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A Study on the Mechanism of Calcium Binding Inhibition of Cardiac Sarcoplasmic Reticulum by Oxygen Free Radicals[1]

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ABSTRACT

Mechanism of calcium transport inhibition of cardiac sarcoplasmic reticulum (SR) by oxygen free radicals was examined. Effects of oxygen free radicals generated by xanthine/xanthine oxidase (X/XO) system on isolated porcine ventricle SR were studied with respect to its calcium binding, lipid peroxidation, SH-group content and alteration of membrane protein components.

The results are as follows.

1) Calcium binding of isolated SR was markedly inhibited by X/XO.
2) During the incubation of sarcoplasmic reticulum with xanthine/xanthine oxidase, there were marked increse in lipid peroxidation and reduction of SH-group content.
3) An antioxidant, *p*-phenylenediamine effectively prevented the lipid peroxidation but partially prevented the calcium binding inhibition of X/XO treated SR.
4) The reduction of SH-group content of SR treated with X/XO was partially prevented by *p*-phenylendiamine.
5) When modifying SH-group of SR by treatment with DTNB, the inhibition of calcium binding activity was partially prevented.
6) On gel-permeation chromatography of X/XO-treated sarcoplasmic reticulum, there was an increase of small molecular weight products, probably protein degradation products.
7) Semicarbazide, which prevents the cross-linking reaction of protein components, did not affect the calcium binding inhibition of X/XO-treated SR.

From these results, it is suggested that the inhibition of calcium binding of SR by oxygen free radicals results from the consequence of multiple changes of SR components, which are lipid peroxidation, SH-group oxidation and degradation of protein components.

Key Words: calcium binding, sarcoplasmic reticulum, oxygen free radical

INTRODUCTION

During episode of myocardial ischemia, functions of subcellular organelles have been reported to be significantly deranged. The sarcoplasmic reticulum (SR) isolated from ischemic and anoxic myocardium has been found to be decreased in its calcium binding, uptake and release as well as Ca^{++}-ATPase

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activity (Bornet *et al.*, 1977; Gillette *et al.*, 1979; Hess *et al.*, 1981a; Schwartz *et al.*, 1973). In recent studies, Hess *et al.* (1981b; 1983) have demonstrated that oxygen free radicals generated from xanthine and xanthine oxidase system uncouple calcium transport from ATP hydrolysis in the SR, a finding similar to that found in ischemic myocardium.

In ischemic state of tissues, oxygen depletion causes an intracellular accumulation of reducing equivalents, leading to production of oxygen free radicals (Fridovich, 1975) which are highly reactive and can damage most types of cellular macromolecules. In *in vitro* studies, they have been shown to oxidize unsaturated fatty acids (Kellogg & Fridovich, 1975, 1977) and proteins (Venkatasubramanian & Joseph, 1977), damage nucleic acids (Lavelle *et al.*, 1973) and cleave polysaccharides (McCord, 1974).

In a previous study using xanthine and xanthine oxidase (X/XO) as an oxygen radical generating system, the authors (1984) have demonstrated inhibition of calcium binding of isolated cardiac SR by oxygen free radicals and suggested that the inhibition was mediated by hydroxyl radical and singlet oxygen, which are produced from interation of superoxide anion and H_2O_2 (Haber-Weiss reaction).

The present study was undertaken to explore the possible mechenism of altered calcium transport of cardiac SR by oxygen free radicas. For that purpose, the calcium binding inhibition by oxygen radicals was examined with respect to changes of various components of SR, i.e. lipid peroxidation, oxidation of sulfhydryl groups and protein degradation.

