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In Vitro Lipid Oxidation Modifies Proteins and Functional Properties of Sarcoplasmic Reticulum

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Abstract

Changes in protein and fatty acid compositions of flounder sarcoplasmic reticulum during NADH plus ascorbate-dependent lipid peroxidation *in vitro* were related to the ability of the sarcoplasmic reticulum to sequester Ca^{+2}. Progressive accumulation of high-molecular-weight protein components occurred concomitantly with loss of Ca^{+2}-sequestering activity. Part of this polymerized protein may be the dimer or trimer of Ca^{+2}, Mg^{+2}-ATPase. Loss in Ca^{+2}, Mg^{+2}-ATPase protein could account for over 60% of the polymerized protein. Rate of loss of polyunsaturated fatty acids was C22:6 > C20:4 > C20:5 > C22:5. Loss of polyunsaturated fatty acids and accumulation of thiobarbituric acid-reactive substances occurred concomitantly with protein polymerization.

Key Words: Sarcoplasmic reticulum; calcium uptake; lipid oxidation; polymerized protein.

Introduction

It has been suggested that one of the causes of aging is the oxidation of polyunsaturated lipids in membranes with attendant changes in the chemistry and functional properties of these membranes (Sinex, 1961; Packer *et al.*, 1967). However, little supporting evidence is available for this hypothesis. Chio and Tappel (1969) demonstrated that both sulfhydryl and nonsulfhydryl enzymes could be deactivated by lipid peroxidation. Ribonuclease A, inactivated by either peroxidizing lipids or by malonaldehyde (MDA), produced dimers as well as high-molecular-weight species. Pfeffer and Swislocki (1982) found several cross-linked proteins in erythrocytes which

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had been subjected to a peroxide-generating system. The same type of cross-linked proteins were found in aging erythrocytes. Steck (1972) observed that high-molecular-weight proteins increased progressively when erythrocyte membrane was treated with formaldehyde or malonaldehyde. *In vitro* lipid peroxidation of rat hepatocytes produced fluorescent chromolipids as well as high-molecular-weight proteins (Koster *et al.*, 1982). Hochstein and Jain (1981) showed that the addition of MDA to erythrocytes *in vitro* caused the formation of fluorescent chromolipids and high-molecular-weight protein polymers while decreasing bands 1 and 2 of spectrin. In these last studies with membranes, no attempt was made to link the observations to the functioning of the membranes.

The sarcoplasmic reticulum (SR) fraction of fish muscle contains an enzymic lipid peroxidation system requiring NADH and iron that is stimulated by ADP (McDonald *et al.*, 1979; Slabyj and Hultin, 1982). A somewhat similar system has been observed in the muscle of homeotherms (Lin and Hultin, 1976; Player and Hultin, 1977). It was demonstrated that lipid peroxidation in the SR of chicken breast muscle caused the membranes to become leaky to Ca^{+2} (Player and Hultin, 1978). In this study, we show that in the case of fish muscle SR, *in vitro* peroxidation of the SR is accompanied by an increase in high-molecular-weight proteins and a loss of the ability of the membrane to take up Ca^{+2}.

Materials and Methods

Winter flounder (*Pseudopleuronectes americanus*) which had been iced after catching were purchased from Gloucester day boats a few hours after catching. They were transported to the laboratory on ice and immediately filleted. All chemicals used in this research were of reagent grade.

SR were prepared by a gradient centrifugal procedure (Borhan *et al.*, 1984). The protein concentration of the SR was determined by a modified Lowry procedure (Markwell *et al.*, 1978).

SR at a concentration of 10 mg protein per ml was incubated in a solution containing NADH, 0.1 mM; ADP, 0.1 mM; $FeCl_3$, 0.12 mM; ascorbate, 0.83 mM; KCl, 0.12 M; and histidine, 5 mM, pH 7.3, in a shaking water bath at 7°C. The mixture was sampled periodically for analysis of lipids, proteins, and Ca^{+2}-uptake activity of the SR. Oxidation of the sample was determined by measuring the thiobarbituric acid (TBA) reactive-substances as described by Bidlack *et al.* (1973). Results are expressed as nmol malonaldehyde (MDA) per mg of SR protein using a molar extinction coefficient of $\varepsilon_{1\,cm/532} = 1.56 \times 10^5 cm^{-1} M^{-1}$.

