Singlet Oxygen Interaction With Ca\textsuperscript{2+}-ATPase of Cardiac Sarcoplasmic Reticulum

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We investigated the role of singlet oxygen (generated from photoactivation of rose bengal) on the calcium transport and Ca\textsuperscript{2+}-ATPase activity of cardiac sarcoplasmic reticulum (SR). Isolated cardiac SR exposed to rose bengal (10 nM) irradiated at 560 nm resulted in significant inhibition of Ca\textsuperscript{2+} uptake (from 2.27±0.05 to 0.62±0.05 μmol Ca\textsuperscript{2+}/mg·min [mean±SEM], \(p<0.01\)) and Ca\textsuperscript{2+}-ATPase activity (from 2.08±0.05 to 0.28±0.04 μmol P\textsubscript{i}/min·mg [mean±SEM], \(p<0.01\)). The inhibition of calcium uptake and Ca\textsuperscript{2+}-ATPase activity by rose bengal–derived activated oxygen (singlet oxygen) was dependent on the duration of exposure and intensity of light. Singlet oxygen scavengers ascorbic acid and histidine significantly protected SR Ca\textsuperscript{2+}-ATPase against rose bengal–derived activated oxygen species, but superoxide dismutase and catalase did not attenuate the inhibition. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of SR exposed to photoactivated rose bengal for up to 14 minutes demonstrated complete loss of the Ca\textsuperscript{2+}-ATPase monomer band, which was significantly protected by histidine. The addition of dithiothreitol (5 mM) had a slight protective effect, showing that new disulfide bond formation was not a major cause of aggregation. The results were also confirmed by high-performance liquid chromatography of the SR exposed to irradiated rose bengal. Irradiation of rose bengal also caused an 18% loss of total sulfhydryl groups of SR. On the other hand, superoxide radical (generated from xanthine oxidase action on xanthine) and hydroxyl radical (in the presence of Fe\textsuperscript{3+}-EDTA or 0.5 mM H\textsubscript{2}O\textsubscript{2} plus Fe\textsuperscript{3+}-EDTA) as well as H\textsubscript{2}O\textsubscript{2} (0.25–12 mM) were without any effect on the 97,000-d Ca\textsuperscript{2+}-ATPase band of SR. Generation of radical species (superoxide and hydroxyl radical) from rose bengal was studied by electron paramagnetic resonance spectroscopy using the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The results showed that irradiation of rose bengal formed a 1:2:2:1 quartet, characteristic of the DMPO-OH adduct, which was scavenged by ethanol but not by superoxide dismutase, catalase, or histidine. No radical species could be detected from irradiated rose bengal or irradiated DMPO under the assay conditions used. Peroxy adducts of DMPO might be produced but would be observed only at very low temperatures. Similarly, we could not detect any measurable \(\cdot\text{O}_2^-\) anion from irradiation of rose bengal as indicated by either cytochrome \(c\) reduction at 550 nm or nitro blue tetrazolium reduction at 560 nm. These results show that SR is damaged most likely by singlet oxygen derived from rose bengal. This may represent an important mechanism by which the oxidative injury to the myocardium induces both a loss of tension development and arrhythmogenesis. (Circulation Research 1991;69:1003–1014)
ultrastructural changes (contracture). It is also now well appreciated that the first primary target organ-
elles attacked by the ischemic process is that portion of the excitement–contraction coupling system that regulates calcium delivery (the sarcotubule and sar-
coplasmic reticulum [SR]). These caparable cardiac SR, which regulates intracellular calcium, is one of the major target organelles injured by this process. It is an intracellular organelle that rapidly sequesters and releases calcium, thereby regulating muscle relaxa-
tion and contraction. Sequestration of the released calcium into the SR lumen is mediated by magne-
sium-dependent Ca$^{2+}$-ATPase. Several in vitro stud-
ies from our laboratory have shown that this enzyme is not susceptible to inactivation by ·O$_2^-$ anion.$^{10,11}$ Hydrogen peroxide has an inhibitory effect on this enzyme only at high nonphysiological concentrations (1–10 mM).$^{11}$ Damage to this enzyme by hydroxyl radical has not been clearly demonstrated because this radical causes iron-dependent lipid peroxidation, which might have a role in inhibiting the enzyme activity.$^{11}$