MATERIALS AND METHODS

Adenosine triphosphate(disodium salt), 0-(1,8-dihydroxy-3,6-disulfo-naphthylene-2,7-biazo)-bis-benzenearsonic acid (Arsenazo III), xanthine oxidase, 5,5′-dithiobis-2-nitrobenzoic acid(DTNB), DL-dithiothretiol (DTT), sodium dodecyl sulfate (SDS), 2-thiobarbituric acid, Sephadex G-200, were obtained from Sigma Chemical Co.; xanthine from Merck; sodium deoxycholate from Oxoid Ltd.; *p*-phenylenediamine and semicarbazide from Junsei Chemical Co.; allopurinol from Samil Pharm. Co..

Preparation of cardiac sarcoplasmic reticulum vesicle

SR vesicles were prepared from porcine cardiac ventricles according to the method of Harigaya and Schwartz(1969) with little modification. The final pellet obtained by centrifugation at 100,000g for 45 min was resuspended in 0.25M sucrose and 10mM Tris-maleate, pH 7.0 to make protein concentration of 7-8 mg/ml and stored at −20°C. Under these conditions the isolated SR showed no detectable changes in the calcium binding for about one week. All the procedures were performed at 0-4°C. Protein concentration was measured by the method of Lowry *et al.* (1951). Porcine ventricle was obtained freshly from a slaughter house.

Treatment of sarcoplasmic reticulum with xanthine/xanthine oxidase

SR(0.1-1 mg protein/ml) was incubated with xanthine oxidase (20 munits/ml) in the reaction mixture consisting of 100 mM KC1, 40 mM Tris-maleate, pH7.0 and 4 mM xanthine at 25°C. The reaction was started by addition of xanthine, and stopped by adding either 10 mM allopurinol or 2% sodium dodecyl sulfate or 5% trichloroacetic acid depending on experiments. The incubation was performed for up to 180 min in a Dubnoff shaking water bath with a constant agitation rate of 120/min. At time intervals, aliquots were removed and assayed for calcium binding, sulfhydryl group and lipid peroxidation, and were analyzed by gel-permeation chromatography.

Measurement of calcium binding

Calcium binding of SR was measured with a dual wavelength spectrophotometer(Aminco-Chance,

American Instrument Co., U.S.A.) using arsenazo III as a calcium sensitive metallochromic indicator. SR(0.3-0.5 mg protein/ml) treated with xanthine and xanthine oxidase (X/XO) as described above was incubated at 37°C in a cuvette containing 3ml of reaction mixture consisting of 100 mM KC1, 40 mM Tris-maleate, pH 7.0, 5 mM $MgCl_2$, 5 mM NaN_3, 20 μM $CaCl_2$, 0.2 mM ATP and 100 μM arsenazo III. After 5 min preincubation, the calcium binding was started by addition of ATP and measured by observing at a wavelength pair of 675-685nm. At this wavelength pair the changes of ionized calcium concentration, up to 20-30 μM, produced linear increament of ΔA without interference from Mg^{++} (Scarpa, 1979).

Assay of sulfhydryl group

Sulfhydryl (SH) group of SR were determined spectrophotometrically by Ellman's method(1959). After treatment of SR with X/XO, 1.0 ml of aliquot contining 0.7 mg/ml of SR was mixed with 2.0 ml of 0.5% sodium dodecyl sulfate (SDS) in 100 mM Tris-HCl, pH 8.0. After the mixture became clear, 0.02 ml of 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 100 mM Tris-HCl, pH 7.0 was added. After 10 min incubation at 37°C, absorbance was measured at 412nm. The content of SH-group was estimated from the molar extinction coefficient of p-nitrothiophenol anion, 1.36 x 10^4/M/cm(Ellman, 1959).

Modification of SH-group

SR was treated with DTNB, a reversible SH-group modifying agent. SR(4.0 mg/ml) in 100mM Tris-HCl, pH 8.0 was incubated with 10 mM DTNB for 30 min at 25°C, and then was washed twice with 20 mM Tris-maleate, pH 7.0 at 4°C. To reactivate the modified SH-group, the DTNB-treated SR was reincubated with 10 mM dithiothreitol (DTT) at 25°C for 30 min. After that, the SR was washed twice with 20 mM Tris-maleate, pH 7.0 and used for experiments.

