The Effect of Lipid Peroxidation on the Calcium-Accumulating Ability of the Microsomal Fraction Isolated from Chicken Breast Muscle

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The effect of lipid peroxidation on the Ca^{2+} -accumulating and Ca^{2+} -retaining abilities of the microsomal fraction from chicken breast muscle was investigated. At 25°C, enzymic lipid peroxidation did not seriously affect either of these abilities unless ascorbic acid was present, when both were diminished. At 37°C, Ca^{2+} -concentrating ability was decreased further by the effects of heat damage to the membrane. Membrane lipid peroxidation did not affect microsomal adenosine triphosphatase activity unless the microsomal fraction was subsequently washed with albumin. This effect of albumin is possibly due to removal of lipid-breakdown products. Addition of soya-bean phospholipids to the peroxidized vesicles washed with albumin restored adenosine triphosphatase activity, demonstrating a non-specific phospholipid requirement.

An NAD(P)H-dependent lipid-peroxidation system found in the microsomal fraction of chicken breast muscle has previously been described (Player & Hultin, 1977). This system was shown to be dependent on NAD(P)H, ADP and Fe^{3+} ions for activity. Enzymic lipid-peroxidation systems have been shown to come from a number of sources, e.g. liver microsomal fractions (Hochstein & Ernster, 1963), mitochondria (Pfeifer & McCay, 1972) and electron-transport particles (Takeshige & Minakami, 1975). However, the role of these peroxidation processes and of the lipid peroxides formed in the cell has not been elucidated. It has been suggested that such a system might be connected with membrane turnover (Fong *et al.*, 1973), or with a possible control mechanism for cell division (Wolfson *et al.*, 1956).

With the discovery of an enzymic lipid-peroxidation system in the skeletal-muscle microsomal fraction, it was decided to investigate the effects of peroxidation on recognized sarcoplasmic-reticulum activities, i.e. Mg^{2+} -activated ATPase and Ca^{2+} transport. A phospholipid requirement has been shown for both ATP hydrolysis (Kielley & Meyerhof, 1950) and Ca^{2+} transport (Martonosi *et al.*, 1968). Several workers have indicated that the fatty acid composition of the membrane may vary considerably without affecting sarcoplasmic-reticulum activities (Newbold *et al.*, 1973; Tume *et al.*, 1973; Martonosi *et al.*, 1968; Fleischer *et al.*, 1962). However, Seiler & Hasselbach (1971) found that increasing amounts of

fatty acids of the oleic acid family and decreasing those of the linoleic acid family decreased Ca^{2+} -permeability of the membrane, giving rise to a fall in Ca^{2+} -dependent ATPase activity and a higher Ca^{2+} -concentrating capacity.

Inesi & Scales (1974) state that the requirement for detergents for the solubilization of the ATPase protein indicates that hydrophobic interactions contribute to its stabilization within the membrane structure. Tam & McCay (1970) showed that lipid peroxidation of rat liver microsomal fraction resulted in altered phospholipids, which were considerably more polar than normal microsomal lipids. The products of the reaction included water-soluble aldehydes and phospholipids, the 2-acyl groups of which contained carbonyl functions.

The present paper describes the effect of lipid peroxidation and of incubation temperature on the Ca^{2+} uptake and ATPase activities of the microsomal fraction isolated from chicken breast muscle. We have demonstrated that peroxidation in itself does not inactivate the ATPase, but washing the peroxidized samples with 1% albumin does result in inactivation. Activity may be restored by the addition of soya-bean phospholipid.

Experimental

Materials

ADP, ATP and NADPH were obtained from P-L Biochemicals, Milwaukee, WI, U.S.A. Phospholipase A (bee-venom) was from Worthington Biochemical Co., Freehold, NJ, U.S.A., bovine serum albumin was from Calbiochem, San Diego, CA,

* Abbreviation used: ATPase, adenosine triphosphatase.

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U.S.A., and thiobarbituric acid was from Eastman Organic Chemicals, Rochester, NY, U.S.A. $^{45}\text{CaCl}_2$ and Aquasol Universal Liquid Scintillation 'cocktail' were supplied by New England Nuclear Corp., Boston, MA, U.S.A. Asolectin (purified soya-bean phosphatides) were bought from Associated Concentrates, New York, NY, U.S.A. All other chemicals used were the purest available commercially.

