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The Oxidative Inactivation of Sarcoplasmic Reticulum Ca^{2+}-ATPase by Peroxynitrite

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The oxidative inactivation of rabbit skeletal muscle Ca^{2+}-ATPase in sarcoplasmic reticulum (SR) vesicles by peroxynitrite ($ONOO^-$) was investigated. The exposure of SR vesicles (10 mg/ml protein) to low peroxynitrite concentrations (≤0.2 mM) resulted in a decrease of Ca^{2+}-ATPase activity primarily through oxidation of sulfhydryl groups. Most of this deactivation (ca. 70%) could be chemically reversed by subsequent reduction of the enzyme with either dithiothreitol (DTT) or sodium borohydride ($NaBH_4$), indicating that free cysteine groups were oxidized to disulfides. The initial presence of 5 mM glutathione failed to protect the SR Ca^{2+}-ATPase activity. However, as long as peroxynitrite concentrations were kept ≤0.45 mM, the efficacy of DTT to reverse Ca^{2+}-ATPase inactivation was enhanced for reaction mixtures which initially contained 5 mM glutathione. At least part of the disulfides were formed *inter*molecularly since gel electrophoresis revealed protein aggregation which could be reduced under reducing conditions. The application of higher peroxynitrite concentrations (≥0.45 mM) resulted in Ca^{2+}-ATPase inactivation which could not be restored by exposure of the modified protein to reducing agents. On the other hand, treatment of modified protein with $NaBH_4$ recovered all SR protein thiols. This result indicates that possibly the oxidation of other amino acids contributes to enzyme inactivation, corroborated by amino acid analysis which revealed some additional targets for peroxynitrite or peroxynitrite-induced processes such as Met, Lys, Phe, Thr, Ser, Leu and Tyr. Tyr oxidation was confirmed by a significant lower sensitivity of oxidized SR proteins to the Lowry assay. However, neither bityrosine nor nitrotyrosine were formed in significant yields, as monitored by fluorescence spectroscopy and immunodetection, respectively. The Ca^{2+}-ATPase of SR is involved in cellular Ca^{2+}-homeostasis. Thus, peroxynitrite mediated oxidation of the Ca^{2+}-ATPase might significantly contribute to the loss of Ca^{2+}-homeostasis observed under biological conditions of oxidative stress.

Key words: Ca^{2+}-ATPase, peroxynitrite, oxidation, thiols, disulfide, nitrotyrosine

Abbreviations: ROS, reactive oxygen species; DTT, dithiothreitol; SR, sarcoplasmic reticulum; ATP, adenosine triphosphate; MOPS, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethylether)-N,N′-tetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 2,4-DNPH, 2,4-dinitrophenylhydrazine; LDS, lithium dodecyl sulfate; SDS, sodium dodecyl sulfate; MDA, malondialdehyde; NEM, N-ethylmaleimide.

INTRODUCTION

Reactive oxygen species (ROS) have been implied in many pathophysiological processes such as,

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e.g, atherosclerosis, ischemia-reperfusion injury, and hyperoxia.[1–3] Besides many pathological effects, however, ROS also play a natural role in the biological defense mechanisms provided by neutrophils,[4] e.g. during inflammatory processes. There is as yet no conclusive evidence as to whether particular ROS are of importance under a specific condition of oxidative stress, or if the deleterious processes are carried out by a mixture of different species acting simultaneously or even synergistically. In general, a most efficient biological damage would require a species which selectively reacts with specific targets with high reactivity. In recent years, the nitric oxide-derived peroxynitrite [$ONOO^-$; recommended nomenclature: oxoperoxonitrate (–1)] has been forwarded as a potential candidate for a highly reactive but still selective reactive oxygen species responsible for biological damage under conditions of oxidative stress.[5–17] Peroxynitrite forms via diffusion-controlled reaction of nitric oxide ($NO^\bullet$) with superoxide ($O_2^{\bullet-}$) (reaction 1; $k_1=6.7\times10^9\ M^{-1}s^{-1}$),[18] or possibly via reaction of the nitroxide anion (NO^-) with molecular oxygen.[19]

$$NO^\bullet + O_2^{\bullet-} \rightarrow O{=}N{-}O{-}O^- \qquad (1)$$

In its protonated form, peroxynitrite (*cis*-form: $pK_a=6.8$, *trans*-form: $pK_a=8$[20]) is a strong oxidant which oxidizes several aromatic[7,8,21] and sulfur-containing amino acids,[22–24] lipids,[6] ascorbate[25,26] and deoxyribose,[27] and induces luminol chemiluminescence.[28] Furthermore, its *in vivo* formation, e.g. by activated macrophages and stimulated endothelial cells has been confirmed.[9,29]

An early biological event associated with oxidative stress is the loss of calcium homeostasis.[30] Several rationales were suggested including the oxidative inactivation of the voltage-dependent calcium channels and the plasma membrane Ca^{2+}-ATPase.[31] In addition, it was demonstrated that the Ca^{2+}-ATPase of sarcoplasmic reticulum constitutes a major target for ROS both *in vitro*[32] and *in vivo* under conditions of ischemia-reperfusion.[33] It was proposed that the oxidation of protein sulfhydryl groups was sufficient for the inactivation of the protein[34] although other amino acid residues have not been examined in detail.

Cysteine and protein sulfhydryl groups have been shown to be a major target of peroxynitrite.[22] Peroxynitrite might, therefore, be a likely candidate for affecting biological Ca^{2+}-homeostasis by oxidative inactivation of cysteine residues of the SR Ca^{2+}-ATPase. The present study was undertaken in order to investigate (i) the potential of peroxynitrite to inactivate SR Ca^{2+}-ATPase in purified SR vesicles, and (ii) the distribution of chemical products obtained upon reaction of peroxynitrite with SR Ca^{2+}-ATPase at physiological pH. A direct physiological role for peroxynitrite induced inactivation of specifically SR Ca^{2+}-ATPase may soon evolve since it has been reported that human skeletal muscle contains high levels of nitric oxide synthase (NOS).[35,36] Thus, nitric oxide and potentially peroxynitrite are likely to be produced in skeletal muscle.

EXPERIMENTAL

Materials

The following chemicals were obtained from Fisher Scientific Co. (St. Louis, MO): NaOH, H_2O_2 (30% in H_2O), $NaNO_2$, HCl (all metal grade), KH_2PO_4, K_2HPO_4; 2,4-dinitrophenylhydrazine (2,4-DNPH) was from Eastman Kodak Company (Rochester, NY); Catalase (bovine liver, EC 1.11.1.6), N-ethylmaleimide (NEM), tetranitromethane (TNM), 4-chloro-1-naphtol, sodium borohydride ($NaBH_4$), sodium arsenite ($NaAsO_2$), and dithiothreitol ($HSCH_2CH(OH)CH(OH)CH_2SH$; DTT) were from Sigma Chemical Company (St. Louis, MO). Anti-nitrotyrosine monoclonal antibodies were supplied by Upstate Biotechnology, Inc. (Lake Placid, NY) and goat anti-mouse IgG(H + L) HRP conjugate was from Pierce (Rockford, IL). All reagents for gel electrophoresis and prestained protein markers were obtained from BIORAD (Richmond, CA). The

phosphate buffer for performing the peroxynitrite mediated oxidation of SR protein was treated with Chelex 100 (BIORAD; 5 g/ 100 ml) in order to remove transition metal contaminations.

