurements from a single experiment, \( \pm \)S.E. Similar results were obtained from two repeat experiments.

**Fig. 6.** Incubation of SERCA2b-containing microsomes with saturated phospholipids inhibits SERCA2b activity. 100 μg of microsomal membranes from SERCA2b-transfected HEK293 cells were incubated with various amounts of PC vesicles consisting of 14:0–18:0 PC (circles) or 18:1–18:1 PC (diamonds). The membranes were re-isolated by centrifugation and then assayed for Ca\(^{2+}\)-ATPase activity. Each point represents duplicate measurements, which varied by <5%. SUV, small unilamellar vesicles.
Restriction of these conformation changes in a more ordered membrane is a plausible mechanism accounting for the decrease in SERCA activity in cholesterol-enriched ER. This hypothesis is consistent with the frequency domain phosphorescence spectroscopy data of Hunter et al. (53) and Whiting et al. (54), and with the saturation-transfer electron paramagnetic resonance data of Squier et al. (55). While separation of lipid phases is an additional mechanism that might account for SERCA inhibition, we did not find evidence for this phenomenon in ER membranes as the cholesterol level was changed. Nonetheless, this negative result does not rule out the possibility that such domain formation occurs.

Previous studies have explored the effect of cholesterol enrichment of membranes and vesicles containing SERCA1 from the sarcoplasmic reticulum of skeletal muscle, although there have been potential methodological problems and, in some cases, contradictory results. All of these studies used DPPC-cholesterol vesicles to enrich membranes with cholesterol, often without re-isolating the membranes. As shown by the data

### Table I

Parameters obtained from nonlinear least-squares fitting of ESR spectra of 16-doxyl-PC in ER-enriched membranes (component 1) at 22 and 37 °C

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<thead>
<tr>
<th>Sample</th>
<th>FC:PL*</th>
<th>T (°C)</th>
<th>R₂</th>
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<tr>
<td>I</td>
<td>0.12</td>
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<td>37</td>
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<tr>
<td>II</td>
<td>0.21</td>
<td>22</td>
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<td></td>
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<td>37</td>
<td>2.08 × 10⁶</td>
<td>0.14</td>
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<tr>
<td>III</td>
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<tr>
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<td></td>
<td>37</td>
<td>2.21 × 10⁶</td>
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* FC:PL, FC:phospholipid molar ratio.

Restriction of these conformation changes in a more ordered membrane is a plausible mechanism accounting for the decrease in SERCA activity in cholesterol-enriched ER. This hypothesis is consistent with the frequency domain phosphorescence spectroscopy data of Hunter et al. (53) and Whiting et al. (54), and with the saturation-transfer electron paramagnetic resonance data of Squier et al. (55). While separation of lipid phases is an additional mechanism that might account for SERCA inhibition, we did not find evidence for this phenomenon in ER membranes as the cholesterol level was changed. Nonetheless, this negative result does not rule out the possibility that such domain formation occurs.

Previous studies have explored the effect of cholesterol enrichment of membranes and vesicles containing SERCA1 from the sarcoplasmic reticulum of skeletal muscle, although there have been potential methodological problems and, in some cases, contradictory results. All of these studies used DPPC-cholesterol vesicles to enrich membranes with cholesterol, often without re-isolating the membranes. As shown by the data.
of Fig. 6 in the current study, DPPC itself would likely influence SERCA2a activity. In one of these studies, DPPC-cholesterol was found to inhibit SERCA1 activity (56), but in another study this result was not obtained, and the authors attributed the previous finding to an artifact related to the absence of dithiothreitol in the incubation mixture (57). Moreover, neither of these studies reported membrane fluidity measurements. In a more recent study, both DPPC-cholesterol and progesterone enrichment of SERCA1-containing membranes increased membrane order as measured by fluorescence polarization, but only cholesterol inhibited SERCA1 activity (54). Cheng et al. (58) reconstituted SERCA1 in synthetic vesicles containing PC, phosphatidylethanolamine, and cholesterol. Vesicles with a high cholesterol:phospholipid ratio demonstrated decreased fluidity and decreased ATPase activity, but calcium uptake was either not affected or actually increased, depending on the relative amounts of phosphatidylcholine and phosphatidylethanolamine. Overall, these studies tend to support the idea that skeletal muscle SERCA1 ATPase is adversely affected by cholesterol-induced membrane stiffening, which is consistent with the SERCA2b data in this report, but the caveats and inconsistencies outlined above are notable. Moreover, a physiologic or pathophysiological context in which the cholesterol content of muscle sarcoplasmic reticulum might be increased has not been demonstrated.

Interest, other ER membrane proteins involved in intracellular cholesterol metabolism appear to be affected more by direct cholesterol-protein interactions than by cholesterol-induced changes in membrane fluidity. Cholesterol-mediated regulation of the sterol regulatory element-binding protein pathway involves a conformational change in sterol regulatory element-binding protein cleavage-activating protein (SCAP). In this scenario, sterol specificity data are consistent with a direct sterol-SCAP interaction causing this effect (59, 60). Indeed, SCAP contains a consensus “sterol-sensing domain” that likely mediates this interaction (44), although direct sterol binding to this protein has not yet been demonstrated. In another scenario, Chang and colleagues (61) have shown that a number of sterols activate ACAT by a mechanism thought to involve an allosteric sterol-enzyme interaction, although ACAT may actually be suppressed when the order of the ER membrane is increased by other lipids (62).

As mentioned above, FC loading of cultured macrophages leads to ER calcium depletion, UPR induction, and apoptosis, all of which are completely dependent on FC trafficking to the ER (9, 12). Depletion of ER calcium stores is a known inducer of the UPR (13, 14), and we have shown that the UPR is necessary for FC-induced apoptosis. Thus, ER calcium depletion, which is an early event in macrophage FC loading, could be a key upstream event leading to macrophage death. Interestingly, Rodriguez and colleagues (63) have reported that macrophages used in the current study. For example, advanced leishmanial macrophages are known to be FC-loaded (7), and we have shown that they express the UPR effector CHOP (9). Moreover, a genetic manipulation that blocks FC trafficking to the ER in vitro protected leishmanial macrophage from apoptotic death (12).

If ER calcium depletion is an important inducer of these events, then inhibition of SERCA2b by cholesterol-induced or-

dering of the normally fluid ER membrane might represent a biophysical explanation of why arterial wall cholesterol is associated with advanced atherothrombotic disease. On a broader scale, the findings in this report would imply that other integral membrane proteins in the ER, particularly those with multiple membrane-spanning regions and whose mechanism involves changes in protein conformation, might also be adversely affected in advanced, FC-loaded leishmanial macrophages. Indeed, recent work has shown that cholesterol enrichment of ER membranes in vitro can inhibit the translocation of newly synthesized proteins (64). The testing of these ideas must await the development of new strategies in which calcium depletion in particular and ER membrane fluidity changes in general could be prevented in FC-loaded macrophages. Such strategies might also form the basis of novel therapeutic approaches to the problem of the unstable atherosclerotic plaque.

Acknowledgments—We thank Dr. Andrew Marks for helpful discussions and advice related to ER calcium channels, Dr. Rebecca Juliano for assistance with the ESR studies, and Inge Hansen for the GC analysis.

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