Effects of peroxynitrite on sarcoplasmic reticulum Ca\(^{2+}\) pump in pig coronary artery smooth muscle

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Peroxynitrite is a reactive product of NO and superoxide and has been implicated in vascular dysfunction. However, the effects of peroxynitrite on the sarcoplasmic reticulum Ca\(^{2+}\) pump in coronary smooth muscle are not known. We tested the effect of peroxynitrite on the SERCA2 pump in pig coronary artery with sarcoplasmic reticulum-enriched vesicular membranes. Peroxynitrite treatment of these membranes resulted in a concentration-dependent inhibition of Ca\(^{2+}\) uptake and acylphosphatase formation, associated with a 34% decrease in the half-maximal inhibitory concentration of ATP. Peroxynitrite also cross-linked the 110-kDa SERCA2 pump. Inhibition of Ca\(^{2+}\) uptake and PMCA activity in pig coronary artery vascular smooth muscle was also inhibited by peroxynitrite, but pretreatment with DTT abolished the inhibitory effect of peroxynitrite. Peroxynitrite is a powerful oxidant and therefore is likely to damage the pump. We conclude that peroxynitrite is a potent inhibitor of the SERCA of coronary artery smooth muscle, and its action is mediated by covalent modification of the pump.

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cellular membrane fractionation as previously reported (22). The tissue used in the membrane preparation was predominantly smooth muscle devoid of endothelium, adventitia, erythrocytes, and cardiac myocytes (22). On the basis of the biochemical markers previously described, the gradient fraction F2 (band just above 28% sucrose) is enriched in PM, and gradient fraction F3 (band just above 40%) is rich in SR (22).

Peroxynitrite pretreatment. Aliquots of peroxynitrite (Calbiochem) under nitrogen were stored at −80°C and used within 3 mo. The peroxynitrite concentration was determined by its absorbance at 302 nm (extinction coefficient = 1.670 M⁻¹·cm⁻¹) in 0.1 M NaOH immediately before use. Peroxynitrite was used as a solution in 0.1 M NaOH, and all the control samples also contained the same concentration of peroxynitrite. Peroxynitrite was used as a solution in 0.1 M NaOH, and all the control samples also contained the same concentration of peroxynitrite.

RESULTS

Comparison of effects of peroxynitrite on PM- and SR-enriched membrane fractions. Two membrane fractions were used in the initial work, F2 and F3. Previous characterization of these fractions using biochemical markers shows that F2 is enriched in PM and F3 in SR (22). We used PMCA- and SERCA-selective antibodies in Western blots to confirm that F2 is more enriched in PMCA and F3 in SERCA (not shown). Figure 1 shows the ATP-dependent azide-insensitive (nonmitochondrial) Ca²⁺ uptake by F2 and F3 fractions. Because both phosphate and oxalate prevent back flux of Ca²⁺ by promoting its crystallization inside the vesicles but oxalate works preferentially in the SR membranes, we examined the uptake in the presence of 5 mM phosphate with F2 and 5 mM oxalate with F3. Figure 1 shows that the uptake was linear with time for up to 120 min for F2 and F3 in control and peroxynitrite (200 μM)-pretreated membranes. The pretreatment with 200 μM peroxynitrite decreased the uptake by 75 ± 2% (from 13.4 ± 0.5 to 3.3 ± 0.3 nmol·mg protein⁻¹·min⁻¹) in the F3 fraction but by only 30 ± 5% (from 2.0 ± 0.1 to 1.4 ± 0.1 nmol·mg protein⁻¹·min⁻¹) in F2. An uptake time of 60 min was used in all subsequent experiments even though the uptake was linear up to 120 min.

