Oxidation of Membrane Cholesterol Alters Active and Passive Transsarcolemmal Calcium Movement

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Oxygen free radicals have the ability to oxidize cholesterol. However, nothing is known about the effects of cholesterol oxidation on ion transport in isolated myocardial membranes. The purpose of the present study was to investigate the effects of in situ oxidative modification of sarcolemmal cholesterol on Ca\(^{2+}\) flux. Cholesterol oxidase was used to oxidatively modify membrane cholesterol. After incubation of cardiac sarcolemmal vesicles with cholesterol oxidase, cholest-4-en-3-one (cholestenone) was the predominant species of oxidized cholesterol produced. Cholesterol oxidase inhibited sarcolemmal Na\(^{+}\)-Ca\(^{2+}\) exchange in a concentration-dependent manner. Both the \(V_{\text{ma}}\) and \(K_{\text{m}}\) of the reaction were altered after cholesterol oxidase treatment. Extensive treatment of the sarcolemmal membranes with cholesterol oxidase increased the passive permeability characteristics of the membrane. Passive Ca\(^{2+}\) efflux from the sarcolemmal vesicles was stimulated by increasing the concentration of cholesterol oxidase. ATP-dependent Ca\(^{2+}\) uptake was also inhibited after cholesterol oxidase treatment, but it was not as sensitive as the Na\(^{+}\)-Ca\(^{2+}\) exchange. Conversely, passive Ca\(^{2+}\) binding to sarcolemmal vesicles was strikingly stimulated by cholesterol oxidase treatment. The results demonstrate that oxidative modification of sarcolemmal membrane cholesterol can directly affect ionic interactions with the sarcolemmal vesicle and provide potentially important mechanistic information for the molecular basis of the effects of free radicals on ion flux and function in the heart. (Circulation Research 1991;68:18–26)

The formation of reduced oxygen intermediates has been demonstrated to have the capacity to cause contractile dysfunction and cellular damage in the heart. These reduced oxygen intermediates, or oxygen free radicals, have been implicated mechanistically in a number of pathophysiological processes including the oxygen paradox, calcium paradox, adriamycin-induced cardiomyopathy, and ischemic/reperfusion injury.\(^1-3\) Because the underlying defect in these pathologies is likely to be alterations in ion transport in the heart, research attention has focused on the effect of oxygen free radicals on ion flux. Exposure of cardiac membranes to free radicals results in increased permeability characteristics,\(^4\) depressed Na\(^{+}\)/K\(^{+}\) and Ca\(^{2+}\) pump activities,\(^5,6,7\) and altered Na\(^+\)-Ca\(^{2+}\) exchange.\(^8-10\)

Several sites of action within the myocardial cell have been proposed to explain the deleterious effects of oxygen free radicals. Oxidation of specific amino acid residues in membrane-bound proteins\(^7,11\) and peroxidation of unsaturated fatty acids in the membrane\(^5,6\) are the two sites of attack by oxygen free radicals that are most frequently cited. However, another cellular site of attack for free radicals that may be altering ion flux is membrane cholesterol. Oxygen free radicals are known to have the capacity to oxidatively modify cholesterol.\(^12,13\) However, it is unknown what effects cholesterol oxidation may have on the myocardium. It is clear that sarcolemmal membrane cholesterol is important in modulating Na\(^+\)-Ca\(^{2+}\) exchange and Ca\(^{2+}\) pump activity in the heart.\(^10\) It would also appear that the structure of the cholesterol molecule is an important factor in regulating ion transport in isolated membrane systems.\(^14\) It is possible, therefore, that oxidative modification of membrane cholesterol in situ may affect ionic interactions with the membrane.

The purpose of the present study was to determine the effects on ion transport of in situ cholesterol...
oxidation in isolated sarcolemmal membranes. Cholesterol oxidase was used to selectively oxidize membrane cholesterol residues. Our results demonstrate that extensive oxidation of membrane cholesterol residues can significantly increase membrane permeability characteristics. Less extensive oxidation of membrane cholesterol can directly inhibit sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchange and, to a lesser extent, Ca\(^{2+}\) pump activity. Passive Ca\(^{2+}\) binding to the membranes is, however, strikingly stimulated. Our results demonstrate that oxidation of membrane cholesterol can significantly alter ionic interactions with the myocardial cell and may represent an important mechanism whereby free radicals exert their effects on ion flux pathways.