Synthesis of peroxynitrite

Peroxynitrite was synthesized by the reaction of ozone with ice-cooled aqueous sodium azide at pH 12, essentially as described[37] (precautions for the handling of ozone are described in this reference). This method of preparation ensures that the peroxynitrite-solutions are free of H_2O_2[37] (which is not the case for peroxynitrite solutions prepared by quenched-flow reaction of acidic H_2O_2 with $NaNO_2$). Final peroxynitrite concentrations of about 90 mM were obtained, as quantified by its UV-spectrum with λ_{max} = 302 nm (ε_{302} = 1670 M^{-1} cm^{-1}[38]). For SR protein oxidations, the actual concentration of peroxynitrite was determined right before the experiment.

Membrane preparations

Native SR vesicles (longitudinal 'light' fraction) were prepared from rabbit skeletal white (fast twitch) muscle, essentially as described.[39] Electron microscopy studies of our preparations indicated that we obtained highly purified, right oriented, intact SR vesicles.

Determination of Ca^{2+}-ATPase content in our preparations

According to gel electrophoresis (see below), the content of Ca^{2+}-ATPase in our preparations was (72 ± 3.6)% based on the total protein content. Taking a molecular weight for SR Ca^{2+}-ATPase of 110 kDa, a preparation of 10 mg protein/ml will then contain an overall concentration of [Ca^{2+}-ATPase] = 65 μM.

Oxidation of Ca^{2+}-ATPase by peroxynitrite

The oxidation reactions were carried out using SR vesicles containing 10 mg/ml SR protein, 10 mM phosphate buffer, and 100 mM NaCl at 25°C in a total volume of 250 μl. The starting pH was adjusted to pH 7.3 and shifted less than 0.2 units through the addition of the peroxynitrite-stock solution. As a control experiment, an aliquot of peroxynitrite was added to a reaction medium containing only 10 mM phosphate buffer, pH 7.3, and 100 mM NaCl but no SR vesicles. The peroxynitrite was allowed to decompose in this reaction mixture before SR vesicles were added (reverse order-of-addition experiment).[10] Generally, there was no significant loss of the Ca^{2+}-ATPase activity after incubation of SR vesicles in the reverse order-of-addition experiment.

Functional assay of Ca^{2+}-ATPase

Calcium-dependent ATPase activity was measured at 25°C by a colorimetric determination of inorganic phosphate[40] released from vesicles made leaky to calcium by the addition of the ionophore A23187. ATPase activity was measured at 0.05 mg/ml SR protein, 5 mM MgCl, 6 μM A23187, 25 mM MOPS (pH 7.0), 5 mM ATP, and 1.0 mM EGTA or 0.1 mM $CaCl_2$.

Fluorescence measurements

The fluorescence measurements were performed on a Perkin Elmer MPF-44B spectrofluorometer. The intrinsic fluorescence of the native and oxidized protein was determined by excitation at 275 nm (emission between 300 and 400 nm) in 10 mM phosphate buffer, pH 7.3. The potential formation of bityrosine (emission at 420 nm[41]) and formylkynurenine (emission at 435 nm[42]) was assessed by excitation at 325 nm and measuring the emission spectrum between 400 and 500 nm.

Fluorescence quenching was employed in order to assess potential peroxynitrite-induced changes of the solvent accessibility of the Trp residues of SR proteins. Native and modified SR vesicles were examined by measuring the steady-state intrinsic fluorescence as a function of the added fluorescence quencher acrylamide [Q] (λ_{ex} = 275 nm, λ_{em} = 330 nm). The slope K_{SV} of the

Stern-Volmer equation $F_0/F = 1.0 + K_{SV}[Q]$, plotted as F_0/F versus [Q] can be used as a measure for changes in solvent accessibility[43] [fluorescence in the absence (F_0) and presence (F) of quencher Q].

Determination of protein sulfhydryl groups

The sulfhydryl content of the Ca^{2+}-ATPase was determined by measuring the increase of absorbance at 412 nm after reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).[44,45] SR protein (100 μg) was incubated for 30 minutes in guanidine-HCl (6 M) in 50 mM potassium phosphate, pH 9.0. Thereafter a final concentration of 2 mM DTNB was added into a total volume of 1 ml. The absorbance at 412 nm ($\varepsilon_{412} = 1.36 \times 10^4$ M^{-1} cm^{-1}) was measured after 45 minutes of incubation at room temperature.

Information about the chemical nature of the sulfhydryl oxidation products can be obtained by the reductive recovery of oxidized sulfhydryls with various reducing agents: $NaBH_4$[46] and dithiothreitol (DTT)[22] will reduce disulfide bonds to free thiols whereas $NaAsO_2$[22] will reduce sulfenic acid (RSOH) to free thiol. Aliquots of 1 mg/ml SR Ca^{2+}-ATPase were incubated with either sodium arsenite (final concentration of 37 mM) or sodium borohydride (final concentration 26 mM) for 45 minutes at 37°C in 20 mM MOPS, pH 7.0, before the samples were assayed for their sulfhydryl content in the presence of guanidine-HCl (see above). For the reduction by DTT an aliquot of 1 mg/ml oxidized SR protein was incubated with 2–5 mM DTT for 30 minutes at room temperature. The excess DTT was removed by overnight dialysis at 4°C against 1 L of 20 mM MOPS, pH 7.0, before the protein sulfhydryl content was assayed as described above.

Determination of protein carbonyl content

The carbonyl content of the SR was measured after reaction with 2,4-DNPH in analogy to the procedure reported by Levine *et al.*[47] A sample of 0.5 mg/ml native or modified SR was centrifuged for 30 minutes at 100 000 g_{max} before redissolving the sediment in 0.1 ml of solubilization buffer (1% LDS, 0.1 M Li_2SO_4, 0.05 M Li acetate, pH 4.5) and 0.1 ml 12% LDS. Then each sample was divided into two aliquots. One aliquot of each sample was treated with 10% TFA (blank) and the other was treated with two equal volumes of 20 mM 2,4-DNPH in 10% TFA. Both samples were incubated 10–15 minutes at room temperature before the mixture was neutralized with 2.0 M Tris. Subsequently, 100 μl aliquots of the samples were subjected to size-exclusion chromatography, essentially as described by Barrabin *et al.*[48] Size-exclusion chromatography was carried out on a Varian HPLC-System, equipped with a TSK G3000 SW column (Toso Haas; Montgomeryville, PA) which was eluted at room temperature with the solubilization buffer at 0.4 ml/minute. The column was calibrated with the following molecular weight standards (from Sigma): β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (28 kDa) and cytochrome c (12.4 kDa). The eluent was monitored at two wavelengths, 280 and 360 nm, respectively. The carbonyl content of the peak of monomeric Ca^{2+}-ATPase was calculated by equation I, taking $\varepsilon_{hydrazon,360} = 22{,}000$ M^{-1} cm^{-1} and $\varepsilon_{Ca\text{-}ATPase,280} = 160{,}000$ M^{-1} cm^{-1} [49].

$$\text{mol carbonyl/mol protein} = [(\varepsilon_{Ca\text{-}ATpase,280})(\text{area}_{360})]\,/\,[22{,}000\,(\text{area}_{280} - 0.43\,\text{area}_{360})] \quad \text{(I)}$$

Response of native and oxidized protein to the Folin-Lowry reagent

Typical samples of 10 mg/ml SR protein in 10 mM potassium phosphate, pH 7.3, and 100 mM NaCl were exposed to 0–0.45 mM peroxynitrite before aliquots of 20 μl were subjected to the Lowry assay.[50]

Amino acid analysis

Amino acid analysis was done after acid (20 hours, 110°C, 6 N HCl) and alkaline (16 hours, 110°C, 1 N NaOH) hydrolysis of the protein; services

were provided by Commonwealth Biotechnologies, Inc. (Richmond, VA).