Measurement of lipid peroxidation

Lipid peroxidation was followed by measuring malondialdehyde with thiobarbituric acid method (Bidlack and Tappel, 1973). One ml aliquots of reaction mixutres containing 0.1 mg protein/ml of SR were mixed with 0.5 ml of distilled water and 0.5 ml of cold 30% trichloracetic acid. The mixtures were centrifuged at 2,000g for 20 min. The supernatant was added to an equal volume of aqueous 0.67% thiobarbituric acid and the chromophore was developed in a boiling water bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm. The concentration of malonaldehyde was expressed as nmoles/mg protein using the molar extinction coefficient of 1.52 x 10^5/M/cm(Placer et al., 1966).

Chromatographic analysis of sarcoplasmic reticulum treated with xanthine-xanthine oxidase

Two ml of X/XO-treated SR (1.0 mg/ml) was mixed with 0.5 ml of 10% sodium dodecyl sulfate and 0.1 ml of mercaptoethanol, and boiled for 3min. The mixture was then applied on Sephadex G-200 column (2.5x15cm) equilibrated with eluting buffer containing 0.5% sodium deoxycholate, 100 mM NaCl, 0.2 mM EDTA and 10 mM Tris-HCl, pH 7.8. The effluent was monitored continuously with a spectrophotometer (Hitachi-Perkin Elmer, UV-VIS) at 280 nm. All the chromatographic procedures were operated at room temperature and the flow of column effluent was maintained in a constant rate of 17ml/h with a peristaltic pump.

RESULTS

Effects of xanthine/xanthine oxidase on sulfhydryl group

During the incubation of SR with X/XO, the content of SH-group was rapidly decreased in the first 60 min and in a slower rate thereafter. At 60 min of incubation, the SH-group content was reduced

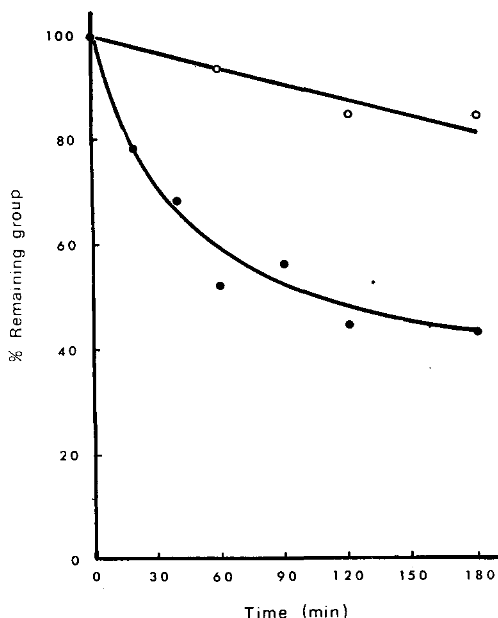

Fig. 1. Change in SH-group content of SR treated with xanthine/xanthine oxidase. SR(0.7 mg protein/ml) was incubated with 20 munits/ml xanthine oxidase in the absence(o) or presence (●) of 4 mM xanthine in the solution of 100 mM and 40 mM Tris-maleate, pH 7.4 at 25°C. Total volume was 8.0 ml. At various intervals, aliquots (1.0 ml) were mixed with 0.3 ml of 5% SDS and 1.7 ml of 100 mM Tris-HCl, pH 8.0, and assayed for sulfhydryl group as described under the Materials and Methods. The SH-group content at zero time was 53.0 nmoles/mg protein which was taken as 100%.