Materials and Methods

Materials

All chemicals and reagents were purchased from Sigma Chemical Co., St. Louis. Cholesterol oxidase (Pseudomonas fluorescens) was purchased from Sigma. All chemicals were of standard reagent grade.

Membrane Preparation

Sarcolemmal membrane vesicles were isolated from canine left ventricular tissue as described in detail previously.\(^{10,15,17}\) The vesicles exhibited relatively high activities of enzymes commonly associated with the sarcolemmal membrane. For example, Na\(^+\),K\(^+\)-ATPase activity in the membranes (\(n=9\)) was 26.4±4.1 and 131.5±17.5 μmol P/mg/hr in the absence and presence, respectively, of 12.5 μg alamethicin/ml reaction medium. K\(^+\)-dependent para-nitrophenyl phosphatase activity was 26.1±1.8 μmol phenol/mg/hr, which represented an enrichment of 107±22-fold over homogenate values. Cross-contamination of this preparation with other subcellular organelles is minimal.\(^{16,17}\)

Assay Procedures

All assays were carried out at 37°C. Na\(^+\)-Ca\(^{2+}\) exchange was measured as described in detail previously.\(^{10,16}\) Exchange was measured as the rate of intravesicular Na\(^+\)-dependent Ca\(^{2+}\) uptake with 45Ca as the radioisotopic tracer. When cholesterol oxidase was included in the reaction medium, catalase was also present at 75 times (units:units) the cholesterol oxidase concentration. This catalase concentration was in excess of the amount required to quench the \(\text{H}_2\text{O}_2\) produced by the reaction of cholesterol oxidase with the membrane cholesterol, assuming that 1 mol \(\text{H}_2\text{O}_2\) was produced for each mole of cholesterol oxidized\(^{18}\) and all of the membrane cholesterol was oxidized. Catalase itself had no effect on the Na\(^+\)-Ca\(^{2+}\) exchange reaction. In preliminary experiments, it was discovered that La\(^{3+}\) caused large increases in background counts in the presence of Ca\(^{2+}\) and the cholesterol oxidase. Similar problems have been encountered by others.\(^{19}\) We have circumvented this problem by eliminating the use of La\(^{3+}\) to stop the reaction and instead used 0.03 ml of 140 mM KCl, 10 mM EGTA, and 20 mM MOPS (pH 7.4) delivered with a rapid quenching device described elsewhere.\(^{20}\) A 1-ml aliquot of ice-cold 140 mM KCl, 1 mM EGTA, and 20 mM MOPS (pH 7.4) was then immediately added to the reaction tube, and 1 ml was removed for filtration. The filters were then washed with 2x3 ml of this solution. This modified quenching and wash solution has been used previously with success,\(^{19}\) and we confirm its utility here. Background counts were reduced to approximately 5% of total counts, and activity was similar in control experiments whether La\(^{3+}\) or EGTA was used as the stop/wash solution. Our results were also qualitatively similar using another cholesterol oxidase preparation (Nocardia erythropolis).

Passive Ca\(^{2+}\) efflux was measured as described previously.\(^{10}\) Vesicles were allowed to accumulate 45Ca via Na\(^+\)-Ca\(^{2+}\) exchange and then were diluted into a medium to allow 45Ca efflux in the absence or presence of cholesterol oxidase (plus catalase, as above). Values were corrected for ATP-independent Ca\(^{2+}\) binding by subtracting blank values obtained by using uptake medium that provided no transsarcolemmal Na\(^+\) gradient and, therefore, did not allow vesicular loading with 45Ca to proceed.