Experimental animals for the investigations were female chickens (*Gallus americanus*) and were obtained from the Department of Veterinary and Animal Sciences at the University of Massachusetts, Amherst. The birds varied in breed and age, and were fed *ad libitum* on a commercial diet (H. K. Webster Co., Lawrence, MA, U.S.A.).

Methods

Preparation of the microsomal fraction from the chicken breast muscles (pectoralis major and pectoralis minor) has been described previously (Player & Hultin, 1977).

Malonaldehyde production was measured as an estimate of lipid peroxidation by the thiobarbituric acid test. The method was essentially that of Bidlack *et al.* (1973). A molar absorption coefficient ($\epsilon_{532}^{1\text{cm}}$) of 1.56×10^5 was used. Protein determination was by the biuret method of Gornall *et al.* (1949).

Microsomal Ca^{2+} accumulation was determined in a medium consisting of 0.12M-KCl, 5mM-histidine (adjusted to pH 7.25 with dilute NaOH), 5mM-MgCl₂, 5mM-potassium oxalate, 5mM-ATP and 0.1mM- $^{45}\text{CaCl}_2$. A protein concentration of 0.01–0.025mg/ml was generally used. Incubations were conducted at 23°C for 15 min in a shaking water bath and terminated by Millipore filtration (Martonosi & Feretos, 1964). The amount of bound Ca^{2+} was determined by liquid-scintillation counting of the filtrates and appropriate standards. Samples (0.5ml) of the filtrates were added to 14ml of Aquasol Universal Scintillation 'cocktail' and counted for radioactivity in a Nuclear-Chicago scintillation counter (series 720). Data and gate voltages used were 1000 and 1300 respectively.

Ca^{2+} release from vesicles was measured by a modification of the method of Fiehn & Hasselbach (1970). Microsomal fractions (7mg of protein) were loaded with calcium oxalate in 4ml of medium containing 0.12M-KCl, 5mM-histidine (pH 7.25), 5mM-potassium oxalate, 5mM-ATP, 5mM-MgCl₂ and 1mM- $^{45}\text{CaCl}_2$. Incubation conditions were 25°C for 15min in a shaking water bath. After this time 26ml of various dilution media was added, the compositions of which are described in the Results section 2. Samples (2ml) were taken at intervals and filtered through Millipore type HA filters. Samples (0.5ml) of the filtrate were taken for scintillation counting.

ATPase activity of the microsomal fractions was

determined essentially by the method described by Greaser *et al.* (1969). The incubation medium was 0.12M-KCl/5mM-histidine (pH 7.25)/5mM-MgCl₂/5mM-ATP/0.2mM- CaCl_2 . The final protein concentrations used were 0.1–0.5mg/ml. The medium was equilibrated to 25°C for 5min and the reaction started by the addition of the microsomal fraction. Samples (0.5ml) were removed periodically and placed in 0.5ml of cold 20% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation for 20min at maximum speed (setting 7) in a model C.L. International Clinical centrifuge. Samples (0.2ml) of the supernatant fraction were taken for inorganic phosphorus determination by the method of Rockstein & Herron (1951).

The ability of lipid-peroxidation-breakdown products to support sarcoplasmic-reticulum ATPase activity was investigated as follows. Samples (2.5mg of microsomal protein/ml) were incubated in the presence of a peroxidation system consisting of 0.12M-KCl, 5mM-histidine (pH 7.25), 0.4mM-NADPH, 4mM-ADP, 0.015mM-FeCl₃ and 0.25mM-ascorbic acid, for 75min at 25°C. Each sample was then halved and to one half was added 4vol. of 0.1M-KCl/5mM-histidine (pH 7.25)/5mM-MgCl₂/0.2mM- CaCl_2 . To the other half was added 4vol. of the same medium to which 1% albumin was added. The mixtures were centrifuged at 105000g_{av.} for 45min in a no. 40 rotor (Beckman model L ultracentrifuge). The sedimented fractions were resuspended in 0.12M-KCl/5mM-histidine (pH 7.25)/5mM-MgCl₂/0.2mM- CaCl_2 and centrifuged at 105000g_{av.} for 45min to remove residual albumin and were finally resuspended in the same medium before ATPase determination.