Table 1. Effect of SH-group protection with DTNB on the inhibition of calcium binding of SR treated with xanthine/xanthine oxidase

Experiments	DTNB treatment	X/XO treatment (min)	DTT treatment	Calcium binding (%)
1	–	0	–	100
2	+	0	–	2.1
3	+	0	+	95.8
4	–	30	–	59.7
5	+	30	+	79.7

SR (4.0 mg/ml) was incubated with 10 mM DTNB for 30 min in 100 mM Tris-HCl, pH 8.0 at 25°C, and centrifuged at 100,000 g. The resulting pellet was washed twice with 20 mM Tris-maleate, pH 7.0 by centrifugation at 100,000 g, and then treated with X/XO for 30 min in the same way as in Fig. 1. The reaction mixture was further incubated with 5 mM DTT for 30 min. SR pelleted and washed as described above was assayed for calcium binding.

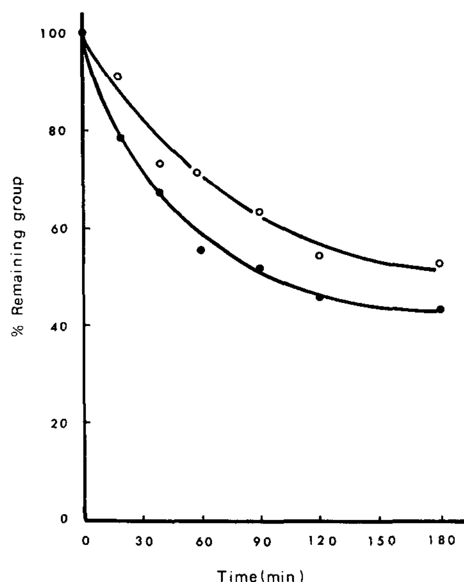

Fig. 2. Effect of *p*-phenylenediamine on SH-group content of xanthine/xanthine oxidase-treated SR. SR was treated with X/XO in the absence (o) or presence (●) of 50 μM *p*-phenylenediamine. All the experimental conditions were the same as in Fig. 1.

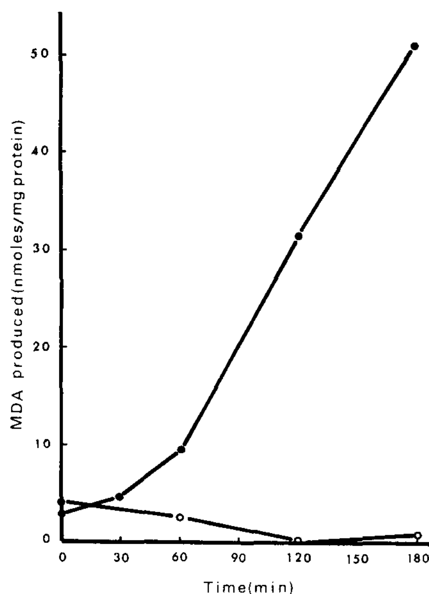

Fig. 3. Malondialdehyde production from SR treated with xanthine/xanthine oxidase. SR (0.1 mg protein/ml) was incubated with xathine oxidase in the absence (o) or presence (●) of xanthine in the same way as in Fig. 1. Aliquots (1.0 ml) taken at intervals indicated were assayed for malondialdehyde as described under the Materials and Methods.

to 52% of zero time level (53.0 nmoles/mg protein). Neither xanthine nor xanthine oxidase alone affected the level of SH-group (Fig. 1). Since this reduction of SH-group content in X/XO-treated SR was considered a result of oxidation reaction by oxygen radicals, preventive effect of an antioxidant was observed. By addition of an antioxidant, *p*-phenylenediamine into the incubation mixture containing SR and xanthine-xanthine oxidase, the reduction of SH-group was partially prevented (Fig. 2).