ATP-dependent Ca\(^{2+}\) pumping of the sarcolemmal vesicles was carried out as described in detail\(^{10,16}\) except the stop/wash solution was as described above for the Na\(^+\)-Ca\(^{2+}\) exchange. ATP-independent (passive) Ca\(^{2+}\) binding was measured as described previously.\(^{10,16}\) Briefly, 10 μg sarcolemmal membranes that had been suspended in 140 mM KCl and 20 mM MOPS (pH 7.4) were incubated in this same medium that also contained varying CaCl\(_2\) concentrations, 1.25 μCi of 45CaCl\(_2\) and 1 μM A23187. The A23187 was included to permeabilize the membranes to Ca\(^{2+}\) and allow free access of the ion to both sides of the sarcolemmal vesicles. Thus, even if cholesterol oxidase treatment of the sarcolemma increased vesicular permeability, this change in permeability could not account for the alteration in passive Ca\(^{2+}\) binding capacity of the membranes. The reaction was carried out for 1 minute at 37°C and was terminated by filtration. Nonspecific Ca\(^{2+}\) binding was corrected for, as described previously.\(^{10,16}\) Vesicular protein was measured as described previously.\(^{10}\)

High-performance liquid chromatography (HPLC) was used to identify the presence of oxidated cholesterol species in the membranes after treatment with cholesterol oxidase. Sarcolemmal vesicles (15 μl, ~30 μg) were suspended on the side of a ground-glass homogenization vessel, and a reaction was initiated by vortexing with 7 units of cholesterol oxidase (+catalase) in 140 mM KCl and 20 mM MOPS (pH 7.4) (total volume was 250 μl). All reactions were carried out at 37°C and were terminated at specified times with the addition of 2 ml of 2:1 (vol/vol) chloroform:methanol. The sample was further disrupted by homogenization with a glass pestle; then the vessel was rinsed with 2 ml plus 1 ml of the above
solution. Lipids were extracted from membranes in a 2:1 (vol/vol) chloroform:methanol solution as described\textsuperscript{21} and were then evaporated under a stream of nitrogen and suspended in 100 \textmu l methylene chloride for separation via HPLC. For the separation of oxidized cholesterol species, a modification of the technique of Sevanian and McLeod\textsuperscript{12} was used. A 20–50 \textmu l aliquot was injected into an HPLC system (Beckman Instruments, Inc., Fullerton, Calif.) fitted with a 5-\textmu m particle size Beckman Ultrasphere silica column (4.6 mm×15 cm). Flow rate was maintained at 1.0 ml/min, and the mobile phase was 95:5 (vol/vol) hexane:isopropanol. Ultraviolet detection of the peaks was carried out at an absorbance of 208 nm. Appropriate standards (Sigma; Steraloids Inc., Wilton, N.H.) were run to identify the cholesterol species.

Statistical Analysis

Data were subjected to analysis of variance testing followed by Duncan’s multiple range test to determine statistical significance.

Results

After treatment of sarcolemma with cholesterol oxidase, cholest-4-en-3-one was generated (Figure 1) in a rapid, time-dependent manner in the myocardial membrane vesicles (Figure 2). The oxidation product was observed as early as 1.5 seconds after incubation and appeared to exhibit saturation kinetics after 5–10 minutes of exposure. Generation of cholestenone from cholesterol was also dependent on the cholesterol oxidase concentration (Figure 3).

Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange was measured as Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake in the isolated cardiac sarcolemmal vesicles. The reaction was linear through the first 2 seconds of the reaction, then exhibited saturation kinetics by 30–60 seconds. This pattern is similar to results reported previously, as is the absolute activity demonstrated here (Figure 4).\textsuperscript{10,16,19} Inclusion of 2.0 units/ml cholesterol oxidase in the reaction medium inhibited Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange. As shown in the inset graph of Figure 4, this inhibition was particularly striking (67%) during the early linear part of the reaction (0.5 seconds), then lessened as the reaction progressed. Approximately 30% inhibition was observed during the nonlinear phase of the reaction (5–60 seconds).