One species of activated oxygen, singlet oxygen, has received little attention in the past, especially in relevance to ischemia and reperfusion damage in cardiac muscle. Singlet oxygen is an electronically excited state of oxygen that results from the promotion of an electron to higher energy orbitals. It is a short-lived species with a lifetime of 10$^{-6}$ seconds, and its energy above the ground-state level is 22.5 kcal.$^{12}$ Unlike ·O$_2^-$ or ·OH radical, evidence for the formation of singlet oxygen in vivo does not exist at present for two reasons. First, singlet oxygen has a very short life, and second, unlike ·O$_2^-$ and ·OH radicals, there are no highly specific traps available to detect this species in vivo. However, some of the sources that can theoretically generate free radicals in vivo, such as catecholamine oxidation,$^{13}$ prostaglandin pathways,$^{14,15}$ mitochondria,$^{16}$ xanthine ox-
idase (which is not thought to be present in human heart),$^{17,18}$ and invading leukocytes,$^{19}$ may potentially form singlet oxygen too, but the quantitative aspects of their physiological feasibility remains uncertain. Recent studies from Hearse’s group$^{20,21}$ have shown that singlet oxygen generated from photoactivated rose bengal can lead to ultrastructural changes that resemble those associated with severe injury during myocardial ischemia. Stuart and Abramson$^{22}$ have recently reported that the photoactivation of rose bengal can induce calcium release from isolated SR vesicles, and this can be inhibited by caffeine. In studies with isolated papillary muscles it has been demonstrated that singlet oxygen induces a transient positive inotropic response followed by negative inotropic effect and the development of contracture.$^{23}$ These findings suggest that singlet oxygen is also able to induce damage similar to ischemia and reperfu-
sion–induced injury. The purpose of the present investiga-
tions is 1) to show that singlet oxygen is capable of inducing damage in cardiac SR and 2) to compare singlet oxygen–mediated damage to that caused by ·O$_2^-$, H$_2$O$_2$, and ·OH radical. Using sodium dodecyl sulfate–polyacrylamide gel electro-
phoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) techniques, we show here that singlet oxygen severely damages SR Ca$^{2+}$-
ATPase and that the singlet oxygen scavenger histi-
dine demonstrated a significant protective effect.

**Materials and Methods**

**Generation of Singlet Oxygen ·O$_2^-$ Anion and ·OH Radical**

Singlet oxygen was generated by photoexcitation of the light-sensitive dye rose bengal, which is one of the most efficient sources of singlet oxygen generation.$^{24,25}$ Rose bengal is a fluorescein derivative that absorbs light strongly in the range of 530–590 nm, with two major absorptions (caused by the xanthene chromophore) at approximately 530 and 558 nm (depending on solvent and pH). It is a tetrachloridate of 4,5,6,7-
tetrachlorofluorescein converted to potassium salt. The system consisted of a cable delivering 530–590-nm light. The intensity of light delivered in the calcium uptake bath was controlled by a rheostat. The light intensity was measured by Lux Meter (model Lx-101).

Superoxide anion was generated by xanthine oxidase action on xanthine and was quantitated spectrophotometrically by superoxide dismutase (SOD)—inhibitable cytochrome c reduction at 550 nm or nitro blue tetrazo-
lum reduction at 560 nm.

Hydrogen peroxide was purchased commercially (Sigma Chemical Co., St. Louis, Mo.), and its concen-
tration was estimated spectrophotometrically by measuring its absorbance at 240 nm using the extinction coefficient ($\varepsilon = 81 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Hydroxyl radical was generated either by xanthine oxidase (0.05 units/ml) action on xanthine (100 μM) plus Fe$^{3+}$-EDTA (100 μM) or by H$_2$O$_2$ (0.5 mM) in the presence of Fe$^{2+}$-EDTA. Both of these systems generate ·OH radical by Fenton’s reaction as follows:

\[
\begin{align*}
\text{xanthine oxidase} & \rightarrow \text{urate}^- + \cdot \text{O}_2^- \\
\cdot \text{O}_2^- + \cdot \text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
\cdot \text{O}_2^- + \text{EDTA-Fe}^{3+} & \rightarrow \text{O}_2 + \text{EDTA-Fe}^{2+} \\
\text{H}_2\text{O}_2 + \text{EDTA-Fe}^{2+} & \rightarrow \text{EDTA-Fe}^{3+} + \text{OH}^- + \cdot \text{OH}
\end{align*}
\]

SR protein (200 μg/ml) was incubated with the ·OH radical–generating systems for 45 minutes before it was subjected to SDS-PAGE.

**Electron Paramagnetic Resonance Spin Trapping**

The spin trapping studies of free radicals were performed by mixing rose bengal (100 nM) with 100 mM of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and illuminating for 10 minutes with light at a wavelength of 560 nm. Care was taken to keep the DMPO solution covered to prevent light-
induced degradation. The DMPO was purchased from Aldrich Chemical Co., Milwaukee, Wis., and further purified by filtration through charcoal.

Electron paramagnetic resonance spectra were recorded in flat cells at room temperature with an IBM-Bruker ER 300 spectrometer operating at X-band using 100 kHz modulation frequency and with TM 110 cavity. The microwave frequency and magnetic field were precisely measured using a microwave frequency counter (model 5342A, Hewlett-Packard Co., Palo Alto, Calif.) and Bruker ER 035M NMR Gaussmeter. Spectral simulations were performed using simulation programs that assume isotropic g and A tensors, written in either BASIC or ASYST as described previously.

Isolation of Sarcoplasmic Reticulum

Cardiac SR from dog left ventricle was isolated according to the method published previously with modifications. Briefly, the left ventricle was minced and homogenized in imidazole buffer and centrifuged at 4,000g for 20 minutes. The supernatant was saved and the pellet was again homogenized and centrifuged. The combined supernatants were filtered and centrifuged at 31,000g for 2 hours. The pellet was homogenized and layered on top of a discontinuous sucrose gradient and centrifuged overnight at 50,000g. The SR vesicles were harvested, diluted, and centrifuged again. Protein was measured by the method of Lowry et al.