Figure 2A shows a summary of five experiments on the effects of different concentrations of peroxynitrite on the Ca²⁺ uptake by F2 and F3 fractions. The inhibition was more severe for F3 than for F2. The best fit for the inhibition data for F3 gave an inhibition constant (Kᵢ) of 56 ± 4 μM and a Hill coefficient of 1.49 ± 0.15. For F2, the data fit best with a model in which F2 contains some SR as an impurity, and the inhibition observed in this fraction stems solely from this impurity, i.e., has the same Kᵢ and Hill coefficient values but only a small fraction of the uptake can be inhibited by peroxynitrite (Fig. 2A). To confirm this finding, we inhibited the Ca²⁺ uptake due to the SERCA pump with 1 μM thapsigargin. This concentration of thapsigargin inhibited the Ca²⁺ uptake in the F3 fraction by
93%. Using 10 μM thapsigargin does not produce significantly greater inhibition \((P > 0.05)\). In the F2 fraction, however, the thapsigargin inhibition is not as pronounced (Fig. 2B). Thapsigargin inhibits by \(60\%\) with the thapsigargin-insensitive component being \(40\%\). When or without the treatment, the \(\text{Ca}^{2+}\) uptake was linear over the 120-min period \((r^2 = 0.9785\) and 0.9867, respectively) in the F3 fraction. The \(\text{Ca}^{2+}\) uptake in samples treated without peroxynitrite was taken as 100%, and all other values for F2 were compared with this value. Similarly, the mean value of the \(\text{Ca}^{2+}\) uptake by the F3 fraction treated without peroxynitrite was taken as 100%, and all other values for F3 were compared with this value. The data did not fit well when maximum inhibition was fixed to \(100\%\) and \(K_i\) and the Hill coefficient were varied. These data did not fit well when maximum inhibition was fixed to \(100\%\) and \(K_i\) and the Hill coefficient were varied.

The \(\text{Ca}^{2+}\)-dependent formation of acylphosphates from ATP is the first step in the reaction cycle of both the PMCA and SERCA pumps, but the acylphosphates formed from the PMCA are 140 kDa compared with 110 kDa formed from the SERCA pump.
kDa band. Thus, at these concentrations, peroxynitrite inhibited mainly the SERCA pump. Hence, all subsequent experiments were conducted on the F3 fraction.

Other ROS as possible intermediates. Peroxynitrite at neutral pH can spontaneously decompose to produce superoxide, catalase, and low concentrations of hydroxyl radicals (15, 31). Therefore, we determined whether the inhibition of Ca$^{2+}$ uptake in F3 by peroxynitrite was due to the generation of hydrogen peroxide, superoxide, or hydroxyl radicals formed during the reaction. We have shown previously that catalase plus SOD can overcome the effect of superoxide and that catalase alone can abolish the effect of hydrogen peroxide in this system (17, 20). Including excess catalase plus SOD with or without mannitol (to quench hydroxyl radicals) does not prevent the inhibition due to peroxynitrite ($P > 0.05$).

Table 1. Catalase activity after preincubation with peroxynitrite

<table>
<thead>
<tr>
<th>Peroxynitrite, µM</th>
<th>Catalase Activity, %Control</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>250</td>
<td>108 ± 8</td>
</tr>
<tr>
<td>500</td>
<td>109 ± 4</td>
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Values are means ± SE of 3 experiments on different days, normalized to the catalase activity in the 0 µM control as 100. The values at any of the concentrations did not differ from the control ($P > 0.05$). See METHODS for details.
an increased leakiness. Acylphosphates formed from P_i gave a major band at 105–110 kDa (reverse reaction, Fig. 5B). Peroxynitrite treatment also inhibited the formation of acylphosphates from P_i. However, the inhibition was poorer than that of the Ca^{2+} uptake (Fig. 5E). Further analysis also showed that the inhibition of the acylphosphates from P_i was poorer than that from ATP (P < 0.05).

We also examined the effects of peroxynitrite treatment on the Ca^{2+} pump protein in Western blots by using a SERCA2-specific monoclonal antibody, 4G5. Samples treated without peroxynitrite gave a major band at 105–110 kDa as expected (Fig. 5C). However, the treatment with peroxynitrite gave an additional very high molecular mass band (Fig. 5C). The expected size of the dimer would be 210–220 kDa. However, the oligomers moved considerably slower than the 250-kDa molecular mass marker, indicating their size to be much larger than dimers. We analyzed the cross-linked samples in 6% acrylamide gels, running the gels for 4 h even though the dye band had left the gels at 2.5 h and using laminin (200 and 400 kDa) as an additional marker. The oligomer had a mobility similar to that of the 400-kDa laminin subunit. The intensity of the oligomers increased with the increasing peroxynitrite concentration (Fig. 5C). Figure 5C also shows that no dimers (220 kDa) were observed even at low concentrations of peroxynitrite when only a small fraction of the SERCA2 protein had formed the oligomers. For each lane we computed the reactivity of the oligomers with the antibody as a percentage of the total reactivity with oligomers plus the monomer (Fig. 5F). This value increased with the inhibition of the Ca^{2+} uptake observed in these membranes, but its slope was significantly less than 1 (P < 0.05). The slope of the percent oligomers formed was also significantly different from the acylphosphates formed from ATP but not those formed from P_i.