Susceptibility of Ca^{2+}-ATPase to tryptic hydrolysis

The susceptibility of oxidant-exposed membranes to tryptic digestion was assessed by incubation of 1.25 mg/ml protein with 0.025 mg/ml trypsin at 30°C in 50 mM ammonium carbonate buffer, pH 8.5, 200 μM $CaCl_2$ and 1.0 mM DTT. At different times within 0 and 15 minutes a final volume of 5% trichloroacetic acid was added to the reaction mixture and the residual protein precipitated by centrifugation for 30 minutes at 6,000 × g_{max}. Trichloroacetic acid soluble peptides were measured using the Pierce BCA assay (Rockford, Il).

Measurement of lipid peroxidation

Lipid peroxidation was monitored by HPLC analysis of 2,4-DNPH derivatives of malondialdehyde (MDA) according to the method recently applied by Cordis *et al.*[51] An aliquot of SR corresponding to 1 mg/ml protein was derivatized with 3 mmol of 2,4-DNPH, followed by extraction with hexane/methylene chloride (80:20, v/v). The extracts were evaporated, reconstituted with 0.1 ml of a hexane/acetonitrile mixture (60:40, v/v), and an aliquot of 0.01 ml subjected to HPLC analysis on an ISCO C18 column (4.6 × 250 mm, 5 μ). The malondialdehyde hydrazone was monitored at 307 nm, and eluted with t_R = 4.6 minutes.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed using 7.5% separating gels, according to the method of Laemmli.[52] The samples were dissolved in a solution of 2% SDS, 50 mM Tris-HCl (pH 6.8), 15% sucrose, and 0.05% bromophenol blue (i) in the presence of 2.5% β-mercaptoethanol (heated for 5 minutes at 100°C; reducing conditions) or (ii) in the absence of β-mercaptoethanol without heating (non-reducing conditions). The latter samples were not heated in order to avoid heat-induced aggregation in the absence of reductants.There was no difference between gels of samples which were run in the described way and gels of samples which had been alkylated for 30 minutes with 5 mM NEM. All samples were applied to the gels and stained for protein with Coomassie Blue R-250. The relative amount of monomeric and aggregated Ca^{2+}-ATPase was determined from densitometric measurements (Hewlett Packard Scan Jet IIp) of the protein bands. After electrophoretic separation, the transfer to nitrocellulose and immunodetection of nitrotyrosine were carried out by standard techniques (see below).[53]

Electrophoretic transfer for immunoblot analysis of SR proteins

The protein bands were transferred to nitrocellulose sheets (BIORAD, 0.45 μm pore size) using a Mini Trans Blot electrophoretic transfer cell (BIORAD). The transfer buffer contained 25 mM Tris, 192 mM glycine, and 20% methanol at pH 8.0. The transfer was achieved after 2 hours at 95 V in ice-cooled medium.

Immunostaining of nitrocellulose sheets

After electrophoretic transfer, the nitrocellulose sheets were dried and then incubated for at least 2 hours at room temperature with 6% non-fat dry milk in Tris-buffered saline (TBS), containing 20 mM Tris-HCl and 250 mM NaCl at pH 7.4. The sheets were then incubated for 2 hours at room temperature with a 1:1000 dilution of primary antibodies (anti-nitrotyrosine) in 3% albumin-TBS. After washing repeatedly with TBS-0.05% Tween 20 solution, the nitrocellulose sheets were incubated for 1 hour at room temperature with HRP-conjugated anti-mouse IgG, diluted 1:10,000 in 3% TBS. The reaction was terminated by washing the sheets repeatedly for 5 minutes with TBS-0.05% Tween 20 and TBS. The bound conjugated IgG was visualized by reaction for 15 minutes with a solution containing 0.04% 4-chloro-1-naphtol in methanol and 0.1 ml 30% H_2O_2 in 20 mL

of TBS. When the bands became visible, the nitrocellulose sheets were rinsed with water, photographed, and dried at room temperature.

Nitrated reference proteins

For obtaining reference proteins we nitrated SR vesicles with TNM analogous to a described procedure for calmodulin[54] (1 mg/ml SR protein in 10 mM phosphate buffer, 100 mM NaCl, pH 7.3, 650 μM TNM). From the high molecular weight of the TNM-exposed SR proteins (see Figure 4) we conclude that TNM does not only nitrate but also induces aggregate formation of the SR proteins (most probably via thiol oxidation). In addition we nitrated superoxide dismutase (SOD) with peroxynitrite, analogous to a procedure reported by Ischiropoulos *et al.*[7] (150 μM SOD, 4.5 mM $ONOO^-$, 50 mM phosphate buffer, pH 8).

RESULTS

Inactivation, aggregation, and fragmentation of the protein

Table 1 and Figure 1a demonstrate that the incubation of SR vesicles (10 mg SR protein/ml, corresponding to 65 μM SR Ca^{2+}-ATPase; see *Experimental*) with increasing concentrations of peroxynitrite led to a gradual inactivation of the SR Ca^{2+}-ATPase. Most of the enzyme activity was lost at peroxynitrite concentrations ≤0.9 mM, corresponding to a ratio of [peroxynitrite]/[Ca^{2+}-ATPase] ≤13.8. The activity of the oxidized enzyme was measured against control samples which were subjected to the reverse order-of-addition experiments (see *Experimental*).

The incubation of 10 mg/ml SR protein to peroxynitrite concentrations ≤0.2 mM led to an overall loss of up to 21% of the Ca^{2+}-ATPase activity. A large part (ca. 70%) of this deactivation can be chemically reversed by the subsequent reduction of the modified protein with either 26 mM $NaBH_4$ or 2.0 mM DTT (we did not observe any significant differences between 2.0 and 5.0 mM DTT and, therefore, performed all subsequent experiments with only 2.0 mM DTT). However, a more extensive inactivation of Ca^{2+}-ATPase by higher concentrations of peroxynitrite (≥0.45 mM) could be only partially reversed by subsequent reduction.

In order to closer mimic biological conditions under which such oxidation reactions may occur, we subjected 10 mg/ml SR protein to the oxidation by peroxynitrite in the additional presence of a representative concentration of 5 mM glutathione (GSH). As displayed in Figure 1b, the peroxynitrite-induced inactivation of SR Ca^{2+}-ATPase in the presence of 5 mM GSH was comparable to the inactivation observed in the absence of GSH. However, some differences between the systems initially containing GSH and systems initially lacking GSH were apparent for exposures to peroxynitrite concentrations ≤0.45 mM: When, subsequently to the completion

TABLE 1 Effect of peroxynitrite on skeletal muscle SR Ca^{2+}-ATPase activity.

[Peroxynitrite], mM	w/o reductants	+ 26 mM $NaBH_4$	+ 2 mM DTT
0.00	3.45 ± 0.15	3.46 ± 0.13	3.60 ± 0.14
0.10	2.87 ± 0.15	3.20 ± 0.02	3.67 ± 0.13
0.20	2.72 ± 0.07	3.22 ± 0.09	3.20 ± 0.11
0.45	2.05 ± 0.09	1.95 ± 0.02	2.35 ± 0.40
0.90	1.01 ± 0.03	1.69 ± 0.09	1.75 ± 0.25
1.80	0.87 ± 0.09	1.59 ± 0.04	1.45 ± 0.09

SR vesicles (10 mg/ml) were incubated with peroxynitrite (0.1–1.80 mM) for 4 min at room temperature in 0.25 ml of 0.01 M potassium phosphate buffer, pH 7.4. Then, the reductants were added to aliquots of the reaction mixture and incubated for 45 min at 37°C or 30 min at room temperature (DTT). Samples were assayed for Ca^{2+}-ATPase activity as described in 'Experimental procedures'. The values are expressed as μmol P_i/min/mg SR protein. The numbers indicate mean ± S.D., n = 3.