Phototirradiation of Sarcoplasmic Reticulum Membranes

For studies involving SDS-PAGE, SR membranes (1 mg/ml) were suspended in 10 mM imidazole buffer (pH 7.0) and 50 nM rose bengal in a borosilicate test tube placed in a 37°C water bath. The water bath was irradiated with 530–590-nm light from the bottom for the time periods indicated in the figure legends. Except for the rose bengal and protein concentrations, all other experimental conditions were similar unless indicated otherwise in the figure legends.

Oxalate-Supported Calcium Uptake

The rate of oxalate-supported uptake was estimated by assay at various times for the disappearance of $^{45}$Ca from filtrates of the reaction bath using 0.45 μm filters (Millipore Corp., Bedford, Mass.). The reaction bath was maintained at 37°C and contained 104 mM KCl, 10 mM imidazole buffer, 10 mM potassium oxalate, 5 mM Na₂ATP, 0.18 mM added CaCl₂, and 0.0375 mg/ml cardiac SR.

Measurement of Ca²⁺-ATPase Activity

The Ca²⁺-ATPase activity of SR was measured by the coupled enzyme assay described earlier. The reaction mixture was kept at 37°C and contained 100 mM KCl, 20 mM MOPS (pH 7.0), 2 mM MgCl₂, 0.1 mM CaCl₂, 0.213 μmol NADH, 1 mM phosphoenolpyruvate, 17.5 units pyruvate kinase, 25 units lactate dehydrogenase, and 1 μM ionophore A23187. The SR concentration was 15–20 μg/ml. The rate of disappearance of NADH was monitored at 340 nm, and the activity was expressed as micromoles P₁ liberated per minute per milligram. The extinction coefficient used for NADH was 6.2 mM⁻¹. The reaction rate in the absence of 1 mM EGTA was subtracted from the rate after the addition of EGTA to obtain Ca²⁺-ATPase activity.

Fragmentation and Aggregation of Ca²⁺-ATPase by Irradiated Rose Bengal

Aggregation and fragmentation of the Ca²⁺-ATPase monomer band by exposure to singlet oxygen was measured by SDS-PAGE. These experiments use 7% SDS in the absence of urea with 50 μg SR protein per lane. The SR was incubated with 50 nM rose bengal with and without exposure to light for various times (2–14 minutes). The gels were stained with Coomassie brilliant blue.

High-Performance Liquid Chromatography of Ca²⁺-ATPase

Because results from SDS gels depend heavily on the ability of proteins to stain with Coomassie brilliant blue, additional experiments were performed in which the SR exposed to singlet oxygen was subjected to gel filtration on a molecular sieve HPLC column. The protein concentration in the effluent was monitored by the optical density at 280 nm, and thus the assay did not involve the bias of Coomassie brilliant blue staining. Gel filtration chromatography was carried out essentially as described by Barrabin et al. Samples of SR exposed to irradiated rose bengal with or without histidine were prepared by dissolving the protein in elution buffer containing 1% lithium doxyl sulfate and 0.05 M lithium acetate, pH 4.5. The samples were filtered through 0.45-μm Millipore filters, and 20 μl of the filtered sample was injected into an HPLC system (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) equipped with a size exclusion column type TSK G 3000SW (Tojo Sada). The column was eluted with elution buffer at 0.5 ml/min at room temperature. The eluate was monitored for optical density at 280 nm using an LKB 2138 Uvicord S.

Determination of Thiol Groups

The sulfhydryl group oxidation of SR by singlet oxygen was determined using DTNB (5,5'-dithiobis[2-nitrobenzoic acid] or Ellman's reagent). This reagent has been used extensively to determine sulfhydryl concentration at pH 8.0. DTNB provides a convenient optical assay for studying sulfhydryl group oxidation because it does not interact with disulfides and exhibits a large change in absorption at 412 nm on reacting with sulfhydrys. SR vesicles were incubated with rose bengal (50 nM) and irradiated for 15 minutes at 37°C. After exposure, the SR (200 μg) was added to the reaction mixture (2 ml) containing 0.1 M Tris-HCl buffer (pH 8.0), 1 mM EDTA, 1% SDS,
1 mM DTNB, and 200 μg/ml SR. At the end of 10 minutes, absorbance at 412 nm was measured.

Results

Effect of Singlet Oxygen, Superoxide Radical, and Hydrogen Peroxide on Ca\(^{2+}\)-ATPase and Calcium Uptake

