



Original Contribution

Inhibition by 4-hydroxynonenal (HNE) of Ca^{2+} transport by SERCA1a: Low concentrations of HNE open protein-mediated leaks in the membrane

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ABSTRACT

Exposure of sarcoplasmic reticulum membranes to 4-hydroxy-2-nonenal (HNE) resulted in inhibition of the maximal ATPase activity and Ca^{2+} transport ability of SERCA1a, the Ca^{2+} pump in these membranes. The concomitant presence of ATP significantly protected SERCA1a ATPase activity from inhibition. ATP binding and phosphoenzyme formation from ATP were reduced after treatment with HNE, whereas Ca^{2+} binding to the high-affinity sites was altered to a lower extent. HNE reacted with SH groups, some of which were identified by MALDI-TOF mass spectrometry, and competition studies with FITC indicated that HNE also reacted with Lys⁵¹⁵ within the nucleotide binding pocket of SERCA1a. A remarkable fact was that both the steady-state ability of SR vesicles to sequester Ca^{2+} and the ATPase activity of SR membranes in the absence of added ionophore or detergent were sensitive to concentrations of HNE much smaller than those that affected the maximal ATPase activity of SERCA1a. This was due to an increase in the passive permeability of HNE-treated SR vesicles to Ca^{2+} , an increase in permeability that did not arise from alteration of the lipid component of these vesicles. Judging from immunodetection with an anti-HNE antibody, this HNE-dependent increase in permeability probably arose from modification of proteins of about 150–160 kDa, present in very low abundance in longitudinal SR membranes (and in slightly larger abundance in SR terminal cisternae). HNE-induced promotion, via these proteins, of Ca^{2+} leakage pathways might be involved in the general toxic effects of HNE.

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From the structural and functional points of view, sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA1a), an ATP-dependent Ca^{2+} pump that plays a major role during skeletal muscle relaxation, is the best

characterized member of the family of ion-translocating P-type ATPases [1,2]. Its crystal structure has been determined and comprises 10 transmembrane α -helices, connected to small luminal loops and to a large cytoplasmic head containing, in particular, the nucleotide-binding domain (N), to which the adenine moiety of ATP binds, and the phosphorylation domain (P) with the transiently phosphorylated active site (Asp³⁵¹) [2]. SERCA pumps provide useful models for studying oxidative stress-induced dysfunction of a membrane protein involved in Ca^{2+} homeostasis [3–11], and related ion pumps have indeed been found to be impaired during various kinds of oxidative stress, because of the formation of reactive oxygen species (ROS) and/or the lipid peroxidation-induced modification of the lipid bilayer structure [12–19].

It has been suggested that the cytosolic head of SERCAs was a target for various ROS. For instance, exposure of rat SERCA1a to peroxynitrite results in chemical modification of several Cys residues, mainly present in the cytoplasmic domain of SERCA [5–7]. Similarly, the inhibitory effect of hydroxyl radicals on rabbit SERCA1a (from skeletal muscle) or SERCA2a (from heart) is due to direct attack on the ATP binding site in the cytoplasmic domain [8]. On the other hand, peroxidation of membrane

Abbreviations: HNE, 4-hydroxy-2-nonenal; SR, sarcoplasmic reticulum; SERCA1a, sarcoplasmic reticulum Ca^{2+} -ATPase isoform 1a; Ca^{2+} -ATPase, calcium-dependent adenosine triphosphatase (EC 3.6.1.38); ROS, reactive oxygen species; GSH, reduced glutathione; DTNB, 5,5'-dithiobis(2-nitrobenzoate); DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; A23187, calcimycin; BSA, bovine serum albumin; FITC, fluorescein 5-isothiocyanate; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene; DNP, 2,4-dinitrophenylhydrazine; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; HCCA, α-cyano-4-hydroxycinnamic acid; C₁₂E₈, octaethylene glycol mono-*n*-dodecyl ether; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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lipids during oxidative stress in biological tissues is known to result in formation of several different aldehydic compounds as secondary oxidation products, e.g., 4-hydroxy-2-nonenal (HNE), malonaldehyde, and related aldehydes [20–26]. HNE, in particular, because it is a potent electrophile with three functional groups (a C1 carbonyl group, a double bond in C2, and a C4 hydroxyl group), is considered the most cytotoxic aldehyde produced in lipid peroxidation processes, because of its high chemical reactivity. HNE also has a half-life (ranging from minutes to hours) considerably longer than that of free radical species and can diffuse over longer distances than the precursor ROS (therefore behaving as a secondary toxic messenger to propagate and amplify oxidative injury). In addition, HNE is an amphiphilic compound and may accumulate within membranes in response to oxidative insult [20–26].