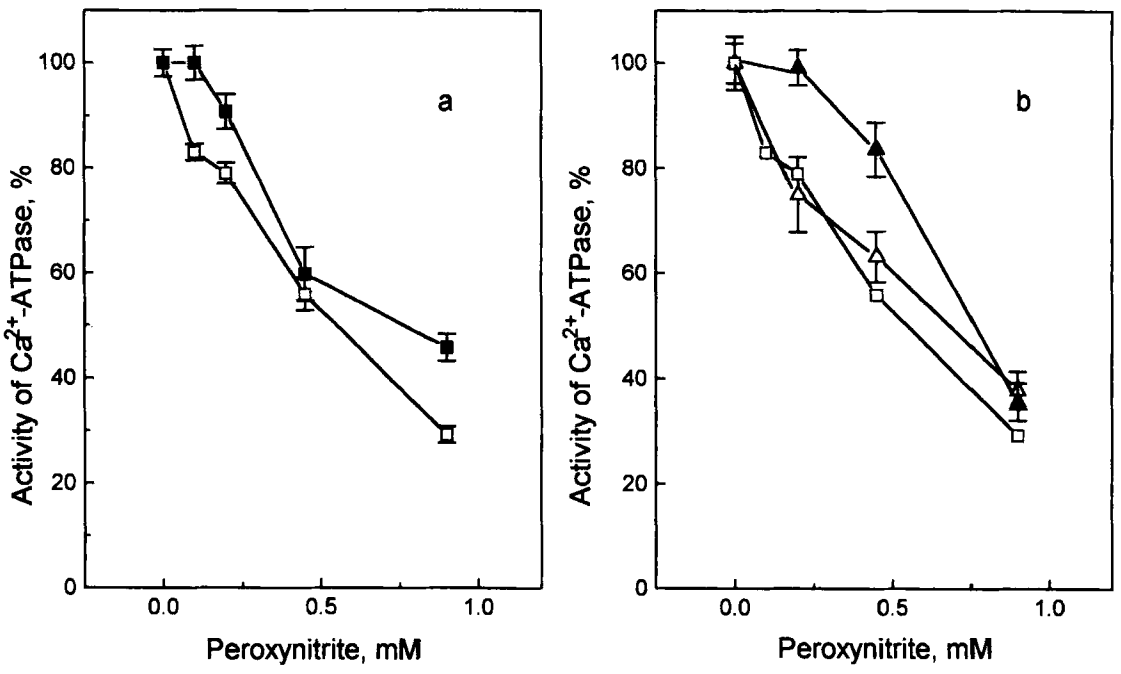

FIGURE 1 Effect of peroxynitrite on the Ca^{2+}-ATPase activity of SR vesicles. (a) SR vesicles (10 mg/mL protein) were treated with 0–0.9 mM peroxynitrite and the Ca^{2+}-ATPase activity measured before (□) and after (■) subsequent reduction of the reaction mixtures with 2 mM DTT. (b) SR vesicles (10 mg/mL protein) were treated with 0–0.9 mM peroxynitrite in the absence (□) and in the presence (△, ▲) of 5 mM GSH. Ca^{2+}-ATPase activity was measured before (□, △) and after (▲) subsequent reduction of the reaction mixtures with 2 mM DTT.

of the peroxynitrite-induced oxidation, the reaction mixtures were exposed to 2 mM DTT (as described above), there was a more efficient reversal of the protein inactivation in systems which initially contained 5 mM GSH (compare top curves in Figures 1a and 1b, respectively). For example, after exposure of 10 mg/mL SR protein to 0.45 mM peroxynitrite, 2 mM DTT were able to recover 20% of the lost Ca^{2+}-ATPase activity in systems initially lacking GSH, and 55% in systems initially containing GSH. This more efficient reversal of lost Ca^{2+}-ATPase activity clearly vanishes for the exposure of SR proteins to peroxynitrite concentrations >0.45 mM.

The exposure of 10 mg/ml SR protein to peroxynitrite did not cause any significant protein fragmentation but gradually caused aggregation of the Ca^{2+}-ATPase, indicated by the formation of higher molecular weight species during gel electrophoretic separation. Figure 2 representatively displays the gels obtained for experiments in which SR vesicles were exposed to no (A) and 0.2 mM peroxynitrite (B). The positions of the Ca^{2+}-ATPase monomer and dimer were calibrated against molecular weight standards and are indicated by the arrows. A densitometric analysis of the gels run under non-reducing conditions (lanes 2) revealed that higher molecular weight species of Ca^{2+}-ATPase accounted for the following percentages relative to total protein: 0 (no $ONOO^-$), 6.9 ± 0.13 (0.1 mM $ONOO^-$), 14.0 ± 5.2 (0.2 mM $ONOO^-$), and 24.5 ± 3.8 (0.45 mM $ONOO^-$). Comparably less Ca^{2+}-ATPase dimers were detected on gels run under reducing conditions: 0 (no $ONOO^-$), 0 (0.1 mM $ONOO^-$), 1.9 (0.2 mM $ONOO^-$), and 7% (0.45 mM $ONOO^-$) (all values compared to native Ca^{2+}-ATPase and corrected for some dimerization caused by the heating procedure prior to gel

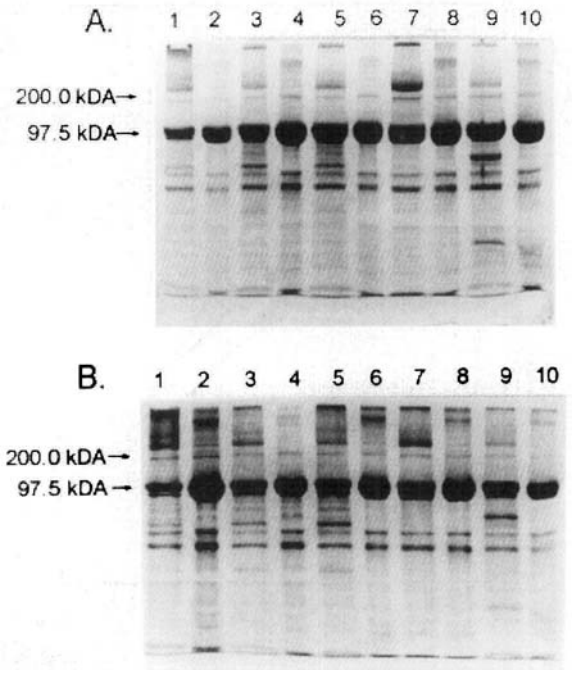

FIGURE 2 Effect of peroxynitrite on Ca^{2+}-ATPase association. SR vesicles (10 mg/ml protein) were treated with (A) 0 and (B) 0.2 mM peroxynitrite and applied to gel electrophoretic separation. Lanes 1,3,5,7, and 9 represent gels run under reducing conditions (see *Experimental*). Lanes 2,4,6,8, and 10 represent gels run under non-reducing conditions. Lanes 1 and 2: SR vesicles which were not further treated after incubation with peroxynitrite; lanes 3 and 4: + 2 mM DTT after incubation with peroxynitrite; lanes 5 and 6: SR vesicles were heated for 45 minutes at 37°C after incubation with peroxynitrite; lanes 7 and 8: + 37 mM $NaAsO_2$ at 37°C after incubation with peroxynitrite; lanes 9 and 10: + 26 mM $NaBH_4$ at 37°C after incubation with peroxynitrite.