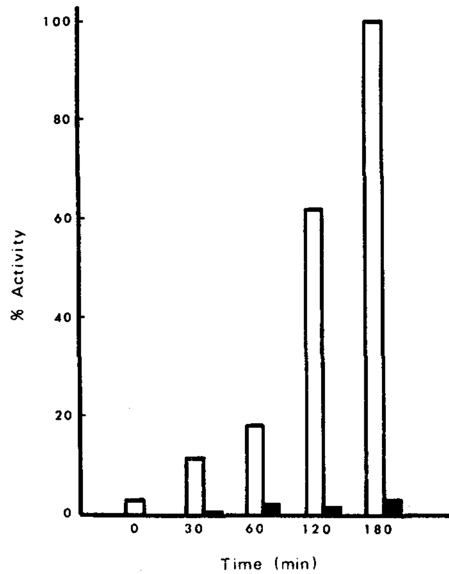

Fig. 4. Effect of *p*-phenylenediamine on malondialdehyde production from SR treated with xanthine/xanthine oxidase. SR was treated with X/XO in the absence (open bar) or presence (solid bar) of 50 μM *p*-phenylenediamine as in Fig. 3.

Table 2. Protective effect of *p*-phenylenediamine on the inhibition of calcium binding of xanthine/xanthine oxidase-treated SR

Time (min)	% Remaining activity	
	−PPDA	+PPDA*
0	100	100
30	80.5	94.1
60	60.3	79.1
120	28.9	59.1
180	13.9	36.6

SR (2.0 mg/ml) was treated with X/XO in the absence or presence of 50 μM *p*-phenylenediamine. The conditions for experiments and calcium binding assay were the same as in Fig. 6. Calcium binding at zero time (35.7 nmoles/mg protein) was taken as 100%. **p*-phenylenediamine

Considering the essential role of SH-group for catalytic activities of various enzymes and membrane functions, the reduction of SH-group may cause an inhibition of calcium transport in sarcoplasmic reticulum. To examine the possibility, an attempt was made to protect SH-groups against oxygen radical attack. As shown in Table 1, when SR was treated with a SH-modifying agent, DTNB, calcium binding of SR was almost completely abolished (Experiment 2). And with reactivation of this modified SH-groups by DTT treatment, the binding activity was restored to the original control level (Experiment 3). However, when the DTNB-treated SR was incubated with X/XO, the recovery of the calcium binding activity after DTT teratment was not so complete (Experiment 5).

Effects of xanthine/xanthine oxidase on lipid peroxidation

With X/XO treatment of SR, peroxidation of the membrane lipids was also observed as indicated

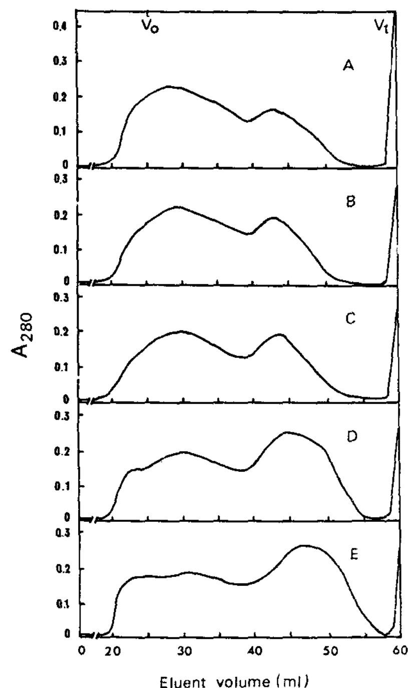

Fig. 5. Gel-permeation chromatography of xanthine/xanthine oxidase-treated SR. SR (1.0 mg protein/ml) was incubated in five reaction mixtures (2.0 ml each) with X/XO as described in Fig. 1. At time intervals indicated, each reaction mixture was mixed with 0.5 ml of 10% SDS and 0.1 ml of mercaptoethanol and boiled for 3 min. 2.0 ml aliquot from the resulting solution was placed on Sephadex G-200 column (2.5 x 15 cm) equilibrated with eluting buffer containing 0.5% sodium deoxycholate, 100 mM NaCl, 0.2 mM EDTA and 10 mM Tris-HCl, pH 7.8. The eluent flowing at 17 ml/h was continuously monitored at 280 nm. Panels A, B, C, D and E; 0, 30, 60, 90 and 120 min of incubation, respectively.