The preparation of Asolectin suspensions was modified from the procedure of Racker (1972). Suspensions were made up in 0.12M-KCl/5mM-histidine (pH 7.25)/1.6% (w/v) cholic acid at a concentration of 25mg of Asolectin/ml. This was sonicated in 30s bursts for 15min with a Branson Sonifier (setting 8), and was continuously cooled in an ice bath. The resulting suspension was centrifuged at 78000g_{av.} for 45min in a no. 30 rotor (Beckman model L preparative ultracentrifuge). The sedimented undispersed phospholipid was discarded, and the suspension was either stored in the refrigerator or used immediately. The final Asolectin suspension concentration was determined to be 10mg/ml.

Results

Effect of peroxidation on Ca^{2+} uptake

To investigate the effects of lipid peroxidation on the Ca^{2+} -accumulating ability of the microsomal fraction, incubations were carried out in the presence of the peroxidation system described in Table 1, at

7 and 34°C. Ascorbic acid was included in the medium to ensure a high degree of lipid peroxidation (Player & Hultin, 1977). Samples were taken periodically from the peroxidation medium and then incubated with Ca^{2+} -uptake medium. Malonaldehyde production was assayed simultaneously.

Table 1. *Effect of peroxidation at 7°C (a) and 34°C (b) on Ca^{2+} uptake*

The peroxidation system consisted of 0.12M-KCl, 5mM-histidine (pH 7.25), 0.4mM-NADPH, 4mM-ADP, 0.015mM- FeCl_3 , 0.25mM-ascorbic acid and 2.5mg of microsomal protein/ml. The data shown for Ca^{2+} uptake are the means $\pm$ s.d. of three experiments. The values for malonaldehyde production are the averages of two separate experiments.

Incubation time (h)	Ca^{2+} uptake ($\mu\text{mol}/\text{mg}$ of protein)	Malonaldehyde production (nmol/mg of protein)
(a)		
0	1.32 ± 0.16	0.63
1	1.21 ± 0.16	0.53
2	1.16 ± 0.10	0.95
3	1.14 ± 0.20	0.84
4	1.12 ± 0.10	0.95
5	1.12 ± 0.14	1.05
(b) (min)		
0	1.12 ± 0.15	1.9
10	1.08 ± 0.18	5.9
20	0.88 ± 0.09	8.9
35	0.78 ± 0.10	9.6
50	0.74 ± 0.09	11.4

The results in Table 1 show that at 34°C incubation with the peroxidation system stimulated malonaldehyde production. There also appeared to be a decline ($P < 0.05$ by Student's *t* test) in the ability of the microsomal fraction to accumulate Ca^{2+} . At 7°C no great increase in malonaldehyde production or significant change in Ca^{2+} -accumulating ability was apparent over a 5 h period.

Effect of incubation temperature during peroxidation on Ca^{2+} uptake

The effect of peroxidation at three incubation temperatures, 6, 25 and 37°C, on Ca^{2+} -accumulating ability was investigated. The incubation systems used are described in Table 2.

The results indicate that at an incubation temperature of 37°C considerable membrane damage occurred, whether or not a peroxidation system was present. When ascorbic acid was present with the peroxidation system at 25°C, a marked decrease in Ca^{2+} accumulation was observed. Under these latter conditions, therefore, it appears that peroxidation may be partially responsible for the decrease in the ability to take up Ca^{2+} and heat damage is not the only factor. At 6°C there appeared to be no significant decrease in the ability of the microsomal fraction to accumulate Ca^{2+} , in either the control system or the peroxidation systems.