A separate series of experiments was conducted to determine the concentration dependency of the cholesterol oxidase effects. From 1 to 20 units/ml cholesterol oxidase, Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange (1.5-second reaction time) was inhibited in a concentration-dependent manner (Figure 5). Twenty units of cholesterol oxidase almost eliminated exchange activity.

The dependence of the initial rate of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange on [Ca\textsuperscript{2+}] in untreated and cholesterol
oxidase–treated sarcolemmal vesicles is shown as a double reciprocal plot in Figure 6. Reaction time was 1.0 second, and the cholesterol oxidase concentration was 2.5 units/ml. The $K_m$ for the control preparations ($n=4$) was $21.73 \pm 1.85 \mu M$, which is similar to values reported previously.$^{10,16,19}$ Cholesterol modification in the membrane vesicles strikingly increased the $K_m$ to $84.03 \pm 10.94 \mu M$ ($n=4$). The $V_{max}$ for the reaction was $15.87 \pm 1.40$ and $24.58 \pm 2.72$ nmol/mg/sec in the untreated and cholesterol oxidase–treated vesicles, respectively ($n=4$).

It is possible that the depression in exchange was due to an increase in the permeability properties of the membrane. Thus, experiments were carried out to test the relative permeability characteristics of the untreated and cholesterol-modified membranes to Ca$^{2+}$ flux. Vesicles were loaded with $^{45}$Ca$^{2+}$ via the Na$^+$-Ca$^{2+}$ exchange (initial load was $7.42 \pm 0.67$ nmol/mg), then diluted into a medium with or without cholesterol oxidase, which was optimum to allow for the passive efflux of $^{45}$Ca$^{2+}$ from the vesicles. As shown in Figure 7, after 30 seconds of efflux, vesicular Ca$^{2+}$ content had declined about 10% in the control vesicles. Inclusion of cholesterol oxidase in the efflux medium up to 2.5 units/ml had no significant effect on this efflux rate. However, 10 units/ml did significantly enhance the passive efflux of Ca$^{2+}$ from the sarcolemmal vesicles, indicating that the passive permeability characteristics of the membrane had been compromised. Further study of the time dependency of the efflux reaction (Figure 8) demonstrated that cholesterol oxidase could increase passive Ca$^{2+}$ efflux from the vesicles even in the presence of low cholesterol oxidase concentrations (1.5 units/ml) if the incubation time was substantially increased up to 5 minutes. This effect was accentuated in the presence of 10 units/ml cholesterol oxidase.

For the purpose of comparison, the effects of in situ cholesterol modification on another transsarcolemmal ion transport system, the Ca$^{2+}$ pump, was studied. ATP-dependent Ca$^{2+}$ uptake by the cardiac sarcolemmal vesicles was studied as a function of reaction time in the presence or absence of 4 units/ml cholesterol oxidase (Figure 9). The absolute activity for the control vesicles was $5.48 \pm 0.71$ nmol Ca$^{2+}$ taken up/mg/min, which is in the same range as values reported elsewhere.$^{10,16}$ As shown in the inset graph of Figure 9, cholesterol oxidase inhibited ATP-dependent Ca$^{2+}$ uptake by 22%, 36%, and 52% at 15, 30, and 60 seconds of reaction time, respectively.

The concentration dependence of the inhibition of ATP-dependent Ca$^{2+}$ uptake by cholesterol oxidase
was examined over the 60-second reaction time (Figure 10). Cholesterol oxidase concentrations above 1.0 units/ml significantly depressed this transport pathway. Cholesterol oxidase (10 units/ml) could almost reduce uptake completely.