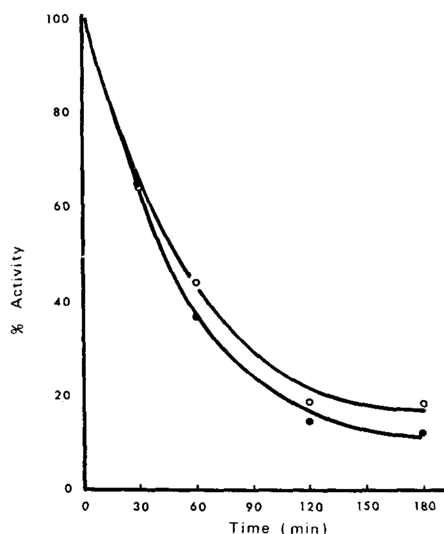

Fig. 6. Effect of semicarbazide on calcium binding of xanthine/xanthine oxidase-treated SR. SR (0.3 mg protein/ml) was treated with X/XO in the absence (●) or presence (o) of 100 mM semicarbazide as described in Fig. 1. Aliquots (1.0 ml) taken at intervals indicated were assayed for calcium binding as decribed under the Materials and Methods. The calcium binding at zero time was 37.1 nmoles/mg protein which was taken as 100%.

by the increase of a lipid peroxidation product, malondialdehyde. As shown in Fig. 3, the extent of lipid peroxidation increased throughout 180 min of incubation. In the first 60 min the rate of production of malondialdehyde was slow, but thereafter it was increased rapidly. This increase of malondialdehyde production was almost completely prevented by addition of *p*-phenylenediamine (Fig. 4). With these findings, to examine the possible relation between calcium binding inhibition and lipid peroxidation, effect of the antioxidant calcium binding activity of X/XO-treated SR was also observed. As presented in Table 2, in spite of almost complete prevention of malondialdehyde production, the calcium binding inhibition of X/XO-treated SR was only partially prevented by *p*-phenylenediamine.

Chromatographic analysis of xanthine/xanthine oxidase-treated sarcoplasmic reticulum

To investigate the probable alterations of protein components which are also essential for membrane functions, SR treated with X/XO was analyzed by gel-permeation chromatography. Fig. 5 shows the chromatographic profiles of X/XO-treated SR applied on Sephadex G-200 column. Protein components of SR were not resolved clearly, rather polydispersed. They were grouped into two major peaks on this chromatogram. With prolongation of the incubation, the first peak, representing proteins of higher molecular weight, decreased in its size, while the second peak of smaller molecular weight increased. This result suggests that some protein components of SR membrane are degraded into smaller fragments. In addition to degradation, it is possible for polymerization of proteins to occur. One mechanism for that is known to be cross-linking of proteins owing to malonaldehyde produced by lipid peroxidation. In the present study, however, no evidence of polymerization was observed on Sepharose 2-B (2.5x15cm) chromatogram (data not shown).

Funthermore, semicarbazide which was shown to prevent cross-linking of proteins occuring in lipid peroxidation of membrane did not show any significant effect on the inhibition of calcium binding of X/XO-treated SR (Fig. 6).

DISCUSSION

Calcium transport of SR has been reported to be significantly reduced in myocardial ischemia. Recently, several investigators (Hess *et al.*, 1981a & 1983; Kim *et al.*, 1984) have suggested the possible involvement of reactive oxygen free radicals in the reduced calcium transport of cardiac SR. In their studies, the investigators have observed that oxygen free radicals produced enzymatically from xanthine and xanthine oxidase inhibited significantly the calcium transport of isolated cardiac sarcoplasmic reticulum vesicle. However, the precise nature of mechanism of oxygen radical inhibition in calcium transport of SR has not been elucidated yet. The present results suggest that calcium transport inhibition of cardiac SR by oxygen radical may be a result of combined effects on lipids, SH-group and protein components of SR membrane.