Lipid was extracted from the SR membrane as described by Folch *et al.* (1957). Methyl esters were prepared by the procedure of Ackman and

Eaton (1971). The fatty acid methyl esters were separated using a 15% OV-275, chrom W column (2 m × 3.2 mm), pre-packed (Supelco, Inc., Bellefonte, Pennsylvania).

SDS-polyacrylamide gel electrophoresis was performed with a gel containing 10% of acrylamide : bisacrylamide (100 : 1) using the modified buffer systems of Porzio and Pearson (1977). Suspensions of SR were prepared for electrophoresis by disolving them in an equal volume of buffer containing 25 mM Tris-glycine, 8 M urea, 2% SDS, 4% mercaptoethanol, 50% glycerine, and 0.002% bromomethyl blue. A 5-mA electric current was passed through each tube (10 cm × 1.3 cm) until the tracking dye migrated to within 0.5 cm of the bottom of the gel. Gels were fixed in a solution containing 25% isopropanol and 10% acetic acid for 3 h. They were then stained overnight in a solution containing 0.1% Coomassie brilliant blue, 10% acetic acid, and 50% methanol. Destaining was accomplished by soaking the gels in a 10% acetic acid : 50% methanol solution until the background of the gel was clear. The gels were then scanned in a densitometer at a wavelength of 660 nm.

Ca^{+2}-uptake assays were performed using the method of Inesi and Scarpa (1972) with the addition of oxalate. This method utilizes the metallochromic indicator, murexide, whose absorbance changes when it binds Ca^{+2}. The absorbance of an equilibrated reaction medium of 3 ml containing 20 mM Tris, pH 6.8, 10 mM $MgCl_2$, 50 mM KCl, 200 μM $CaCl_2$, 5 mM potassium oxalate, and 100 μM ammonium murexide was measured in a dual wavelength spectrophotometer at 540 nm against 507 nm. The absorbance decreased when Ca^{+2} was removed from the reaction medium by the addition of SR vesicles and 0.3 ml of 3 mM ATP solution to a final pH of 6.8. ATP was used as the energy source to start the accumulation reaction. Various concentrations of EGTA in 0.6 ml of water (pH 6.8) were added to the reaction medium to obtain the standard curve.

Results and Discussion

In vitro lipid peroxidation of the SR from winter flounder was performed at 7°C in the presence of NADH, ADP, Fe^{+3}, and ascorbate. The production of TBA-reactive substances (MDA) is shown in Fig. 1. The rate of production of MDA was approximately linear for the first 90 min and then leveled off. Samples of SR incubated in the absence of any of the cofactors or in the presence of ADP-Fe^{+3} but not NADH showed no change in MDA during an incubation period of 135 min. Also shown in Fig. 1 is the decrease of accumulation of Ca^{+2} in the SR as the lipids of the membrane were oxidized. The observed decrease could be due to a decrease in Ca^{+2} uptake and/or an increase in passive permeability to Ca^{+2} causing leakage of accumulated

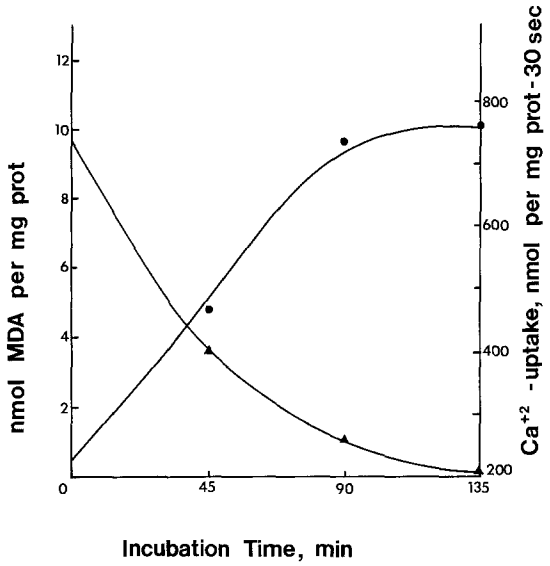

Fig. 1. Formation of TBA-reactive substances (●) (expressed as MDA) and loss of Ca^{+2}-sequestering activity (▲) in peroxidizing muscle SR. The conditions of incubation were as described in Materials and Methods. Data for TBA-reactive substances are the averages of duplicate determinations and those for Ca^{+2}-uptake are averages of triplicate determinations.