Passive (ATP-independent) Ca\(^{2+}\) binding to cardiac sarcolemma was examined in the presence of varying concentrations of cholesterol oxidase. Because we were aware of the possibility that cholesterol oxidase may make the vesicles leaky and therefore expose more potential Ca\(^{2+}\) binding sites on the intravesicular surface of the sarcolemma, 1 \(\mu\)M A23187, a Ca\(^{2+}\) ionophore, was included in the reaction medium for both control and oxidase-treated membrane preparations. Thus, artifactual increases in sarcolemmal Ca\(^{2+}\) binding produced by the increased membrane permeability to Ca\(^{2+}\) were not a factor in the present series of experimental results. As shown in Table 1, cholesterol oxidase concentrations of 1–10 units/ml produced significant increases in sarcolemmal Ca\(^{2+}\) binding capacity. This peaked at 5.0 units/ml, where Ca\(^{2+}\) binding capacity almost doubled from control values. Extensive oxida-

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Time dependency of Na\(^{+}\)-Ca\(^{2+}\) exchange in cardiac sarcolemmal vesicles with (●) or without (▲) cholesterol oxidase (2.0 units/ml) treatment. Values are mean±SEM (n=3). Insert graph depicts same data presented as a percent of untreated values. Absolute values in the absence of treatment were 1.78±0.16, 5.97±0.19, 12.53±0.85, 22.04±2.17, and 22.70±2.16 nmol Ca\(^{2+}\)/mg for 0.5, 5, 30, and 60 seconds of reaction time, respectively.

![Figure 5](http://circres.ahajournals.org/)

**Figure 5.** Na\(^{+}\)-Ca\(^{2+}\) exchange examined in the presence of varying concentrations of cholesterol oxidase. Values represent mean±SEM of three to five experiments. Control values (100%) in the absence of cholesterol oxidase treatment were 4.14±0.78 nmol Ca\(^{2+}\)/mg/sec.

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Lineweaver-Burke plot of Na\(^{+}\)-Ca\(^{2+}\) exchange in untreated (●) and cholesterol oxidase-treated (▲) sarcolemmal vesicles. Absolute values for Na\(^{+}\)-Ca\(^{2+}\) exchange in untreated sarcolemma were 5.08±0.33, 7.22±0.39, 9.77±0.66, and 13.87±1.32 nmol Ca\(^{2+}\)/mg/sec at 10, 20, 40, and 100 \(\mu\)M Ca\(^{2+}\), respectively. Values represent mean±SEM of four experiments. If the standard error bar is not present, the data point was larger than the standard error.


FIGURE 7. Effects of varying concentrations of cholesterol oxidase on passive efflux of $^{45}$Ca$^{2+}$ from cardiac sarcolemmal vesicles. Efflux time was 30 seconds. Results are mean±SEM of four separate experiments. Values are presented as a percent of total vesicular $^{45}$Ca$^{2+}$ content at time 0 before initiation of efflux. Cholesterol oxidase, if present, was included only during the efflux period.

Sarcolemmal passive Ca$^{2+}$ binding was examined in the absence or presence of 2.0 units/ml cholesterol oxidase over a range of Ca$^{2+}$ concentrations (0.01–5 mM). At all [Ca$^{2+}$] examined, cholesterol oxidase treatment stimulated sarcolemmal Ca$^{2+}$ binding capacity (Figure 11).

Discussion

The results from the present study indicate that oxidation of in situ membrane cholesterol depressed two important transsarcolemmal Ca$^{2+}$ transport pathways in the heart: Na$^+-$Ca$^{2+}$ exchange and ATP-dependent Ca$^{2+}$ uptake. Cholest-4-en-3-one was the only oxidated cholesterol species produced in the membrane after incubation with cholesterol oxidase. The inclusion of excess catalase in the medium whenever cholesterol oxidase was present would rule out the possibility that hydrogen peroxide effects may have caused the alterations in ion flux. Further, large concentrations of H$_2$O$_2$ (5 mM) are required before an effect (30% inhibition) on Na$^+-$Ca$^{2+}$ exchange is produced.$^{10}$ This H$_2$O$_2$ would not be achieved under our assay conditions. Other nonspecific effects of including cholesterol oxidase in the assay medium are unlikely for two reasons. First, pretreatment of sarcolemmal membranes with cholesterol oxidase, and then centrifuging the membranes, resuspending the pellet, and assaying for Na$^+-$Ca$^{2+}$ exchange (now in the absence of cholesterol oxidase) resulted in a similar inhibition as including the enzyme in the reaction medium directly. Second, the use of a different preparation of cholesterol oxidase elicited similar effects on Na$^+-$Ca$^{2+}$ exchange. Thus, the effects of cholesterol oxidase on ion flux appear to be due to the generation of oxidated cholesterol in situ in the sarcolemmal membrane.