The unsaturated fatty acids of membrane lipids are susceptible to oxygen free radical attack, and it has been suggested (Freeman & Crapo, 1982; Meerson *et al.*, 1982) that the membrane lipid peroxidation and ensuing biochemical changes may alter activity of membrane-bound enzymes and also membrane permeability leading to disturbances in cellular functions. The occurrence of lipid peroxidation and the resulting damage by oxygen free radical have been documented in liposomes (Pederson and Aust, 1973), erythrocytes (Kellog and Fridovich, 1977) and cardiac sarcolemmal and microsomal preparations (Kramer *et al.*, 1984). In the present study, significant increase of a lipid peroxidation product, malonaldehyde, was also observed during incubation of SR vesicles with X/XO, an oxygen radical generating system (Fig. 3).

When oxygen radicals react with stable compounds, they generates radicals which lead to chain reactions in which many of stable molecules are oxidized subsequently. Such chain reactions can be terminated by antioxidants, which react preferentially with chain propagating radicals to yield

non-propagating products (Aust and Svingen, 1982). According to this concept, if oxygen free radicals initiate the oxidation of lipid components of SR, antioxidants should limit the lipid peroxidation and resulting impaired function of the membrane. Indeed, an antioxidant, *p*-phenylenediamine effectively inhibited malondialdehyde production from X/XO-treated SR (Fig. 4). But inconsistent with this, the inhibition of calcium binding by X/XO was only partially prevented by the antioxidant (Table 2). Considering the essential role of lipid components in membrane integrity and function, it was rather unexpected result.

As an alternative to see further the involvement of lipid peroxidation in the calcium binding inhibition, the ability of phospholipids to regenerate calcium binding activity of X/XO-treated SR was tested. But the binding activity was not restored either by the phospholipids used, phosphatidylcholine, phosphatidylserine and sphingomyelin (date not shown). Thus it is suggested that the lipid peroxidation can be in part a contributing factor, but not a main mechanism responsible for the calcium binding inhibition of SR by oxygen radicals.

It has been generally accepted that ATP-dependent calcium transport of SR occurs mainly through the membrane enzyme, Ca^{++}-ATPase (Dupont, 1977; Shigekawa *et al.*, 1978). Many kinds of membrane enzymes including ATPase contain SH-groups which is essential for the catalytic actions of the enzymes (Chan and Rosenblum, 1969). In the present study, the essential role of sulfhydryl group in calcium binding of SR was also confirmed by observing the total loss of calcium binding by modifying sulfhydryl groups with DTNB treatment and complete recovery by reduction of the modified SH-groups with DTT (Table 1).

SH-group is known to be an effective hydrogen donor and to react rapidly with free radicals (Pryor, 1982). Akera and Brody (1970) reported that chloropromazine free fadical inhibited ATPase activity of brain microsomal membrane possibly through the interaction of SH-groups with this radical. In the present study, when SR vesicles were incubated with X/XO, the content of SH-groups was also significantly decreased, and this reduction of the content was regarded to be due to oxidation of SH-groups by reactive oxygen radicals. Considering these findings and the generally accepted concept that SH-groups play an essential role in enzyme functions, protection of SH-groups from oxidation should prevent decrease in SH-group content as well as impaired calcium binding activity of SR treated with X/XO. But inconsistently, an antioxidant, *p*-phenylenediamine or DTNB which can protect sulfhydryl group from oxidation did only partially prevent both the reduction of SH-group content and the inhibition of calcium biniding of SR treated with X/XO (Tables 1 and 2). Therefore, it is considered that the decrease in SH-group is not solely responsible for the calcium binding inhibition of SR by oxygen radicals.