It must be remembered that these results only indicate the total amount of Ca^{2+} retained by the microsomal fraction. The experiment does not give information as to whether a decrease in Ca^{2+} retention

Table 2. *Effect of incubation temperature and peroxidation on the Ca^{2+} -accumulating ability of the microsomal fraction*
The 'control' system consisted of 2.5mg of microsomal protein/ml in 0.12M-KCl/5mM-histidine (pH 7.25) solution. The 'peroxidation' system consisted of 2.5mg of microsomal protein/ml in 0.12M-KCl/5mM-histidine (pH 7.25)/0.4mM-NADPH/4mM-ADP/0.015mM- FeCl_3 . The 'peroxidation + ascorbate' system comprised the peroxidation system + 0.25mM-ascorbic acid. The values shown for Ca^{2+} uptake are the means $\pm$ s.d. of three experiments. The values for malonaldehyde production are the averages of two separate experiments.

Incubation temperature (°C)	Incubation time (min)	Ca^{2+} uptake ($\mu\text{mol}/\text{mg}$ of protein)			Malonaldehyde production (nmol/mg of protein)		
		Control	Peroxidation system	Peroxidation + ascorbate system	Control	Peroxidation system	Peroxidation + ascorbate system
6	0	1.86 ± 0.15	1.90 ± 0.08	1.40 ± 0.15	—	—	—
	20	1.90 ± 0.18	1.88 ± 0.14	1.28 ± 0.11	0.00	0.15	0.50
	40	1.90 ± 0.14	1.92 ± 0.11	1.20 ± 0.14	0.15	0.55	0.85
	60	1.90 ± 0.20	1.90 ± 0.14	1.15 ± 0.15	0.50	0.65	0.85
25	0	1.61 ± 0.14	1.60 ± 0.15	1.20 ± 0.15	—	—	—
	20	1.60 ± 0.20	1.60 ± 0.19	1.03 ± 0.10	0.50	1.25	3.25
	40	1.61 ± 0.14	1.52 ± 0.13	0.90 ± 0.11	0.75	2.00	7.00
	60	1.55 ± 0.14	1.50 ± 0.13	0.80 ± 0.15	0.50	2.25	10.20
37	0	1.51 ± 0.25	1.60 ± 0.32	1.12 ± 0.24	—	—	—
	20	1.13 ± 0.22	1.30 ± 0.22	0.85 ± 0.14	0.50	2.40	6.25
	40	0.87 ± 0.12	1.00 ± 0.15	0.54 ± 0.14	0.90	7.00	15.20
	60	0.70 ± 0.16	0.72 ± 0.17	0.47 ± 0.16	0.95	12.80	18.50

is due to a decrease in Ca^{2+} uptake or to a decrease in the ability of the membranes to retain Ca^{2+} .

Effect of peroxidation and phospholipase A on membrane permeability

Experiments were conducted to compare the effects of phospholipase A and peroxidation on the Ca^{2+} -retaining ability of the microsomal fraction. Samples were loaded with calcium oxalate, the details of which appear in the Experimental section. Addition of the dilution medium containing phospholipase A to the loaded vesicles brought about an extremely rapid release of accumulated Ca^{2+} (Fig. 1). This may be explained by the fact that hydrolysis of the membrane phospholipids leads to the formation of leaks in the membrane through which the accumulated Ca^{2+} can diffuse. These results are in agreement with the findings of Fiehn & Hasselbach (1970).

Fig. 1. Effect of phospholipase A on Ca^{2+} release from loaded vesicles

After loading the microsomal vesicles with calcium oxalate, 26 ml of the following dilution media was added: ○, 0.12 M-KCl/5 mM-histidine (pH 7.5)/5 mM- MgCl_2 ; ●, 0.12 M-KCl/5 mM-histidine (pH 7.25)/5 mM- MgCl_2 /0.01 mg of phospholipase A/ml. $^{45}\text{Ca}^{2+}$ release was measured in sample filtrates after Millipore filtration. Values represent the average of two separate experiments and results for each experiment varied by less than 5%.

The experiment was repeated, but phospholipase A in the dilution medium was replaced by a peroxidation system including ascorbic acid. The incubation period during Ca^{2+} release was extended to 60 min. Fig. 2 shows the effect of such a treatment. Although this peroxidation system caused a slower release of Ca^{2+} from the vesicles to the surrounding medium in the early stages of the incubation than resulted from phospholipase A treatment, after 60 min there was almost total release of the accumulated Ca^{2+} . It is therefore a reasonable assumption that peroxidation of the membrane lipids, under the conditions used, also results in 'leaky' membranes.