Effect of hydrogen peroxide. We have reported previously that hydrogen peroxide treatment inactivates the SERCA pump in the coronary artery smooth muscle (18, 20). However, it is not known if DTT can reverse the effect of this treatment, how this treatment affects the formation of acylphosphates from P_i, and whether it causes protein aggregation. Therefore, we repeated the experiment as in Fig. 5 but used only one concentration (500 μM) of hydrogen peroxide. Sodium azide was included in the reaction mixture to prevent the breakdown of peroxide by intrinsic catalase or myeloperoxidase. Peroxide treatment produced an inhibition of the uptake and 1 mM DTT added after incubation with peroxide did not reverse this effect (Fig. 6A). There was also a parallel inhibition of the acylphosphate formation from ATP (Fig. 6B) and inhibition of the acylphosphate did not appear to lag behind significantly (Fig. 6C). The treatment also produced an ag-
aggregation of the pump protein. The aggregates had the same pattern and size as those produced on the peroxynitrite treatment. Percent protein in the aggregate did not appear to lag behind the inhibition of the uptake (Fig. 6D).

DISCUSSION

The results show that 1) peroxynitrite treatment inhibits the SERCA pump preferentially over the PMCA pump; 2) this effect is not prevented by including catalase, SOD, and mannitol with the peroxynitrite; 3) inhibition of the Ca\(^{2+}\) uptake parallels that of the acylphosphate formation from ATP; 4) inhibition of the acylphosphate formation from P\(_i\) is slightly poorer; 5) the treatment leads to formation of oligomers with a molecular weight significantly higher than that of a dimer; 6) formation of the oligomers is slightly poorer than the inhibition of the Ca\(^{2+}\) uptake; 7) treatment with hydrogen peroxide also leads to inhibitions of Ca\(^{2+}\) uptake and the acylphosphate formation, and it gives oligomers of the same molecular weight as the peroxynitrite pretreatment; and 8) these effects of peroxynitrite or hydrogen peroxide cannot be reversed with excess DTT. Below, we focus on the methods used in this study, their possible implications on the pump structure and function, and the biological implications of these findings.

Because pure PM or SR membranes cannot be obtained from coronary artery smooth muscle, we used the effect of oxalate on the inhibition of back flux of Ca\(^{2+}\) to identify the activity in the SR, the acylphosphates from ATP to distinguish between the effects on the PM (140 kDa) and SR (110 kDa), and antibodies to verify these results. We also confirmed, using thapsigargin in the F2 fraction, that SERCA pump is more sensitive to peroxynitrite than is the PMCA pump. The membrane fractions used contain high levels of interfering ATPases and much smaller amounts of the SERCA pump protein compared with tissues such as cardiac and skeletal muscle. Therefore, direct assays of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPases are not possible. Hence, we have verified our results at the levels of acylphosphate formation in forward and reverse directions to establish that the observed effects of peroxynitrite are on the pump rather than on membrane leakiness. Furthermore, in experiments using catalase, SOD, and mannitol we show that the effect of peroxynitrite on the pump does not involve peroxide, superoxide, or hydroxyl radicals. Also, these effects are not reversed by excess DTT, suggesting that the reaction goes beyond simple disulfide bond formation or S-nitrosylation. It is attractive to think of a tyrosine nitration pathway, because Y294, Y295, and Y793 may be nitrated in SERCA2a by peroxynitrite (36). More recently, it has been shown that tyrosine nitration has also been demonstrated in the SERCA pump in aortic smooth muscle (1). However, hydrogen peroxide also produced inhibition of the pump that was not reversed by DTT, and it produced oligomers similar to those produced by peroxynitrite. Peroxynitrite may produce protein cross-linking via ditryptophan (32), but dityrosine is also formed upon free radical treatment of SERCA1 (32, 37). Thus reactions with hydrogen peroxide and peroxynitrite may occur via different pathways but produce similar end results.