electrophoresis). This indicates that part of the covalent dimers are formed via *inter*molecular disulfide bridges. This becomes also evident from the comparison of lanes 2, 4, 8 and 10 of Figure 2B, obtained under non-reducing gel electrophoretic conditions. They represent samples which were not further treated (lane 2), or exposed to 2 mM DTT (lane 4), 37 mM $NaAsO_2$ at 37°C (lane 8), or 26 mM $NaBH_4$ at 37°C (lane 10) subsequent to the oxidation of the SR vesicles by peroxynitrite and prior to gel electrophoresis. Reaction with the reductants resulted in a weakening of the bands of the Ca^{2+}-ATPase dimer and in particular of the bands of the higher molecular weight aggregates, as expected if they were formed by disulfide bond formation. As a control for the experiments displayed in lanes 8 and 10, lane 6 contains the bands obtained after heating of the oxidized SR samples at 37°C in the absence of either $NaAsO_2$ or $NaBH_4$, respectively.

Lipid peroxidation

The addition of peroxynitrite to the SR vesicles did not result in significant lipid peroxidation, as assessed by measurement of MDA. A constant content of 1.2 nmol MDA/1 mg SR protein was determined for SR vesicles subjected to 0–0.45 mM peroxynitrite, suggesting that the enzyme inactivation at this peroxynitrite concentrations is caused by a chemical modification of the protein rather than a change in the lipid environment.

Chemical modification of cysteine residues

According to Brandl *et al.*,[55] native SR Ca^{2+}-ATPase contains 24 Cys residues. The titration of protein sulfhydryl groups of our SR preparations with DTNB in the presence of guanidinium hydrochloride revealed the overall presence of 22.65 ± 0.6 sulfhydryl groups / 1.53×10^5 g native SR protein, as calibrated against Cys standards (see Table 2).

The results summarized in Table 2 show that the exposure of 10 mg/ml SR protein to 0–0.45 mM peroxynitrite revealed a gradual loss of ca. 8 protein sulfhydryl groups / 1.53×10^5 g SR protein. Higher concentrations of peroxynitrite (>0.45 mM) did not significantly further affect the protein sulfhydryl content. On the other hand, they continued to inactivate the Ca^{2+}-ATPase (as shown in Table 1). After exposure to [peroxynitrite]≤0.45 mM, the oxidatively modified SR protein sulfhydryl groups could be completely recovered by reduction with $NaHB_4$ whereas only in part by $NaAsO_2$ or DTT, respectively. This finding is interesting with respect to the activity of the Ca^{2+}-ATPase which could not be completely re-

TABLE 2 Determination of protein thiol groups.

[Peroxynitrite], mM	w/o reductants	+ 26 mM $NaBH_4$	+ 37 mM $NaAsO_2$	+ 2 mM DTT
0.00	22.65 ± 0.59	22.65 ± 0.59	22.65 ± 0.59	22.65 ± 0.59
0.10	20.21 ± 0.32	23.17 ± 0.78	20.52 ± 1.35	22.55 ± 0.79
0.20	20.18 ± 0.41	21.79 ± 0.58	19.36 ± 0.20	20.25 ± 0.66
0.45	14.42 ± 1.92	23.28 ± 0.18	19.44 ± 1.80	16.98 ± 1.14
0.90	14.63 ± 1.69	(n.d.)[a]	16.32 ± 1.20	18.06 ± 0.44
1.80	14.04 ± 0.25	(n.d.)[a]	14.34 ± 0.90	15.91 ± 0.16

[a]n.d. = not determined

Samples were prepared as described in Table 1 and then assayed for sulfhydryl content with DTNB. Data represent the means (± S.D.) of four independent determinations. The values are expressed as number of SH groups per 1.53×10^5 g SR protein.

stored by either of the reductants at peroxynitrite concentrations ≤0.45 mM (see Table 1).

From these results we conclude the following: (i) At low peroxynitrite concentrations (≤0.2 mM) ([peroxynitrite]/[Ca^{2+}-ATPase]≤3) the inactivation of the Ca^{2+}-ATPase is predominantly caused by the oxidation of protein cysteine residues to disulfides. (ii) At higher peroxynitrite concentrations (≥0.45 mM) other amino acid residues in addition to Cys may also be affected. In neither case appears lipid peroxidation to be the determinant factor for Ca^{2+}-ATPase inactivation.

Analysis of other amino acid modifications

In order to obtain information about other potential amino acid targets for peroxynitrite, we subjected extensively oxidized SR vesicles to amino acid analysis [2.5 mg/ml SR protein (15.2 μM SR Ca^{2+}-ATPase) with 1.8 mM peroxynitrite ([peroxynitrite]/protein = 118)]. The results are shown in Table 3. It appears that some small fractions of Lys (ca. 6.8 ± 0.8%), Tyr (ca. 6 ± 2%), Phe (ca. 1.9 ± 0.6%), Met (ca. 5%), Ser (ca. 3%), Thr (ca. 4%), and Leu (ca. 2.2%) are lost together with ca. 50% of the protein sulfhydryls (data not shown). Although Lys, Thr, Ser, and Leu have not yet been reported to react with peroxynitrite, they are generally oxidation-sensitive amino acids which can be oxidized by a number of reactive oxygen species.[56] In this respect we shall note that protein chain oxidations can cause the oxidation of a manifold of amino acid residues whenever intermediary protein-bound carbon-centered radicals (and subsequently peroxyl radicals) had been initially present.[57] Such reactions are generally also possible for sulfur-centered radicals of proteins as demonstrated for thiyl radicals in a model reaction of glutathione.[58]

Protein carbonyl derivatives are a common product of protein oxidation.[56] Table 4 shows that the exposure of SR vesicles (10 mg/ml protein) to increasing concentrations of peroxynitrite resulted in a gradual increase of the formation of protein carbonyls. The formation of protein carbonyls had been indicated for the oxidation of Lys, Arg and Pro.[56] The amino acid analysis of our incubations revealed that neither Arg nor Pro are lost upon the reaction of peroxynitrite with SR vesicles (Table 3). However, the observed decomposition of Lys would be qualitatively in agreement with the formation of protein carbonyls. We cannot quantitatively correlate the number of lost Lys residues (Table 3) with the number of formed protein carbonyls (Table 4). This might be rationalized by the additional oxidation of other amino acid residues (such as Thr, Ser, and Leu).

Fluorescence measurements

Stern-Volmer plots for fluorescence quenching by acrylamide of native and peroxynitrite-modified

TABLE 3 Amino acid analysis of native and modified skeletal muscle SR protein.