Isolated cardiac SR calcium uptake when exposed to illuminated rose bengal was significantly inhibited from 2.27±0.05 to 0.62±0.05 μmol Ca\(^{2+}\)/min·mg (mean±SEM, \(p<0.01\)) and was significantly protected by the singlet oxygen scavenger ascorbic acid (Figure 1). The inhibition of calcium uptake was dependent on the intensity of illumination of rose bengal. As shown in Figure 2, the maximum inhibition of calcium uptake was with rose bengal (10 nM) and 6,000 lux of light. Rose bengal or different intensities of light alone were without any significant effect on the calcium uptake. Similarly, Ca\(^{2+}\)-ATPase activity was inhibited from 2.08±0.05 to 0.28±0.04 μmol P/min·mg and was significantly protected by 1 and 10 mM histidine (\(p<0.01\)) (Figure 3). SOD or catalase was without significant protective effects (\(p>0.01\)). The inhibition of Ca\(^{2+}\)-ATPase activity was also dependent on the intensity of illumination in the presence of rose bengal (Figure 4). In contrast, 30 minutes of incubation of SR with \(\cdot\)O\(_2\) or H\(_2\)O\(_2\) generated from xanthine oxidase (0.05 units/ml) action on xanthine was without any significant (\(p>0.01\)) effect on either Ca\(^{2+}\) uptake or Ca\(^{2+}\)-ATPase activity (Table 1). Increasing xanthine oxidase concentrations up to 0.15 units/ml did not have an inhibitory effect either. Also, extended periods of incubation (up to 45 minutes) with xanthine plus xanthine oxidase did not show any inhibitory effect on SR, although some degradation of SR was noticed in the controls. The ineffectiveness of H\(_2\)O\(_2\) or xanthine plus xanthine oxidase in inhibiting Ca\(^{2+}\)-ATPase was not due to endogenous contamination of catalase activity in SR. We have previously shown that H\(_2\)O\(_2\) concentrations (5 mM or less) in the presence of 3-amino-1,2,4-triazole (1 mg/ml), an inhibitor of catalase, failed to inhibit SR function significantly.\(^{10}\) There-
fore, H₂O₂ either produced by the xanthine plus xanthine oxidase system or added exogenously was not destroyed by endogenous catalase.

Effect of Irradiated Rose Bengal on Lipid Peroxidation

Because Ca²⁺-ATPase is a lipid-requiring system, altering the lipid structure by peroxidation of fatty acid moieties by singlet oxygen might be the cause of inactivation of calcium transport and Ca²⁺-ATPase activity. Using the thiobarbituric acid (TBA) technique for detecting malondialdehyde, we made an attempt to learn if singlet oxygen caused SR lipids to undergo peroxidation. We found absolutely no evidence of lipid peroxidation by irradiated rose bengal. Even extended incubation with photosensitized rose bengal did not show any evidence of TBA reactive materials.

**TABLE 1. Effect of \( \cdot \text{O}_2^- \) and H₂O₂ Generated From the Xanthine/Xanthine Oxidase System on Calcium Uptake and Ca²⁺-ATPase Activity of Isolated Cardiac Sarcoplasmic Reticulum**

<table>
<thead>
<tr>
<th></th>
<th>Calcium uptake rate (µmol/min · mg)</th>
<th>Ca²⁺-ATPase activity (µmol/min · mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control SR</td>
<td>2.23±0.05</td>
<td>1.92±0.10</td>
</tr>
<tr>
<td>Control SR (with xanthine only)</td>
<td>2.21±0.06</td>
<td>1.88±0.15</td>
</tr>
<tr>
<td>Control SR (with xanthine oxidase only)</td>
<td>2.22±0.07</td>
<td>1.86±0.17</td>
</tr>
<tr>
<td>SR (with xanthine + xanthine oxidase)</td>
<td>2.20±0.09</td>
<td>1.87±0.11</td>
</tr>
<tr>
<td>SR (with xanthine + xanthine oxidase + SOD)</td>
<td>2.21±0.05</td>
<td>1.86±0.19</td>
</tr>
</tbody>
</table>

Isolated cardiac sarcoplasmic reticulum (SR, 0.037 mg/ml) was incubated with xanthine (100 µM) and xanthine oxidase (0.05 units/ml) in the presence or absence of superoxide dismutase (SOD) at 37°C for 30 minutes. Calcium uptake rate and Ca²⁺-ATPase activity were determined by millipore filtration and coupled enzyme assay. The results are mean±SEM of three to five independent experiments.

**FIGURE 4. Effect of intensity of illumination on inactivation of Ca²⁺-ATPase activity of cardiac sarcoplasmic reticulum.** Sarcoplasmic reticulum was incubated with rose bengal (25 nM) with or without exposure to various intensities of light (500–2,000 lux) for 10 minutes. At the end of incubation, Ca²⁺-ATPase activity was measured using coupled enzyme assay. The results are mean±SEM of three experiments. Note that the enzyme inactivation was nearly complete by 1,000 lux. Rose bengal or light alone was without any effect on the enzyme activity.

**FIGURE 5. Effect of singlet oxygen on sulphydryl group oxidation of cardiac sarcoplasmic reticulum.** Sarcoplasmic reticulum (75 µg/ml) was exposed to rose bengal (RB, 50 nM) in the presence or absence of light (2,000 lux) for 15 minutes. At the end of incubation, the absorbance increase was monitored at 412 nm. The results are mean±SEM of 10–15 independent experiments. Note that there was a decrease (18%) in absorbance after exposure to singlet oxygen (RB+Light).

**Effect of Irradiated Rose Bengal on Sulphydryl Group Oxidation**

Because free radicals are known to oxidize thiol groups, we studied the effects of singlet oxygen on thiol groups of SR by using the DTNB assay. Figure 5 shows that singlet oxygen caused only about an 18% decrease in absorbance at 412 nm.