The possibility exists that the depression in ion movements may be due to the increase in membrane

FIGURE 8. Time dependency of passive Ca$^{2+}$ efflux from sarcolemmal vesicles in the presence of varying concentrations of cholesterol oxidase (n=3). Refer to Figure 4 for further experimental details.

FIGURE 9. Effect of cholesterol oxidase (CO) on sarcolemmal ATP-dependent Ca$^{2+}$ uptake over varying reaction times. CO-treated sarcolemmal Ca$^{2+}$ uptake as a percent of control untreated activity is shown (inset). Values are mean±SEM (n=4).
permeability. This is highly unlikely with respect to the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange reaction for several reasons. First, 2.0 units/ml cholesterol oxidase produced a 35–70% inhibition in the initial rate of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange (Figure 4), but 2.5 units/ml did not alter passive permeability characteristics of the membrane (Figure 7). Second, one would expect the inhibitory effect of cholesterol oxidase on Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange to increase as the reaction progressed in time if membrane permeability were a significant factor. Instead, the most dramatic effects of cholesterol oxidase were at the earliest time points of the reaction (Figure 4, inset). Third, the effects of cholesterol oxidase on exchange were observed after very short reaction times (0.5 second), whereas even after 30 seconds of incubation, passive efflux was unaffected at these lower concentrations of enzyme (Figures 7 and 8). Fourth, because the rate of Ca\textsuperscript{2+} accumulation via exchange is much greater than that lost through passive Ca\textsuperscript{2+} efflux, large increases in membrane leakiness are required before any effect on Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange would be observed.\textsuperscript{22} Thus, the data argue strongly for a direct inhibitory effect of oxidized membrane cholesterol on the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange pathway.

**TABLE 1. Passive Calcium Binding to Isolated Sarcolemmal Membrane Vesicles After Treatment With Varying Concentrations of Cholesterol Oxidase**

<table>
<thead>
<tr>
<th>Cholesterol oxidase (units/ml)</th>
<th>Ca\textsuperscript{2+} binding (nmol/mg)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>50.5±3.0</td>
<td>100</td>
</tr>
<tr>
<td>0.2</td>
<td>54.5±4.0</td>
<td>108±1</td>
</tr>
<tr>
<td>1.0</td>
<td>62.9±4.2</td>
<td>125±2\textsuperscript{*}</td>
</tr>
<tr>
<td>2.0</td>
<td>78.8±7.1</td>
<td>156±5\textsuperscript{*}</td>
</tr>
<tr>
<td>5.0</td>
<td>89.0±10.9</td>
<td>175±9\textsuperscript{*}</td>
</tr>
<tr>
<td>10.0</td>
<td>72.3±13.1</td>
<td>141±15\textsuperscript{*}</td>
</tr>
<tr>
<td>20.0</td>
<td>25.7±5.1</td>
<td>50±6\textsuperscript{*}</td>
</tr>
</tbody>
</table>

Values represent mean±SEM of four separate experiments. Cholesterol oxidase was included in the reaction medium with 1 \( \mu \text{M} \) A23187 and 1 mM Ca\textsuperscript{2+} for the entire reaction time (1 minute) at 37°C. \*p<0.05 vs. control (untreated preparation).

This may not be the case for the ATP-dependent pathway of Ca\textsuperscript{2+} uptake. Because the concentrations of cholesterol oxidase required to increase membrane permeability and inhibit ATP-dependent Ca\textsuperscript{2+} uptake are similar (Figures 8–10), the incubation times are similar (30–60 seconds), and because the rate of ATP-dependent Ca\textsuperscript{2+} uptake is slower than Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange, it is more difficult to separate the effects of cholesterol oxidase on active Ca\textsuperscript{2+} transport from passive Ca\textsuperscript{2+} flux. Certainly, it is clear that Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange is far more sensitive to oxidized cholesterol in the membrane than is the sarcolemmal Ca\textsuperscript{2+} pump.