High levels of HNE-modified proteins have been detected in animal and human tissue under pathological conditions, suggesting that accumulation of HNE-modified proteins was involved in the pathophysiology of degenerative diseases and aging (Supplemental Refs. [1–10]). In vitro, HNE used at concentrations ranging from 10 μ M to 1 mM has been found to strongly inhibit various enzymatic activities (e.g., Supplemental Ref. [8]). In addition to reacting avidly with cellular nucleophiles such as glutathione, HNE reacts with several amino acid residues in proteins, namely cysteine (via its sulfhydryl group), histidine (via the imidazole nitrogens), and/or lysine (via the ϵ -amine) [20], possibly leading to structural modification and functional impairment of these proteins (e.g., Supplemental Refs. [9,10]). HNE has previously been shown to alter the ATPase activity of various P-type ATPases, such as the Ca^{2+} , Mg^{2+} -ATPase of erythrocyte plasma membrane [13,14], the Ca^{2+} -ATPase from human neutrophils [15], or the Na^{+} , K^{+} -ATPase of brain tissue [16,17]. It also remains possible that HNE could alter the dynamics of lipids in membranes, thereby affecting the function of transmembrane proteins [18,19].

In this paper, we have used the well-known Ca^{2+} pump SERCA1a of rabbit skeletal muscle sarcoplasmic reticulum (SR) fragments as a model for studying HNE-induced protein damage and its possible implications for the disruption of calcium homeostasis in cells. Beyond the characterization of HNE effects on SERCA1a, a remarkable finding was that treatment of SR membranes with HNE resulted in the opening of passive leakage pathways, mediated by very minor proteins associated with these membranes and which might be critical for the toxic effects of HNE.

Materials and methods

Preparation and characterization of native or HNE-treated SR vesicles

SR vesicles were prepared from rabbit fast-twitch skeletal muscle as previously described [3,27]. Glycogen phosphorylase was removed from SR vesicles using the procedure described in [28]. Protein concentration was either determined by the method of Lowry using bovine serum albumin as standard or deduced from absorption measurements at 280 nm in the presence of 1% sodium dodecyl sulfate (SDS), as in [29]. Unless otherwise indicated, SR treatment with HNE was performed by incubating 1 mg/ml SR vesicles with HNE at 37 °C, in a medium containing 100 mM KCl, 250 mM sucrose, and 50 mM Tes–Tris buffer at pH 7.4 (our “standard pH 7.4 medium”), in the presence of endogenous and contaminating Ca^{2+} (a few micromolar). In those cases in which partial evaporation of the initial volume of the sample was significant after a few hours of incubation at 37 °C, it was corrected for.

ATPase activity measurements

Ca^{2+} -ATPase activity was measured spectrophotometrically using a coupled enzyme assay (pyruvate kinase/lactate dehydrogenase), in the presence of either ionophore or detergent or in the absence of both [29,30]. For most of the experiments shown in this article (but

not all, check individual legends), the Ca^{2+} -dependent ATPase activity of SERCA1a, before or after treatment with HNE, was measured after dilution of the samples to 5 μ g/ml of SR protein into an assay medium, at 20 °C, consisting of 100 mM KCl, 50 mM Tes–Tris at pH 7.5, 1 mM Mg^{2+} , 5 mM MgATP, 0.1 mM Ca^{2+} , and 0.05 mM EGTA and containing all components of the coupled enzyme assay system (i.e., about 0.05 mg/ml pyruvate kinase, 0.05 mg/ml lactate dehydrogenase, 1 mM phosphoenol pyruvate, and 0.2–0.3 mM NADH). Unless otherwise indicated, this assay medium was also supplemented with 1 mg/ml C_{12}E_8 , as membrane solubilization by C_{12}E_8 results in ATPase activity values close to those measured in the presence of ionophore. In some cases, detergent was not present and ATPase activity was first measured with intact vesicles, before addition of ionophore (the Ca^{2+} ionophore calcimycin, A23187) or detergent.