**Effect of Irradiated Rose Bengal on Fragmentation and Aggregation of Ca²⁺-ATPase**

The mechanism of damage of singlet oxygen on cardiac SR was further investigated by running SDS-PAGE of SR exposed to irradiated rose bengal for up to 14 minutes. Figure 6 shows the time course of the effects of singlet oxygen (photoradiated rose bengal) on the 97,000-d band of Ca²⁺-ATPase of cardiac SR. There was time-dependent disappearance of the Ca²⁺-ATPase. Quantification of the bands by densitometry confirmed that there was significant loss of the band from 6 to 14 minutes after the exposure to irradiated singlet oxygen. Histidine (10 mM) significantly pro-
ected the enzyme (lane 13) as confirmed by densitometry. Addition of dithiothreitol (5 mM) had little protective effect, showing that new disulfide bond formation was not a major cause of aggregation (Figure 7). Denaturing SDS-PAGE in the presence of urea revealed a similar pattern of aggregation for SR Ca2+-ATPase exposed to irradiated rose bengal (not shown).

**High-Performance Liquid Chromatography of Sarcoplasmic Reticulum Exposed to Irradiated Rose Bengal**

Because the results from the SDS gels depend heavily on the ability of proteins to stain with Coomassie brilliant blue, additional experiments were performed in which SR was subjected to gel filtration on a molecular sieve HPLC column after exposure to irradiated rose bengal. The protein concentration in the effluent was monitored by the optical density at 280 nm, and this does not involve the bias of Coomassie brilliant blue staining. The results are shown in Figure 8. The peak marked 2 in Figure 8 was characterized by an Mr of 116,000, was phosphorylated by [γ-32P]ATP, and has been shown to be Ca2+-ATPase. The height of this peak was not significantly different between control SR, rose bengal (nonirradiated), or light alone (without rose bengal) after 15 minutes. The height of peak 1 (dimer Ca2+-ATPase) was significantly increased at the expense of peak 2 (Ca2+-ATPase), which was reduced significantly after 6 and 15 minutes of exposure to irradiated rose bengal (Figure 8). Six minutes of exposure to irradiated rose bengal in the presence of 2 mM histidine had significant protective effect on peak 2, the Ca2+-ATPase. These studies suggest that the damage to cardiac SR Ca2+-ATPase may be due to singlet oxygen because histidine (which is a powerful quenching agent for singlet oxygen) was protective.

**Identification of Hydroxyl Radical by Spin Trapping**

It has also been shown that irradiation of rose bengal generates about 75% singlet oxygen and 20% superoxide anion. However, it is not known whether the ·OH radical is also formed by irradiation of rose bengal, which might have contributed to the damage caused by singlet oxygen. First, we could not detect ·O2− by SOD-inhibitable reduction of either cytochrome c or nitro blue tetrazolium by irradiated rose bengal (even for extended periods). Second, the generation of ·O2− anion and ·OH radical was verified by electron paramagnetic resonance spectroscopy with DMPO as the spin trap. Figure 9A shows that irradiation of rose bengal formed a 1:2:2:1 quartet, characteristic of the ·OH radical DMPO spin adduct. The addition of ethanol resulted in trapping of the ethoxy radical, further confirming the generation of ·OH radical (Figure 9B). This signal could not be abolished by SOD, catalase, or histidine (10 mM) (Figures 9E and 9F). Incubation of DMPO with rose bengal without irradiation or irradiation of rose bengal without DMPO did not produce ·OH radical (Figures 9C and 9D). The time period from mixing of the sample to loading in the flat cell was approximately 30 seconds. It is unlikely that a large DMPO-OOH was missed. The half-life of DMPO-OOH adduct is approximately 60 seconds. It is possible, however, that a small DMPO-OOH adduct signal decayed to the point where it was no longer detectable over the 30-second interval. This was evidenced by the fact that a small amount of DMPO-OOH signal was observed on irradiating the sample inside the cavity (not shown). Alternatively, the photoactivation of rose bengal did not reduce cytochrome c or nitro blue tetrazolium, proving that a sufficient amount was not formed. The failure of
photosensitized rose bengal to reduce cytochrome c and nitro blue tetrazolium was not due to their destruction by singlet oxygen. Cytochrome c and nitro blue tetrazolium exposed to photosensitized rose bengal could be reduced by \( \cdot O_2^- \) generated from the xanthine plus xanthine oxidase system, showing that singlet oxygen did not inactivate these compounds. Therefore, it appears that photosensitized rose bengal makes only a small amount of \( \cdot O_2^- \), which is not detectable by SOD-inhibitable cytochrome c or nitro blue tetrazolium reduction.