Ca^{+2}. The SR lost approximately 40% of the original Ca^{+2} accumulating ability in the first 45 min of incubation and about 65% after about 90 min. A small further loss was observed after 135 min of incubation. Control SR, which were incubated in the absence of the cofactors catalyzing peroxidation or in the presence of ADP-Fe^{+3} alone, showed no loss of calcium-accumulating ability after 135 min of incubation.

Losses in some of the fatty acids of the SR during the incubation are shown in Fig. 2. Results are normalized with respect to C16 : 0. As would be expected, the major losses were observed in the polyunsaturated fatty acids, with C22 : 6 showing the greatest decrease. The data of Fig. 2 are consistent with the production of TBA-reactive substances shown in Fig. 1 in that the loss of most of the fatty acids was complete by 90 min reaction time (only C22 : 6 still decreased after this time period). C20 : 4, C20 : 5, C22 : 5, and C22 : 6 represent 3, 24, 4, and 31% of the total fatty acids of the SR membrane of flounder, respectively (Shewfelt and Hultin, 1983), thus accounting for 62% of the total fatty acids of the membrane. Approximately 10% of these four fatty acids were oxidized in these experiments, and this was accompanied by a loss of approximately 75% of the Ca^{+2}-sequestering activity of the membrane fraction under our conditions. Lipid peroxidation

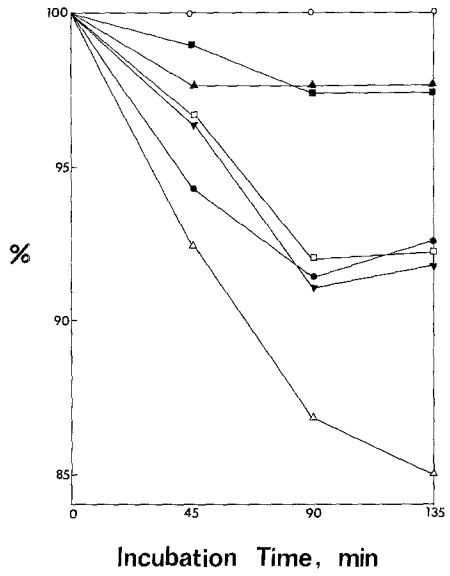

Fig. 2. Decrease in specific fatty acids during lipid peroxidation of SR membrane expressed as % and normalized to the value of C16:0. (○) C16:0; (▲) C18:0; (■) C18:1; (●) C20:4; (□) C20:5; (▼) C22:5; (△) C22:6. The conditions of incubation were the same as in Fig. 1.

of rat liver microsomes was primarily destructive to C20:4 and C22:6 fatty acids (Hogberg *et al.*, 1973). Changes in chemical, physical, and enzymic properties of the membrane were seen before electron microscopically observable changes. Different enzymes of the membrane responded differently to lipid peroxidation, i.e., activity increased, decreased, or remained constant and the response of a given enzyme was dependent on the extent of the peroxidation.

It is not clear why oxidation stopped after 135 min when there was considerable substrate left. It is possible that there was deactivation of the enzyme responsible for the catalysis of the lipid oxidation. Another possibility is that the NADH oxidase activity of the membrane destroyed the cofactor necessary for the reaction (McDonald, 1983).

Concomitant with the production of TBA-reactive substances and loss in Ca^{+2}-sequestering activity of the membrane was an increase in a high-molecular weight protein observed near the top of the electrophoretic gel and shown in the left-hand side of the densitometric scanning diagram (peak A in Fig. 3. Since this high-molecular-weight peptide persisted during SDS-PAGE in the presence of 4% mercaptoethanol, the covalent bonds involved are not disulfide. The molecular weight of this protein was estimated as 250,000 to 300,000 daltons, about 3 times that of the Ca^{+2}, Mg^{+2}-ATPase