Acidic			Alkaline		
amino acid	native	modified	amino acid	native	modified
His	15.0(±0.02)	15.0(±0.2)	His	8.0(±0.03)	8.0(±0.1)
Lys	50.0(± 1.0)	47.0(±1.0)	Lys	26.0(±1.0)	24.0(±1.4)
Met	29.0(±0.7)	30.0(±0.1)	Met	29.5(±0.2)	28.0(±2.0)
Phe	38.0(±0.1)	37.0(±0.4)	Phe	42.0(±0.4)	41.5(±0.5)
Tyr	23.0(±0.2)	22.0(±0.3)	Tyr	26.0(±0.3)	24.0(±0.1)
Ala	79.0(±2.0)	77.0(±0.5)	Trp	8.7(±0.3)	8.1(±0.3)
Arg	44.4(±0.03)	44.0(±0.4)			
Asx[a]	118.0(±3.0)	109.0(±0.5)			
Glx[a]	94.0(±2.0)	107.0(±5.0)			
Gly	67.4(±0.2)	66.0(±0.07)			
Ile	53.0(±0.4)	52.0(±1.0)			
Leu	90.0(±1.0)	88.0(±0.6)			
Pro	50.0(±1.0)	52.5(±0.07)			
Ser	50.0(±1.0)	48.4(±0.4)			
Thr	52.0(±1.0)	50.0(±0.5)			
Val	66.0(±1.0)	65.0(±1.0)			

[a] Asx refers to Asp and Asn, and Glx refers to Glu and Gln.
SR vesicles (15.2 μM of Ca^{2+}-ATPase) were treated with 1.8 mM peroxynitrite at room temperature in 0.25 ml of 0.05 M potassium phosphate buffer, pH 7.4 and then aliquots were lyophilyzed and subjected to amino acid analysis.

SR vesicles revealed no significant differences in the quenching constants for the exposure of SR vesicles to various concentrations of peroxynitrite ($K_{SV,0} = 38.3 \pm 2.6\ M^{-1}$, $K_{SV,0.1} = 46.4 \pm 3.4\ M^{-1}$, $K_{SV,0.2} = 33.2 \pm 2.9\ M^{-1}$, and $K_{SV,0.45} = 46.5 \pm 3.2\ M^{-1}$ for the exposure to 0, 0.1, 0.2, and 0.45 mM peroxynitrite, respectively). Thus, there is no significant change within the microenvironment of the SR protein Trp residues. In addition, we have not observed any significant loss of the characteristic intrinsic fluorescence emission of Trp indicating the fact that this amino acid does not undergo oxidation upon exposure of SR vesicles to peroxynitrite.

By screening the emission spectra between 400 and 450 nm ($\lambda_{ex} = 325$ nm), we have also confirmed that there were no significant yields of either N-formylkynurenine ($\lambda_{em} = 435$ nm) or bityrosine ($\lambda_{em} = 420$ nm).

TABLE 4 Reactivity of the Folin–Lowry reagent towards SR proteins and content of carbonyl groups in SR vesicles treated with peroxynitrite.

[Peroxynitrite], mM	A_{700}[a]	Content of carbonyl groups mol/mol Ca-ATPase
0.00	0.310 ± 0.002	0.38 ± 0.06
0.10	0.306 ± 0.002	4.00 ± 0.09
0.20	0.290 ± 0.005	4.57 ± 0.14
0.45	0.280 ± 0.003	7.60 ± 0.23

[a] By the method of Lowry *et al.*[50] absorbance of samples was determined at 700 nm.
SR vesicles were treated with various concentrations of peroxynitrite and then aliquots were taken for measurements of protein concentration and carbonyl groups. The numbers indicate mean ± ± S.D. (three separate experiments).

Response to Lowry-Folin reagent

The response of a protein to the Lowry-Folin reagent is dependent on the presence of Tyr and Trp in the protein.[42,50] Thus, this reagent provides an additional way of testing the loss of both amino acids. Table 4 shows that the oxidation of 10 mg/ml SR protein with increasing concentrations between 0 and 0.45 mM peroxynitrite resulted in the loss of ca. 10% of the absorbance, measured at 700 nm, as compared to control samples. Amino acid and fluorescence analysis had indicated no significant modification of Trp resi-

dues but of Tyr. The lower sensitivity to the Lowry-Folin reagent is, therefore, consistent with the oxidation of Tyr.

Nitrotyrosine

Nitrotyrosine is a common product of peroxynitrite-induced modification of Tyr residues. As shown in Figure 3, our Western-blot analysis did not reveal any significant formation of nitrotyrosine after exposure of SR vesicles to 0–0.45 mM peroxynitrite (lanes 6–9). If formed, nitro-tyrosine containing Ca^{2+}-ATPase monomers and dimers should have been detectable as bands around 100 kDa and 200 kDa, respectively. On the other hand, nitrotyrosine was detected in our reference proteins, TNM-modified SR proteins (lane 5) and peroxynitrite-modified superoxide dismutase (lane 4). Lane 5 was obtained after application of 20 μg TNM-modified SR protein to the gel, whereas lanes 6–9 contained 60 μg of native and peroxynitrite-modified SR protein, respectively. Lane 4 displays bands for SOD both in its monomeric and dimeric form, obtained after application of 20 μg protein to the gel. Thus, in particular from a comparison of lanes 5–9, we have to conclude that the exposure of SR vesicles to peroxynitrite at [Ca^{2+}-ATPase]/[peroxynitrite] ratios ≤7 does not yield nitrotyrosine as a major reaction product from Ca^{2+}-ATPase.

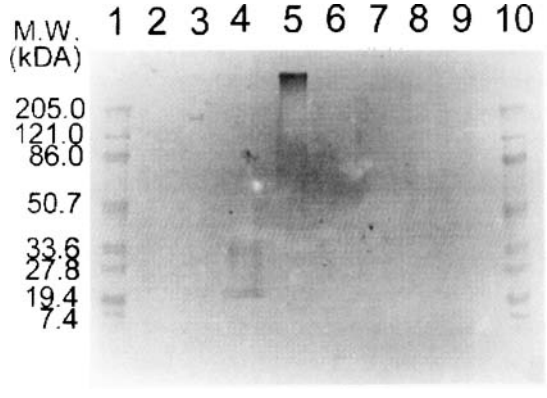

FIGURE 3 Immunodetection of nitrotyrosine in SR vesicles exposed to peroxynitrite. SR vesicles were incubated with 0 (lane 6), 0.1 (lane 7), 0.2 (lane 8), and 0.45 mM (lane 9) peroxynitrite, subjected to electrophoresis on a 7.5% Laemmli gel, and immunostained with an anti-nitrotyrosine antibody (see *Experimental*). Lanes 1 and 10 show protein standard markers; lane 4: nitrated superoxide dismutase; lane 5: SR proteins treated with TNM.

Tryptic hydrolysis

Figure 4 shows that peroxynitrite-modified SR proteins are generally more sensitive to tryptic hydrolysis, as revealed by a linear increase of the rate of hydrolysis versus initial concentration of applied peroxynitrite. Thus, we conclude that the exposure of SR proteins to peroxynitrite results in a more open conformation of these proteins which renders them more susceptible to proteolytic attack. On the other hand, such a potential opening does not go along with any change in the environment of the Trp residues, as demonstrated by the fluorescence quenching results.

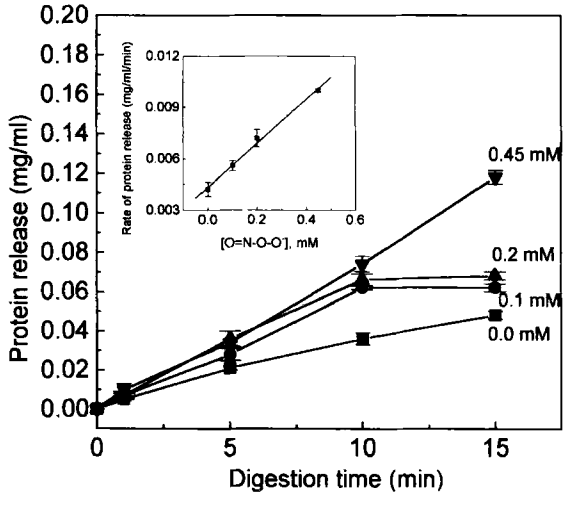

FIGURE 4 Susceptibility of peroxynitrite-treated SR proteins to tryptic digest. SR vesicles (10 mg/ml protein) were exposed to 0–0.45 mM peroxynitrite and subsequently treated with trypsin (12.5 μg/ml; ratio 1:100, w/w) in 50 mM ammonium carbonate, pH 8.5. After incubation for 15 minutes at 30°C the released peptides were monitored as described in *Experimental*. Each value represents the mean ± S.E. of two experiments. Inset: Rate of peptide release as a function of applied peroxynitrite concentration. Symbols: (■) 0, (●) 0.1, (▲) 0.2, (▼) 0.45 mM peroxynitrite.