**Inability of Superoxide Radical, Hydrogen Peroxide, and Hydroxyl Radical to Alter Ca\(^{2+}\)-ATPase Structure**

To demonstrate that the damage to Ca\(^{2+}\)-ATPase enzyme was due to singlet oxygen or any other reactive oxygen intermediate (such as \( \cdot O_2^- \) anion, H\(_2\)O\(_2\), or \( \cdot OH \) radical), we exposed the SR to \( \cdot O_2^- \) (generated from xanthine plus xanthine oxidase), H\(_2\)O\(_2\), and \( \cdot OH \) radical and performed SDS-PAGE. Figure 10 shows such effects of \( \cdot O_2^- \) (xanthine plus xanthine oxidase), \( \cdot OH \) radical (xanthine plus xanthine oxidase plus 50 \( \mu M \) Fe\(^{3+}\)-EDTA), 0.5 mM H\(_2\)O\(_2\), and \( \cdot OH \) radical generated from Fenton’s reagent (0.5 mM H\(_2\)O\(_2\) plus Fe\(^{3+}\)-EDTA). Xanthine and xanthine oxidase were also without any effect on the 97,000-d Ca\(^{2+}\)-ATPase band. Thus there was absolutely no effect of \( \cdot O_2^- \) anion or \( \cdot OH \) radical on Ca\(^{2+}\)-ATPase of SR. H\(_2\)O\(_2\) (0.25–10 mM) was also without any effect on Ca\(^{2+}\)-ATPase activity (Figure 11).

**Discussion**

The present results show that irradiation of rose bengal significantly inhibited Ca\(^{2+}\)-ATPase and calcium uptake of isolated cardiac SR. The damage to SR function appears to be related to the oxidative modification of the Ca\(^{2+}\)-ATPase enzyme. Singlet oxygen caused aggregation of the Ca\(^{2+}\)-ATPase of SR as confirmed by disappearance of the 97,000-d Ca\(^{2+}\)-ATPase band on SDS-polyacrylamide gels of SR or conversion of the monomer 116,000 Ca\(^{2+}\)-ATPase peak to the dimer peak as confirmed by HPLC and significant protection by histidine, the singlet oxygen scavenger. The functional damage of SR was independent of lipid peroxidation because there was no evidence of TBA reactive material formation. There was about an 18% decrease in thiol groups as indicated by decrease in absorbance at 412 nm. SDS-PAGE of SR in the presence of irradiated rose bengal showed a slight protective effect of dithiothreitol, proving that sulfhydryl group oxidation was not a major mechanism of enzyme damage (Figure 7). The damage of cardiac SR appears to be due to singlet oxygen and not due to \( \cdot O_2^- \), H\(_2\)O\(_2\), or \( \cdot OH \) radicals. This is further supported by the following observations: 1) \( \cdot O_2^- \) anion generated from xanthine plus xanthine oxidase was without effect on either the inhibition of SR function (Table 1) or 97,000-d Ca\(^{2+}\)-ATPase, proving this species to be harmless. 2) H\(_2\)O\(_2\) in concentrations ranging from 0.5 to 12 mM was without any significant effect on the Ca\(^{2+}\)-ATPase band but had a significant effect on the inhibition of the enzyme from 1 to 10 mM in a dose-dependent fashion as reported earlier.\(^9\) This inhibition of SR function was related to the oxidation of the sulfhydryl group (unpublished data) similar to other oxidants such as hypochlorous acid\(^8\) and not fragmentation or aggregation of enzyme. 3) Hydroxyl radical generated from xanthine plus xanthine oxidase in the presence of Fe\(^{3+}\)-EDTA or H\(_2\)O\(_2\) plus Fe\(^{2+}\)-EDTA also did not have any effect on fragmentation and aggregation of the enzyme. The xanthine oxidase concentration used in these experiments was 0.05 units/ml, which generated over 0.1 \( \mu M \) \( \cdot OH \) radical (J.L. Zweier, personal communication). Increasing xanthine oxidase up to 0.15 units/ml was also without any effect on the 97,000-d Ca\(^{2+}\)-ATPase...
FIGURE 8. Gel filtration chromatograms of control and singlet oxygen–exposed cardiac sarcoplasmic reticulum (SR). SR (2 mg/ml) in potassium phosphate buffer, pH 7.0, was exposed to rose bengal (RB, 381 nM) in the presence or absence of green light (6,000 lux) for 6 minutes. At the end of exposure, appropriate lithium dodecyl sulfate was added to achieve a concentration of 1%. The samples were dissolved in buffer containing 1% lithium dodecyl sulfate, 0.05 M lithium acetate, and 0.01 M lithium sulfate, pH 4.5. The samples were filtered, and 30 μl was injected into a high-performance liquid chromatography system equipped with a size exclusion column. The column was eluted with the buffer at a flow rate of 0.5 ml/min, and the eluate was monitored for optical density at A280. Peaks were numbered sequentially, and the molecular weight ratios assigned to the peaks were estimated from a standard curve constructed with purified proteins. Panel A: Control; panel B: with RB for 15 minutes; panel C: with light without RB for 15 minutes; panel D: with RB+light for 15 minutes; panel E: with RB+light for 15 minutes; panel F: with RB+light in the presence of the singlet oxygen scavenger histidine (2 mM) for 6 minutes.
protein. Hydroxyl radical significantly inhibited Ca\(^{2+}\) ATPase activity of SR via the lipid peroxidation mechanism reported earlier.\(^{11}\)