Finally, the experiment was conducted in which the dilution medium contained the peroxidation system without ascorbic acid. A preliminary experiment, involving a 60 min incubation of the loaded vesicles after dilution with the peroxidation medium, showed virtually no difference from control values over the same incubation time. Fig. 3 shows that, even after 4 h incubation, the peroxidation system without ascorbic acid caused no more Ca^{2+} leakage than that experienced by the control system, i.e. peroxidation of the membrane lipids under these conditions was not severe enough to form leaks in the membrane through which Ca^{2+} could pass.

Fig. 2. Effect of a peroxidation system (+ ascorbic acid) on Ca^{2+} release from loaded vesicles

The following dilution media were added to the calcium oxalate-loaded vesicles: ○, 0.12 M-KCl/5 mM-histidine (pH 7.25)/5 mM- MgCl_2 ; ●, 0.12 M-KCl/5 mM-histidine (pH 7.25)/5 mM- MgCl_2 /4 mM-ADP/0.4 mM-NADPH/0.1 mM- FeCl_3 /0.25 mM-ascorbic acid. Samples taken periodically were measured for $^{45}\text{Ca}^{2+}$ release. Values represent the average of two separate experiments and results for each experiment varied by less than 10%.

Fig. 3. Effect of a peroxidation system on Ca^{2+} release from loaded vesicles

Loading of the vesicles and measurement of ^{45}Ca release were as described above. The dilution media consisted of: ○, 0.12M-KCl/5mm-histidine (pH 7.25)/5mm-MgCl₂; ●, 0.12M-KCl/5mm-histidine (pH 7.25)/5mm-MgCl₂ / 0.4mm-NADPH / 4mm-ADP / 0.1mm-FeCl₃. Values represent the average of two separate experiments and results for each experiment varied by less than 10%.

Effect of peroxidation on ATPase activity

Experiments were designed to investigate if the presence of breakdown products after peroxidation were important with respect to maintenance of the ATPase activity. After peroxidation for 75 min at 25°C, the sample was washed with a 1% albumin solution before ATPase assay. Experimental details are described under 'Methods'.

Microsomal samples that had been incubated in the presence of the peroxidation medium and then diluted with a solution containing 1% albumin exhibited an ATPase activity of $0.04 \pm 0.02 \mu\text{mol of P}_i/\text{min per mg of protein}$. Samples incubated under the same conditions and diluted with a solution from which albumin was omitted showed an ATPase activity of $0.10 \pm 0.02 \mu\text{mol/min per mg}$ ($P < 0.05$). Results in both cases are means $\pm$ S.D. for three determinations.

The action of albumin in the removal of products formed by the action of phospholipase A on phospholipids was described by Fleischer & Fleischer (1967). Although different end products result from peroxidation, albumin certainly appears to remove some of the membrane constituents in the peroxidized sample that are necessary to support ATPase activity. Later experiments have shown that, if the albumin washing procedure is repeated (i.e. a 'double wash'), the ATPase activity can be eliminated entirely.

Restoration of ATPase activity in peroxidized vesicles by the addition of soya-bean phospholipids

Experiments were undertaken to restore ATPase

Table 3. Effect of Asolectin addition to peroxidized vesicles

The control system consisted of 20mg of microsomal protein in 0.12M-KCl/5mm-histidine (pH 7.25)/0.1mm-dithiothreitol/1% albumin in a total volume of 10ml. The peroxidation system was as the control + 0.4mm-NADPH/4mm-ADP/0.015mm-FeCl₃/0.25mm-ascorbic acid. The reaction systems were incubated at 25°C for 75 min and were stopped by the addition of 4vol. of 0.12M-KCl/5mm-histidine (pH 7.25)/0.1mm-dithiothreitol/1% albumin. Centrifugation followed at 105000g_{av} for 45 min in a no. 40 rotor (Beckman model L ultracentrifuge). The wash was repeated in the same medium and washed a third time with the dilution medium excluding albumin. The sedimented fractions were finally resuspended in 0.12M-KCl/5mm-histidine (pH 7.25) solution. Samples (2ml) of each microsomal suspension were added to Asolectin suspension (2ml) (or 0.12M-KCl/5mm-histidine solution) to give a final microsomal protein concentration of 2.5mg/ml. These were incubated for 4 min at 37°C, then dialysed for 18 h against 0.3M-KCl/5mm-Tris/HCl (pH 7.5) (500vol.) at 4°C. ATPase determination followed, at a final protein concentration of 1mg/ml. The data shown are the means $\pm$ S.D. for three experiments.