DISCUSSION

The mechanism of Ca^{2+}-ATPase inactivation

The exposure of SR vesicles to peroxynitrite results in the loss of Ca^{2+}-ATPase activity. At low ratios of [peroxynitrite]/[Ca^{2+}-ATPase]≤3 ([peroxynitrite] ≤0.2 mM), this inactivation appears to be primarily through the oxidation of protein cysteine residues since large parts of it can be reversed by the subsequent reduction of the modified protein with DTT or $NaBH_4$, respectively. This result was expected based on previous findings that peroxynitrite efficiently oxidizes sulfhydryl groups.[22] Our results with SR Ca^{2+}-ATPase are also consistent with earlier suggestions that the oxidation of sulfhydryl groups by peroxynitrite inhibits sodium transport of Na^+ channels,[11] and affects thiol-containing enzymes of *Trypanosoma cruzi*.[16]

There are some significant differences in the ratios of oxidized protein thiols and the extent of inactivated protein for different peroxynitrite concentrations. The incubation of 10 mg/ml SR protein (65 μM Ca^{2+}-ATPase) with 0.2 mM peroxynitrite leads to an enzyme inactivation of 21%, paralleled only by a total loss of 10% of the SR protein cysteine residues, whereas after the incubation with 0.45 mM peroxynitrite an inactivation of ca. 40% is paralleled by a ca. 36% loss of SR protein thiols. Whereas much of the inactivation through 0.2 mM peroxynitrite is chemically reversible, this is not the case after the incubation with 0.45 mM and higher concentrations of peroxynitrite. With increasing peroxynitrite concentrations, there is a gradually increasing yield of covalent protein aggregates. The fact that these appear on non-reducing gels but only to a lower extent on reducing gels (presence of β-mercaptoethanol) would suggest that part of these aggregates are caused by *inter*molecular disulfide formation. Most interestingly, when SR protein which was modified with 0.45 mM peroxynitrite was subsequently treated with $NaBH_4$, a complete reversal of the sulfhydryl oxidation was observed without, however, a complete reversal of the enzyme activity. This observation may be rationalized by potential structural changes of the enzyme which could not be reversed by chemical reduction of the formed protein disulfides. However, together with the results from the analysis of the individual amino acids, protein carbonyls, fluorescence, and the general sensitivity to the Lowry assay, this observation also suggests the potential involvement of other amino acids in the oxidative protein modification. As such we could possibly suggest Tyr, Lys, Met, Phe, Thr, Ser, and Leu. Recently we found that the oxidation of a Tyr residue of SR Ca^{2+}-ATPase by hydrophilic peroxyl radicals, most probably Tyr_{587} of the cytoplasmic domain, is paralleled by a significant formation of protein aggregates and loss of activity (Viner *et al.*, unpublished results). From the residues listed above, only Tyr, Phe and Met have at present been shown to react directly with peroxynitrite.[7,8,21,23,24] However, redox reactions, occuring subsequently to the initial oxidation of a sulfhydryl group, may lead to the modification of the other residues (see below).

We can clearly discard a significant role of lipid peroxidation in the Ca^{2+}-ATPase inactivation since constant amounts of only 1.2 nmol/1 mg protein were detected for the exposure of SR vesicles to 0–0.45 mM peroxynitrite. In accordance with these results, it has been argued that levels in excess of 16 nmol MDA / 1 mg protein are required for a lipid-based inactivation of SR Ca^{2+}-ATPase.[59]

We shall briefly discuss the possible identities of the peroxynitrite derived protein products. In particular the total recovery of cysteine residues by exposure of a modified protein to $NaBH_4$ seems to indicate the formation of protein disulfides, since $NaBH_4$ was recommended for the selective reduction of protein disulfides.[46] The exposure of modified protein to $NaAsO_2$ was accompanied by the least effective recovery of thiols. This may indicate that sulfenic acids (RSOH) do not constitute a major fraction of stable products derived from the peroxynitrite mediated oxidation of SR vesicles.[22] This result is in line with findings reported on the oxidation of the bovine serum al-

bumine (BSA) sulfhydryl group which as well did not result in the formation of stable sulfenic acids.[22]

There are several possible mechanisms for the oxidation of thiols by peroxynitrite. Recently, it was shown by ESR experiments that thiol oxidation by peroxynitrite resulted in the formation of thiyl free radicals, $RS^{\bullet}$.[17,25] In the presence of molecular oxygen, the latter can form thiyl peroxyl radicals (reaction 2) which have been proposed to yield disulfides by entering reaction pathways 3 and 4.[60,61]

$$RS^{\bullet} + O_2 \rightleftarrows RSOO^{\bullet} \quad (2)$$

$$RSOO^{\bullet} + RSH \rightarrow RSO^{\bullet} + RSOH \quad (3)$$

$$RSOH + RSH \rightarrow RSSR + H_2O \quad (4)$$

This mechanism benefits in particular from the presence of more than one thiol group within the protein, as it is the case for Ca^{2+}-ATPase. On the other hand, a single cysteine residue as in BSA may not easily involve in reaction 3. Instead, reaction 5 may become important in which a thiyl peroxyl radical rearranges to a sulfonyl radical ($k_5 = 2 \times 10^3$ s^{-1} at 37°C)[61] which may undergo subsequent reactions to afford sulfinic and sulfonic acid.

$$RSOO^{\bullet} \rightarrow R^{\bullet}SO(O) \quad (5)$$

There are, however, important competing pathways, in particular when thiol groups are located sufficiently close to interact with each other. In such a case, thiyl radicals can form radical anion complexes with neighboring thiol groups, according to equilibria 6 and 7 (R, R′ = protein backbone).[62,63]

$$RS^{\bullet} + HSR' \overset{(6)}{\rightleftarrows} [RS.\therefore(H)SR'] \overset{(7)}{\rightleftarrows} \underset{\mathbf{1}}{[RS.\therefore SR']^{-}} + H^{+}$$

The radical anion complex **1** is a reducing species which can reduce molecular oxygen to superoxide anion (reaction 8).[64,65]

$$[RS.\therefore SR'] + O_2 \rightleftarrows [RS\text{–}SR'] + O_2^{\bullet -} \quad (8)$$

There is at present no report that superoxide reduces peroxynitrite/peroxynitrous acid, but by analogy to the reaction of superoxide with hypochlorous acid[66] we may expect reaction 9 to be possible.

$$O_2^{\bullet -} + ONOOH \rightarrow O_2 + NO_2^{-} + HO^{\bullet} \quad (9)$$

In addition, the thiolate complex **1** may directly reduce peroxynitrous acid according to reaction 10.

$$\underset{\mathbf{1}}{[RS.\therefore SR']^{-}} + ONOOH \rightarrow RS\text{–}SR' + NO_2^{-} + HO^{\bullet} \quad (10)$$