It has been reported that the quenching of rose bengal by oxygen leads to the production of approximately 75\% singlet oxygen and 20\% \(\cdot\)O_2^- anion.\(^{36}\) Although the evidence for singlet oxygen production is conclusive, the formation of \(\cdot\)O_2^- or \(\cdot\)OH radical by irradiation of rose bengal is in question. Using cytochrome c or the nitro blue tetrazolium reduction assay, we could not detect measurable amounts of \(\cdot\)O_2^- by irradiation of rose bengal. Also, spin trapping of irradiated rose bengal showed clearly the formation of DMPO-OH radical adduct and not \(\cdot\)O_2^-DMPO adduct (Figure 9), which was inhibitable by ethanol but not by SOD. No \(\cdot\)OH radical signal could be detected either with DMPO and rose bengal without irradiation or with irradiation of rose bengal without DMPO. These results suggest that there was formation of \(\cdot\)OH radical in addition to singlet oxygen by irradiation of rose bengal. However, the fragmentation and aggregation of SR Ca\(^{2+}\) ATPase was not due to \(\cdot\)OH radical, as shown by the inability of \(\cdot\)OH radical generating systems (\(\cdot\)H_2O_2 plus Fe\(^{3+}\)-EDTA or xanthine plus xanthine oxidase plus Fe\(^{3+}\)-EDTA) to cause aggregation (Figure 10).

Our data significantly differ from that of Davies and Delsignore,\(^{39}\) who reported that exposure of bovine serum albumin (BSA) to both \(\cdot\)OH and \(\cdot\)O_2^- caused gross structural modification resulting in spontaneous fragmentation. The fragmentation was followed by formation of covalent bonds between BSA molecules, resulting in protein aggregation to higher molecular weight species. Superoxide anion alone was without any effect. In contrast, exposure to \(\cdot\)OH+ \(\cdot\)O_2^- (+O_2) generally produced a dispersed pattern of lower molecular weight protein fragmentation products. More than 98\% of these fragments were larger than Mr 5,000 as judged by PAGE and precipitation studies in 5–10\% trichloroacetic acid. In the present results, there was no formation of smaller fragments or aggregation by \(\cdot\)OH+ \(\cdot\)O_2^- (xanthine plus xanthine oxidase plus Fe\(^{3+}\)-EDTA). Ca\(^{2+}\)-ATPase was not affected by \(\cdot\)O_2^- anion functionally\(^{10}\) as well as structurally. Hydroxyl radical similarly did not cause any structural damage to 97,000-d Ca\(^{2+}\)-ATPase enzyme (Figure 10). We do not quite understand the variability of our results.
peptide chain could cross-link with an amino acid radical in another protein by \( \cdot \) OH radical. The resulting peroxyl radical would further react to produce a peroxide, whose decomposition can cause chain scission to produce a carboxyl and amide. The peptide chain would remain intact during such reactions. A modified version of the scheme of Garrison et al was recently proposed by Schuessler and Schilling. In the Schuessler and Schilling model, BSA is cleaved by \( \cdot \) OH + \( \cdot \) O2− + \( \cdot \) O2 by oxidative destruction of proline residues. Wolff et al have further explored the question of proline attack and have suggested that peptide bond hydrolysis occurs. Whether such a mechanism is possible by singlet oxygen in Ca\(^{2+}\)-ATPase needs further investigation.

Reperfusion injury in the heart is characterized by a unique histological picture with the formation of contraction bands in the contractile proteins and calcific granules within mitochondria as well as cell swelling and disruption of sarcoplasmic and mitochondrial membranes. It has also been proposed that the sudden burst of oxidant stress may injure membrane proteins or lipids and this would lead to a perturbation of ionic balance and consequently instability. In support of this hypothesis, it has been shown that rose bengal photoactivation in the isolated perfused heart can lead to a redistribution of cellular calcium and to ultrastructural changes (contraction) that resemble those associated with severe injury during ischemia and reperfusion. These effects of oxidant stress were attributed to singlet oxygen since histidine, which quenches singlet oxygen, protected against electrophysiological changes and arrhythmias.

Most studies have focused on \( \cdot \) O2−, \( \cdot \) OH, and \( \cdot \) HOCl as mediators of injury and have considered them to be biologically relevant oxygen species. The other active oxygen species is singlet molecular oxygen, which is not a radical. Hydrogen peroxide is the least reactive form of oxygen, and \( \cdot \) OH radical is the most reactive. Singlet oxygen has been thought to be important only in photoreactions. But the sources that are known to produce \( \cdot \) O2−, \( \cdot \) O2, and \( \cdot \) OH during reperfusion injury in the heart may also produce singlet oxygen, although their quantitative significance remains uncertain at present. The following are the biochemical reactions that can theoretically generate singlet oxygen. For example, the myeloperoxidase/H2O2/halide system can produce singlet oxygen as follows:

\[
\text{myeloperoxidase} \\
\text{H}_2\text{O}_2 + \text{H}^+ + \text{Cl}^- \rightarrow \text{H}_2\text{OCl} + \text{HCHO}
\]

\[
\text{H}_2\text{O}_2 + \text{HCOOH} \rightarrow \text{H}_2\text{O} + \text{H}^+ + \text{Cl}^- + \text{O}_2
\]

No singlet oxygen could be detected when polymorphonuclear leukocytes were used. \( \cdot \) O2 interacts with H2O2 through the nonenzymatic dismutation reaction. The Haber-Weiss reaction results in \( \text{O}_2 \) formation as shown below.