Correlation of parameters monitored. The peroxynitrite treatment of the coronary artery F3 fraction produced inhibition of the SERCA pump, acylphosphate formation from ATP and P\(_i\), and also caused oligomerization of the pump protein. However, the inhibition of the acylphosphate formation from P\(_i\) was slightly poorer. There are several possible explanations. The acylphosphate formation from ATP would be inhibited when any one of the following are inhibited: high-affinity Ca\(^{2+}\) binding site, ATP binding site, or the acylphosphate formation site. In contrast, the inhibition of the acylphosphate formation from P\(_i\) would require only one site to be damaged. The reaction with P\(_i\) was carried out in the presence of 40% DMSO, which may have solubilized the lipid annulus of this protein. The treatment may also confer a preference of E2 conformation over the other. Thapsigargin and melittinin

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produce such conformational preferences (3, 39). Aggregation of the pump protein also accompanied the melittinin inhibition, but the size of individual oligomer species was not determined (38). Peroxynitrite treatment of SERCA2a in slow-twitch muscle followed by Western blots also shows the loss of monomeric species (36).

Quaternary structure of the SERCA pump. Current models on interactions between Ca2+ pump subunits are based on skeletal muscle (2, 26). SERCA1, when in the membrane, has been proposed to be a dimer, although in the presence of detergents monomers it may be fully functional (2, 26). Radiation inactivation of SERCA1 in fast-twitch skeletal muscle and of SERCA2a in heart give target sizes consistent with a dimeric structure (2, 9). Cross-linking of SERCA1 also gives mainly dimers but also trimers and tetramers (6). Figure 5 clearly shows that no SERCA2 dimers are formed even upon treatment of the coronary artery membranes at low peroxynitrite concentrations that cause oligomerization of only a fraction of this protein. Only higher molecular weight oligomers are formed, and they appear to be tetramers. This result suggests that the organization of the Ca2+ pump protein in the coronary artery SR differs from that in the skeletal and cardiac SR. In radiation inactivation experiments, the target size of the SERCA pump protein in the coronary artery is much larger than in the cardiac muscle (19).

Therefore, we speculate that the SERCA pump in the coronary artery may be present as a much larger oligomeric complex than in the skeletal or cardiac muscle, but it is not known whether it contains many subunits of this protein or also regulatory proteins.

Physiological implications. The effects of peroxynitrite may be important in aging, inflammation, and atherosclerosis. The SERCA pump activity in rat heart decreases with aging. SERCA2a in rat twitch skeletal muscle shows that nitrated tyrosine residues increase with aging (35, 36). Such data are not currently available for the coronary artery. Protein aggregates formed at high concentrations of peroxynitrite may represent irreversible tissue damage, and hence it should be investigated whether a “denitrase” activity may prevent the loss at an earlier stage (27). The sensitivity of the SERCA pump to peroxynitrite and other ROS in a cell may depend on their levels of antioxidants (such as glutathione and ascorbate), the type of ROS degrading enzymes, and the SERCA isoforms they express. The SERCA pump in endothelium is relatively insensitive to peroxide because endothelium is enriched in catalase and also expresses SERCA3 gene in addition to the SERCA2 gene expressed in the coronary artery smooth muscle (16, 18, 29). The tyrosine residues (Y294 and Y295) shown to be nitrated in SERCA2a are flanked by similar sequences in SERCA2 (GAIYYFKIAV) and SERCA3 (GAVVYFKIAC) (GenBank accession nos. X58291 and AF068220). Similarly, the tyrosine residues (Y753) shown to be nitrosylated in SERCA2a are flanked by similar sequences in SERCA2 (AIYN-NMKQFI) and SERCA3 (AIYSNMKQFI). In HEK-293 T cells overexpressing the pump proteins, SERCA2a protein is more sensitive to peroxide than is SERCA3 (23). It would be of interest to determine whether the difference in susceptibility between SERCA2 and SERCA3 also holds for peroxynitrite, particularly when exposure to this agent is extremely likely for endothelium. Thus the present study has resolved some issues on the effects of peroxynitrite on the coronary artery SERCA pump and raised several other questions of biological and mechanistic importance.

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REFERENCES