Thus, via the potential sequences 9 and 10 there is the possibility for formation of strongly oxidizing hydroxyl radicals. These species could account for the oxidation of Thr, Ser, Leu, and Lys in addition to the traditional peroxynitrite-targets Cys, Met, Tyr, and Phe. An increased yield of hydroxyl radicals has been observed by ESR-spin trapping studies during the decomposition of peroxynitrite in the presence of low molecular weight thiols.[17]

Along these lines we have also to discuss the experiments which additionally contained 5 mM GSH. Initially, this high GSH concentration failed to protect SR Ca^{2+}-ATPase from inactivation by peroxynitrite. However, the incubation of reaction mixtures with 2 mM DTT subsequent to the exposure to ≤0.45 mM peroxynitrite was accompanied by a higher reversal of Ca^{2+}-ATPase activity, suggesting the formation of mixed protein-glutathione disulfides. These protein-glutathione adducts themselves show reduced protein activity. However, since they constitute precursors for efficient chemical conversion into thiols by excess low molecular weight thiols, we have to consider that protein S-thiolation of Ca^{2+}-ATPase would be a protective rather than a destructive mechanism under physiological

conditions. The rate constants for the direct reaction of peroxynitrite with each of the individual 24 thiols of Ca^{2+}-ATPase are not known. A solution of 65 μM Ca^{2+}-ATPase would theoretically contain a maximum concentration of 1.56×10^{-3} M protein thiols, i.e. the concentration of protein thiols would be comparable to the concentration of added GSH (5 mM). Thus, for our experimental system we shall forward at least two possible (free radical) mechanisms for the formation of protein-glutathione disulfides which may start from the initial reaction of peroxynitrite with either GSH or protein-SH. Peroxynitrite may react either with protein thiols (reaction 11) to form protein thiyl radicals, protein-S$^{\bullet}$, or with glutathione (reaction 12) to form glutathionyl radicals, GS$^{\bullet}$.[17] Mechanistically, the formation of mixed protein-glutathione disulfides should then involve the association of either protein-S$^{\bullet}$ with deprotonated glutathione (reaction 13), or of GS$^{\bullet}$ with deprotonated protein thiol (reaction 14).[62,63] The subsequent reduction of molecular oxygen by the unsymmetric radical anion will then yield the mixed disulfide, protein-S-S-G (reaction 15). In our experimental system both pathways are expected to operate to a significant extent. Under physiological conditions, however, we may expect that both potentially formed thiyl radicals, GS$^{\bullet}$ and protein-S$^{\bullet}$, will be trapped by excess GS^{-}. Thus, formation of mixed glutathione-protein disulfides will essentially proceed from initially formed protein thiyl radicals, protein-S$^{\bullet}$, i.e. through reaction sequence 11, 13, and 15.

An alternative non-radical pathway of formation of mixed protein-glutathione disulfides may also proceed via nucleophilic attack of either protein thiol or GSH on peroxynitrous acid[67] (reactions 16 and 17) followed by reaction of the product sulfenic acid with a second unreacted thiol (reactions 18 and 19).

$$\text{ONOOH} + \text{protein-SH} \rightarrow \text{protein-S}^{\bullet} + \text{products} \quad (11)$$

$$\text{ONOOH} + \text{GSH} \rightarrow \text{GS}^{\bullet} + \text{products} \quad (12)$$

$$\text{protein-S}^{\bullet} + \text{GS}^{-} \rightleftarrows [\text{protein-S}\therefore\text{SG}]^{-} \quad (13)$$

$$\text{GS}^{\bullet} + \text{protein-S}^{-} \rightleftarrows [\text{protein-S}\therefore\text{SG}]^{-} \quad (14)$$

$$[\text{protein-S}\therefore\text{SG}]^{-} + \text{O}_2 \rightleftarrows \text{protein-SSG} + \text{O}_2^{\bullet -} \quad (15)$$

$$\text{ONOOH} + \text{protein-SH} \rightarrow \text{protein-SOH} + \text{H}^{+} + \text{NO}_2^{-} \quad (16)$$

$$\text{ONOOH} + \text{GSH} \rightarrow \text{GSOH} + \text{H}^{+} + \text{NO}_2^{-} \quad (17)$$

$$\text{protein-SOH} + \text{GSH} \rightarrow \text{protein-SSG} + \text{H}_2\text{O} \quad (18)$$

$$\text{GSOH} + \text{protein-SH} \rightarrow \text{protein-SSG} + \text{H}_2\text{O} \quad (19)$$

Subsequently, the incubation with DTT will reconstitute protein thiols analogous to reaction 20.

$$\text{protein-SS-G} + \text{DTT} \rightarrow \text{protein-SH} + \text{GSH} + \text{DTT(S-S)} \quad (20)$$

With regard to the potential products from Tyr, we attempted to identify the formation of bityrosine and nitrotyrosine by fluorescence spectroscopy and Western-blots, respectively. Both products do not contribute significantly to product formation. The lack of bityrosine is not surprising, however, since bityrosine formation requires the spatial proximity of two Tyr residues.[42] The absence of significant amounts of nitrotyrosine may be rationalized by the fact that an efficient nitration by peroxynitrite is promoted by transition metals.[8] The latter were removed as good as possible from our reaction systems by treatment with Chelex resin prior to the oxidation reaction.

Other potential products from Tyr may include 3,4-dihydroxyphenylalanine or its oxidation product, the ortho-quinone, but were not specifically analyzed.

The stoichiometry of Ca^{2+}-ATPase inactivation

From the total concentration of 65 μM SR Ca^{2+}-ATPase in our systems (10 mg/ml SR protein, 72% Ca^{2+}-ATPase), and a total titrated amount of 22.65 protein thiol groups/1.53×10^5 g native SR protein, we can attempt to obtain an estimate of the stoichiometry of the peroxynitrite reaction. For example, an exposure of 65 μM Ca^{2+}-ATPase to 100 μM peroxynitrite results in the loss of ca. 10% of the total protein thiol groups. Thus, a bolus addition of 100 μM peroxynitrite oxidizes a total of $(0.1 \times 65 \times 22.65) = 147$ μM protein thiols. This stoichiometry of ≈1.5 oxidized thiols per one equivalent of *added* peroxynitrite may well be the result of reactions 6–10, occuring subsequent to the formation of an initial thiyl radical. However, it shall be noted that the actual fraction of peroxynitrite which reacts with the protein may be significantly smaller than the added peroxynitrite concentration since, in its protonated form, peroxynitrite competitively suffers rapid unimolecular rearrangement into nitrate. Thus, the obtained ratio of [oxidized thiols]/[added peroxynitrite] ≈1.5 represents only a lower limit for the true stoichiometry of the oxidation process. At present we shall not attempt a more detailed mechanistic discussion of these ratios since there are too many unknown variables, in particular with regard to the protein structure, the accessibility, and the oxidation kinetics of the protein thiols.

The physiological significance

The present study demonstrates that peroxynitrite can inactivate the SR Ca^{2+}-ATPase to various extents depending on the peroxynitrite concentration. Other investigators have calculated some biologically relevant rates of peroxynitrite formation by activated macrophages of ca. 0.8 μM/minute in the whole lung or 1.0 mM/minute in the epithelial lining fluid.[9] In particular the latter value is well within the range of peroxynitrite concentrations employed in the present experiments. However, we shall stress that our experiments are still far from simulating oxidative processes in biological cells since they were performed on isolated SR vesicles. Further experiments on the potential mechanisms of peroxynitrite-induced protein oxidation in cells are needed but should benefit from the model studies reported in this paper.

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