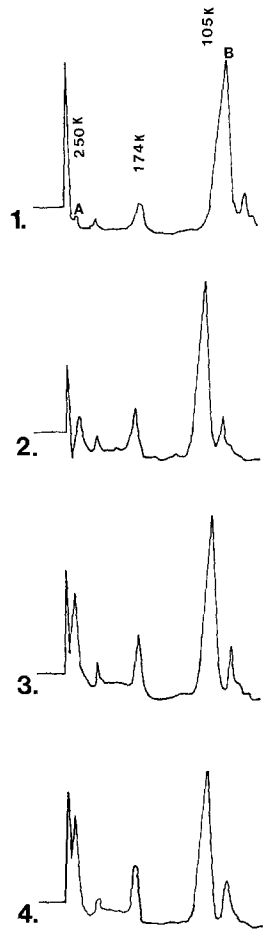

Fig. 3. Densitometric scans of SDS-PAGE gels of SR proteins as a function of time of oxidation. The conditions of incubation were the same as in Fig. 1. The SDS-PAGE is described in Materials and Methods. (A) polymerized protein; (B) Ca^{+2}, Mg^{+2}-ATPase. The scanning diagrams are (1) Control, (2) 45 min, (3) 90 min, and (4) 135 min incubation.

(molecular weight = 105,000 daltons). Peak B (the Ca^{+2}, Mg^{+2}-ATPase) appears in Fig. 3 to undergo some shifts. This was due to slight stretching or shrinking of the gels during handling after electrophoresis.

The areas under the scanned peaks of this high-molecular-weight protein, as well as the Ca^{+2}, Mg^{+2}-ATPase, were measured and plotted against time of incubation in the peroxidizing medium (Fig. 4). There was an essentially linear increase in the high-molecular-weight protein for the first 90 min of incubation, and then the increase slowed down between 90 and 135 min. There was a concomitant decrease in the Ca^{+2}, Mg^{+2}-ATPase

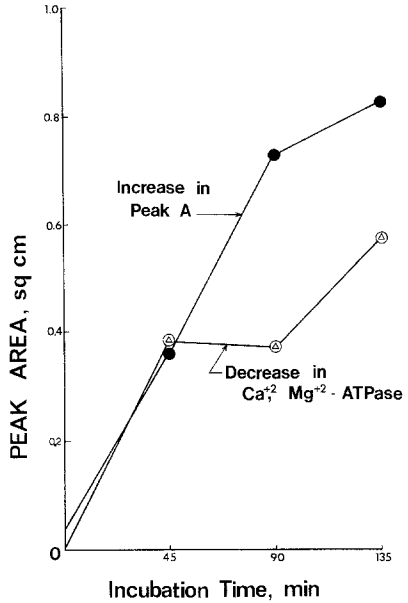

Fig. 4. Increase in high-molecular-weight protein fraction and decrease in Ca^{+2}, Mg^{+2}-ATPase protein as a function of time of oxidation.

protein which could not, however, account for the total increase in the high-molecular-weight fraction. This would indicate that proteins other than the ATPase are involved in the formation of the high-molecular-weight fraction.

Several research groups have reported that microsomal membranes lose their ability to take up Ca^{+2} when the membrane lipids undergo oxidation (Player and Hultin, 1978; Jones *et al.*, 1983; Kagan *et al.*, 1983a; Moore, 1984). Kagan *et al.* (1983b) reported that lipid oxidation of rabbit muscle SR induced both passive channels to calcium in the lipid bilayer as well as active channels in the lipoprotein complexes of the calcium pump. They observed oxidation of sulfhydryl groups as well but indicated that this was not related to membrane permeability. Lebedev *et al.* (1982) demonstrated increased Ca^{+2} permeability of SR after exposure to iron and ascorbate. The relationship that we observed in this study among lipid oxidation, increase in high-molecular-weight protein fraction (nondisulfide linked), decrease in Ca^{+2}, Mg^{+2}-ATPase protein, and loss of the ability of the sarcoplasmic reticulum to accumulate Ca^{+2} suggests that these may be related phenomena. We did not examine changes in sulfhydryl groups which are probably occurring in the Ca^{+2}, Mg^{+2}-ATPase during incubation, and the loss in the ability of the SR to accumulate calcium caused by either an effect on the active transport

system or passive permeability may be affected by these. It will also be important to determine the role of the nondisulfide cross-linked proteins we observed in the calcium transport system. It is of interest that we observed a similar increase in a high-molecular-weight protein fraction not due to disulfide bonding in the sarcoplasmic reticulum of 9-year-old flounder compared to 2-year-old flounder (Luo and Hultin, 1986).

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