Treatment	ATPase activity ($\mu\text{mol of P}_i/\text{min per mg of protein}$)
Control	0.062 ± 0.012
Control + Asolectin	0.084 ± 0.015
Peroxidized	0.025 ± 0.010
Peroxidized + Asolectin	0.086 ± 0.020

activity of peroxidized albumin-washed microsomal samples by the addition of mixed soya-bean phospholipids (Asolectin). The incubation systems, washing procedure and addition of Asolectin are described in the legend to Table 3. The four samples under investigation consisted of control vesicles to which Asolectin had and had not been added, and peroxidized vesicles to which phospholipids had and had not been added. After a dialysis period of 18 h, the samples were assayed for ATPase activity.

It was demonstrated that, although peroxidation and albumin washing, under these conditions, decreased ATPase activity ($P < 0.05$), phospholipids could be applied to the system to restore it. It was noteworthy, however, that the addition of phospholipid to the untreated control sample also caused an increase in ATPase activity.

Discussion

Results from the experiments investigating the effect of temperature on Ca^{2+} uptake in the presence of the peroxidation systems, and from the Ca^{2+} -release experiments described above, indicate that Ca^{2+} uptake in the presence of ATP, and Ca^{2+}

retention in preloaded vesicles, are greatly affected by a peroxidation system including ascorbic acid. Although lipid peroxidation is stimulated by an increase in temperature, care must be taken in interpreting the effects of peroxidation with respect to Ca^{2+} -accumulating ability, because of the labile nature of the membrane towards heat, which has been demonstrated. In the absence of ascorbic acid the peroxidized samples varied very little from the control samples in terms of Ca^{2+} uptake and retention. Such observations raise interesting questions as to the overall effect of ascorbic acid on lipid peroxidation in skeletal muscle, and to the effect of its concentration and localization in the cell. It is also noteworthy that the presence of an enzymic lipid-peroxidation system, which could be involved in such processes as membrane lipid turnover, is not damaging to Ca^{2+} uptake and retention in the absence of ascorbic acid.

The effect of peroxidation in the presence of ascorbic acid may appear to cause gross damage to the membrane in terms of forming leaks, whereby Ca^{2+} may no longer be retained. However, this severe physical damage does not appear to inactivate the ATPase. Inesi & Scales (1974) have stated that hydrophobic interactions contribute to the stabilization of the ATPase protein within the membrane structure. Peroxidation to the extent encountered under the conditions used in our studies, which would result in more highly polar membrane lipid components, does not appear to upset this stabilization. Removal of some of the polar peroxidation end products possibly does upset this stable situation, resulting in enzyme inactivation. These results indicate that, even when the local hydrophobic environment of the membrane is changed through peroxidation, the ATPase remains active. Washing the peroxidized vesicles with albumin, which may be responsible for the removal of water-soluble products, resulting in ATPase inactivation, could indicate a spatial orientation role for the lipid, especially as the activity may be restored on addition of Asolecithin. Martonosi *et al.* (1968) have shown that the phospholipids are not involved as phosphorylated intermediates or cofactors for ATP hydrolysis in the ATPase reaction. Re-activation of the ATPase in peroxidized albumin-washed vesicles by the addition of mixed soya-bean phospholipids demonstrates the relative non-specific phospholipid requirement of the microsomal ATPase isolated from chicken breast muscle.

It should be remembered that this line of experimentation examines the effect of peroxidation in a situation *in vitro*. It is most likely that the microsomal fraction is no longer subject to the protective mech-

anisms operative in the cell towards lipid peroxidation. Such systems would probably include superoxide dismutase, glutathione peroxidase, catalases and peroxidases. It should also be noted that incubations were performed in the presence of air, and that the oxygen supply to the system was therefore not limiting, which is not representative of conditions *in vivo*.

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