In cellular membranes, free radical chain reactions initiated by highly reactive oxygen radicals can degrade or cross-link various protein components (Tappel, 1973). Degradation or cross-linking of membrane components result in alterations of membrane integrity and permeability, ultimately leading to impairment or loss of cellular functions. With reactive oxygen species including ozone, cross linking of proteins in cellular membranes and concurrent alteration of membrane functions have been demonstrated (Chan et al., 1977; Leibovitz and Siegel, 1980). But in this experiment, no evidence of protein crosslinking in X/XO-treated SR was observed by chromatographic analysis (Fig. 5). In addition to this, semicarbazide, which can prevent protein cross-linking by trapping aldehyde products resulting from peroxidation, (Chan *et al.*, 1977), provided no preventive effect on the inhibition of calcium binding of SR treated with X/XO (Fig. 6). However, when the X/XO-treated SR was applied on Sephadex G-200, the chromatographic profile showed the accumulation of low molecular weight products, probably degradation products. (Fig. 5). It is suggested from the these results that the cross-linking of membrane proteins may not be involved in the inhibition of calcium binding of SR by oxygen radical. Instead, degradation of proteins seems to be partly related with the calcium binding inhibition.

In conclusion, the inhibition of calcium binding of sarcoplasmic reticulum by oxygen radicals may be the consequences of oxidative attacks to membrane lipids and essential sulfhydryl groups together

with degradation of membrane proteins.

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=국문초록=

산소대사물에 의한 심장근 Sarcoplasmic reticulum의 칼슘운반 억제 기전에 관한연구

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심근 세포의 칼슘 조절에 중요한 역할을 하는 sarcoplasmic reticulum (SR)의 칼슘운반 능력이 허혈 심근에서 현저히 억제됨이 알려져 있다. 이와같은 허혈 심근에서의 SR 칼슘운반 능력 저하에 유독성 산소 대사물이 관여할 것으로 생각되고 있으나 그 기전에 관하여는 아직 알려진 바 없다. 본 연구에서는 그 기전의 일단을 규명하기 위하여 산틴 산화효소계에 의하여 발생된 유독성 산소대사물이 돼지 심실근에서 추출한 sarcoplasmic reticulum의 칼슘흡수 및 막지질 과산화, sulfhydryl group 그리고 단백질 변성에 미치는 영향을 관찰하여 다음과 같은 결과를 얻었다. 1) 산틴 산화 효소계와 반응시킨 sarcopl smic reticulum의 칼슘흡수는 반응시간 경과에 따라 현저히 억제되었다. 2) sarcoplasmic reticulum 막지질 과산화는 산틴 산화 효소계에 의하여 현저히 증가되었다. 3) 항산화제 β-phenylenediamine은 막지질 과산화의 증가는 효과적으로 억제하였으나, 칼슘흡수 억제는 부분적으로 회복시켰다. 4) 산틴 산화효소계에의하여SH-group은 현저히 감소되었으며, 항산화제 첨가에 의하여 그 감소가 일부 억제되었다. 5) sarcoplasmic reticulum을 DTNB로 처리하여 SH-group을 산소 대사물에 의한 산화반응으로부터 보호했을 경우 칼슘흡수의 억제가 부분적으로 방지되었다. 6) Sephadex G-200 크로마토그라피 상에서 산틴 산화효소계와 반응시킨 sarcoplasmic reticulum의 단백질분해가 관찰되었다. 7) 단백질의 polymerization은 관찰되지 않았으며, 아울러 polymerization을 억제하는 semicarbazide로 칼슘흡수 감소를 방지하지 못하였다.

이상의 결과에서 유독성 산소대사물에 의한 sarcoplasmic reticulum의 칼슘흡수 억제는 sarcoplasmic reticulum의 막지질 과산화, SH-group의 산화 및 막 단백질의 분해 등으로 초래되는 복합적인 기전으로 추정되었다.