ATP-dependent Ca^{2+} uptake

ATP-dependent Ca^{2+} uptake into SR vesicles was measured by filtration through Millipore HA filters as often described previously (e.g., [31]), but here with various protocols, more precisely described in the figure legends and the supplementary material. The medium generally consisted of buffer A (100 mM KCl, 5 mM MgCl_2 , 50 mM Mops–Tris at pH 7 and 20 °C), supplemented with 50 μ M Ca^{2+} plus $^{45}\text{Ca}^{2+}$ at 2 $\mu\text{Ci}/\mu\text{mol}$, and Ca^{2+} uptake was measured after addition of 1 mM MgATP from a concentrated stock solution.

In the simplest case (e.g., Fig. 2A), uptake was measured in the presence of 5 mM oxalate, a Ca^{2+} -precipitating anion. In this case, uptake was measured at a low concentration of protein (e.g., 25 μ g/ml) and aliquots containing only 5 μ g of SR protein (i.e., 200- μ l aliquots) were diluted into a “stop” medium (buffer A supplemented with 1 mM EGTA) before being loaded onto a Millipore HA nitrocellulose filter; the filter was briefly rinsed with stop medium and its radioactivity was counted in a scintillation counter. In the second case (e.g., Fig. 2B), in which uptake was measured in the absence of oxalate, a higher concentration of protein was used (250 μ g/ml) and aliquots (containing a larger amount of SR membranes, 50 μ g) were again diluted into stop buffer and filtered, with short rinsing. In the third case, steady-state uptake was measured without uptake quenching or filter rinsing; this required the use of double labeling (as described below for the measurement of equilibrium $^{45}\text{Ca}^{2+}$ binding, as well as in the legend to Fig. 6) and loading onto the filter of an even larger amount of membranes: 1.1-ml aliquots of 0.25 mg/ml protein samples in buffer A plus 50 μ M Ca^{2+} (plus $^{45}\text{Ca}^{2+}$) and 50 μ M [^3H]glucose were supplemented with 1 mM MgATP, and after 3 min (based on the results in Fig. 2B) 1 ml of this suspension (i.e., 250 μ g) was loaded onto two HA filters on top of each other and perfused with a protein-free but otherwise identical buffer (i.e., containing $^{45}\text{Ca}^{2+}$, [^3H]glucose, and 1 mM ATP, typically 0.7 ml); filters were counted without rinsing.

Ca^{2+} binding at equilibrium in the absence of ATP, ATP binding in the absence of Ca^{2+} , and ATP-derived phosphoenzyme formation in the presence of Ca^{2+}

Equilibrium $^{45}\text{Ca}^{2+}$ binding to SERCA1a in the absence of ATP was measured by filtration through Millipore HA filters, with double labeling (e.g., [32]). A total of 250 μ g of SR protein (in 1 ml) was loaded onto two superimposed filters, quickly rinsed with 1 ml of buffer A supplemented with 20 μ M EGTA (to completely eliminate any previously bound $^{40}\text{Ca}^{2+}$), and then perfused twice with the radioactive ligand-containing medium, namely buffer A supplemented with 50 μ M $^{45}\text{Ca}^{2+}$ (including contaminating Ca^{2+}) and 50 μ M ^3H -labeled glucose. Each perfusion was performed with 0.5 ml of this medium and the overall perfusion period (allowing ligand binding) was a few seconds, which was sufficient to reach equilibrium. Then, the two filters were counted for the two isotopes, *without* preliminary

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