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**Figure 11.** Effect of H2O2 on Ca\(^{2+}\)-ATPase of cardiac sarcoplasmic reticulum. Sarcoplasmic reticulum was incubated with varying concentrations of H2O2 (0.5–10 mM) for 45 minutes at 37°C. At the end of incubation all the samples were run on sodium dodecyl sulfate–polyacrylamide gel. Lane 1, standards; lane 2, control sarcoplasmic reticulum without H2O2; lane 3, sarcoplasmic reticulum+0.25 mM H2O2; lane 4, sarcoplasmic reticulum+0.5 mM H2O2; lane 5, sarcoplasmic reticulum+1 mM H2O2; lane 6, sarcoplasmic reticulum+2 mM H2O2; lane 7, sarcoplasmic reticulum+5 mM H2O2; lane 8, sarcoplasmic reticulum+10 mM H2O2; lane 9, sarcoplasmic reticulum control incubated for 45 minutes at 37°C.

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from that of Davies and Delsignore. One possibility is that the doses of \( \cdot \) OH radical used in their studies may be too high, and another is that this enzyme protein is different from BSA. In our experiments, we used 0.05 units/ml xanthine oxidase, a concentration considered within physiological range. Increasing xanthine oxidase concentration to 0.1 unit/ml also did not have any effect on the Ca\(^{2+}\)-ATPase band.

The data from the present study also unequivocally established the protective effect of histidine on the singlet oxygen–mediated inhibition of calcium uptake and aggregation of Ca\(^{2+}\)-ATPase activity. Yu et al reported that irradiated rose bengal inhibited Ca\(^{2+}\)-ATPase–dependent oxalate and promoted calcium transport and ATP-dependent calcium binding in skeletal muscle SR. They concluded that photooxidation of histidine was responsible for loss of SR function. The functional loss of SR was not attributed to singlet oxygen in their studies.

The process of aggregation by singlet oxygen is not clear from the present studies. However, it has been proposed that \( \cdot \) OH radical causes protein aggregation caused by intermolecular bytyrosine formation. Essentially any amino acid radical formed within a
\[
\begin{align*}
\cdot O_2^- + \cdot O_2^- + 2H^+ & \rightarrow H_2O_2 + O_2 \\
H_2O_2 + \cdot O_2^- & \rightarrow OH^- + O_2^- + H^+
\end{align*}
\]

The Haber-Weiss reaction is very slow unless it is metal-catalyzed or allowed to proceed at acidic pH. It may be relevant during ischemia when the pH of the myocardium drops to 6.4, although no evidence is available to support this theory.

Singlet oxygen generation is also reported during the horseradish peroxidase–catalyzed oxidation of malondialdehyde. Studies on the photoemission associated with lipid peroxidation show that the excited species thereby formed are related to the interaction of lipid-derived radicals formed in the propagation steps of lipid peroxidation. These studies confirmed spectroscopically the generation of singlet oxygen in the termination step of microsomal lipid peroxidation, as a consequence of the self-reaction of lipid peroxyl radicals. Generation of singlet oxygen by enzymatic reactions with xanthine oxidase and lipoxygenase have also been reported, but to what extent these reactions form singlet oxygen in vivo is not known. Most of these sources have been suggested to be the cause of free radical–mediated injury in various injury models.

The above discussion summarizes the possibility of singlet oxygen formation via the reactions of other reactive oxygen intermediates. Organ chemiluminescence is an accepted assay to determine the production rate of singlet oxygen and the steady-state peroxyl radicals under physiological conditions. Using this assay, Ferreira et al demonstrated a significantly higher chemiluminescence in reperfusion biospies. Indirect evidence proved that histidine, the singlet oxygen quencher, afforded significant protection of sarcoplemmal Na⁺,K⁺-ATPase activity during ischemia and reperfusion, suggesting that singlet oxygen is also one of the damaging species during reperfusion injury. We recently obtained further evidence that histidine protected contractile function and reduced arrhythmias. Electron microscopy of the tissue revealed that histidine significantly reduced ultrastructure damage caused by ischemia and reperfusion in a dose-dependent fashion as evidenced by reduction in mitochondrial swelling and preservation of myofibrillar architecture and sarcoplasmic integrity. Similar results were obtained with the singlet oxygen scavenger tryptophan (manuscript in preparation).

Several recent reports from Hearse’s laboratory have shown that photosensitization of rose bengal in isolated perfused rat heart induces arrhythmias similar to those caused by reperfusion-induced damage. The present results are in favor of this argument.

In summary, our results clearly demonstrate the destructive potential of singlet oxygen in cardiac SR. It inhibits the SR function by fragmentation and aggregation of the Ca²⁺-ATPase enzyme, quite independent of lipid peroxidation or to a lesser extent sulfhydryl oxidation. Histidine, which is a strong scavenger of singlet oxygen, was clearly able to protect SR functionally as well as structurally.

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**Key Words** • sarcoplasmic reticulum • free radicals • histidine • singlet oxygen • ischemia/reperfusion injury
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