The results may provide information on the relation of membrane cholesterol with these two sarcolemmal ion flux pathways. Oxysterols are less polar than cholesterol, occupy a higher molecular area in the membrane,\textsuperscript{23,24} and are distributed very differently in the membrane. Cholesterol, for example, tends to form cholesterol-rich domains in the membrane and is most effective on the hydrophobic region of the membrane bilayer. Conversely, oxysterols such as cholestenone are randomly distributed across the bilayer plane and do not form defined domains.\textsuperscript{23} It is clear that higher concentrations of membrane oxysterols will cause a disordering of the membrane lipids leading to an increase in membrane fluidity and permeability characteristics.\textsuperscript{24} This would account for our observations of enhanced passive Ca\textsuperscript{2+} efflux after extensive treatment of the membranes with cholesterol oxidase. However, membrane fluidity has been shown to be unaltered even after 24% of the membrane cholesterol was oxidized.\textsuperscript{25} Thus, although changes in membrane fluidity may explain the increased permeability properties of the membrane, it is unlikely to represent a viable explanation for the effects on Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange. Instead, the disturbance of cholesterol-rich domains in the membrane by the oxidation reaction may better explain the effects demonstrated on the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger. This would be consistent with our previous work,\textsuperscript{10} which also suggested that a cholesterol-rich annulus may be associated with the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange protein.
As stated above, oxidation of in situ cholesterol would alter the hydrophobic region of the membrane. Because cholesterol primarily exerts its effects in the hydrophobic core of the membrane bilayer, our results suggest that the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is more sensitive to this region of the membrane. This interpretation is consistent with the data of Philipson and Ward, who used doxyl-stearates and suggested that the hydrophobic region of the membrane may be the most important area for affecting the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange reaction. However, this does not detract from the significant effects that modification of the hydrophilic head group can have on Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange.

The increase in passive Ca\textsuperscript{2+} binding to the sarcolemma after oxidation of the membrane cholesterol is unclear. The observation of a dissociation between the bulk Ca\textsuperscript{2+} binding to the membrane and Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange (stimulation of binding and inhibition of exchange) is not novel. Other agents have also produced similar qualitative effects, which further strengthen the argument that the two processes are unrelated.

It is important to emphasize that we are not proposing a role for the enzyme cholesterol oxidase in cardiac pathophysiology. It is found in tissues of the body other than the heart. Instead, the enzyme was used to selectively oxidize membrane cholesterol to understand the effects of oxidized cholesterol (which can be generated throughout the body) on ion movements.

Our observations may have implications with regard to the mechanism of oxygen free radical action on the heart. Oxygen free radicals have been demonstrated to play a role in ischemic/reperfusion damage in the heart. A depression in Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange in isolated sarcolemmal vesicles and enhanced membrane permeability have been reported during ischemic/reperfusion challenge. Free radicals can oxidize membrane cholesterol, increase membrane permeability, and inhibit Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange, although the latter may be controversial at this time. As shown in the present study, oxidized membrane cholesterol can depress sarcolemmal Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange and increase membrane permeability. The data are consistent, therefore, with the possibility that free radicals may alter ion movements across the sarcolemmal membrane during ischemic/reperfusion challenge through an oxidation of membrane cholesterol residues as well as other sites on the membrane like target proteins or fatty acid residues. However, further experiments to determine the presence of oxidatively modified membrane cholesterol in ischemic/reperfused hearts is required.

Oxidatively modified lipids contained in low density lipoproteins have been recently proposed to play an important role in atherosclerosis. Although it is clear that oxidized lipids like cholesterol may enter the cell via a low density lipoprotein receptor–independent scavenger pathway, the role of the oxidatively modified cholesterol once it is deposited within the cell is not known. Our data suggest the possibility that this oxidized lipid may alter ionic interactions with the muscle cell.

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