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Studies on The Mechanism of Reactive Oxygen Species Inactivation of Brain Microsomal Na^+-K^+-ATPase†

Myung-Hee Chung††, Jung-Shik Kim*, Young-Sook Son, Kil-Soo Choi*, Jung-Kyoo Lim and Chan-Woong Park

*Department of Pharmacology and *Department of Neurosurgery, College of Medicine, Seoul National University, Seoul 110, Korea*

= Abstract =Effects of xanthine/xanthine oxidase on the brain microsomal fraction were studied with respect to its Na^+-K^+-ATPase activity, lipid peroxidation and SH-group oxidation. The treatment of the membrane fraction with xanthine/xanthine oxidase resulted in the inactivation of Na^+-K^+-ATPase and a proportionate loss of the membrane SH-groups with sparing production of malondialdehyde. The observed amount of malondialdehyde was much less than that by another oxidizing agents, Cu^{++}-H_2O_2 exhibiting the comparable degree of the ATPase inactivation. The inactivated ATPase by xanthine/xanthine oxidase was not recovered with various phospholipids. Among the quenchers, only those which prevented the inactivation of the ATPase recovered the loss of the membrane SH-groups, and the extent of SH-group recovery was correlated to that of the ATPase activity. Furthermore, SH-group-containing compounds (cysteine and glutathione) prevented the ATPase against the inactivation when the membrane fraction was treated with xanthine/xanthine oxidase in the presence of them. Serine showed no effect in this respect. The results obtained suggest that reactive oxygen species inactivate brain microsomal Na^+-K^+-ATPase primarily by interacting with SH-groups, which appear to be more susceptible than membrane phospholipid components to oxidative attack by the reactive oxygen species.

Key Words: ***Na^+-K^+-ATPase, Oxygen radicals and Lipid peroxidation***

INTRODUCTION

Superoxide radical, O_2^- is a common intermediate in the univalent reduction of molecular oxygen, O_2 (Fridovich 1978). It participates in reactions that produce hydrogen peroxide, H_2O_2 (Fridovich 1978; Klebanoff 1980), hydroxyl radical, $OH^\cdot$ (Beauchamp and Fridovich, 1970) and possibly singlet oxygen, $^1O_2$ (Pederson and Aust 1973). These oxygen species are highly reactive and can alter most types of cellular macromolecules. In *in vitro* experiments, they have been shown to oxidize proteins (Venkatasubramnian and Joseph 1977; Kim 1984) and unsaturated fatty acids (Kellogg and Fridovich 1975 and 1977), damage nucleic acids (Lavelle et al. 1973) and cleave polysaccharides (McCord 1974).

There has accumulated evidence that implicates the reactive oxygen species as toxic intermediates in tissue damage in several pathologic conditions. These include inflammation (Fantone and Ward 1982), aging (Leibovitz and Siegel 1980), toxicity of ceratin drugs (Trush et al. 1982) and carcinogenesis (Ames 1983).

Recently, it has been suggested that the reactive oxygen species can be produced in hypoxic condition of various tissues (Taylor 1983). Demopoulos et al. (1980) suggested that pathologic processes

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††To whom reprint requests should be addressed

observed in the ischemic state of brain tissue usually accompanied by cerebral vascular occlusion or impact injury were attributable to free radical reactions initiated by these oxygen species.

Ubiquinone (CoQ) is normally found as a semiquinone free radical during active e^--transport in mitochondria(Ruzica et al. 1975). Under normal circumstances, it is well controlled by tight association with other components of the e^--transport system. When oxygen supply is reduced, a high enough redox potential for e^--transport can not be provided, thus CoQ along with all the other e^--transport substances remain reduced. Then the reduced CoQ reacts with molecular oxygen to form $O_2^{\cdot-}$ which in turn spontaneously disproportionates to form H_2O_2 (Demopoulos et al. 1979; Fridovich 1979). The interaction of $O_2^{\cdot-}$ and H_2O_2 has been known to produce $O_2^{\cdot-}$ and/or $^1O_2$ to act as final mediators to attack tissue components (Fridovich 1978; Klebanoff 1980; Beauchamp and Fridovich 1970; Kellogg and Fridovich 1975 and 1977).

In view of the fact that Na^+-K^+-ATPase takes an important part in the physiological functions of nerve cells, it is of importance to study the effect of the reactive oxygen species on this enzyme and explore the mechanism of their action to understand their roles in causing the functional impairment or loss of nerve cells observed in the hypoxic or ischemic condition of brain tissue.

$O_2^{\cdot-}$ as well as other reactive oxygen species can be generated *in vitro* by the enzymatic action of xanthine oxidase converting xanthine to uric acid (Beauchamp and Fridovich 1970; Greenwald and Moy 1979). For *in vitro* experiment, the use of xanthine oxidase has a advantage of convenience, rapidity and easy control of the reactive oxygen species production. Oh et al.(1982) has demonstrated a specific loss of Na^+-K^+-ATPase activity without significant change in the Mg^{++}-ATPase activity when bovine brain microsomal membranes were exposed to xanthine/xanthine oxidase(X/XO) reaction and showed that $^1O_2$ was involved as a final mediator to cause the enzyme inactivation.

In the present study, to explore primary targets of attack by the reactive oxygen species leading to impairment of cellular function, effects of X/XO reaction on various components of brain microsomal membranes were examined with respect to Na^+-K^+-ATPase activity. The results suggested that sulfhydryl(SH)-group oxidation contributes to the ATPase inactivation as a principal mechanism.

MATERIALS AND METHODS

1. Materials:

Xanthine oxidase (XO), superoxide dismutase (SOD), catalase, DL-dithiothreitol (DTT), 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), glutathione, cysteine, serine, 2-thiobarbituric acid (TBA), phosphatidyl-choline, sphingomyelin and phosphatidylserine were obtained from Sigma Chemical Co.; xanthine (X) from Wako Pure Chem.; 1,4-diazabicyclo(2,2,2)octane(DABCO) from Aldrich Chem. Co.; ouabaine from Mann Research Lab.; allopurinol from Samil Pharmaceut. Co.. XO and SOD were dialyzed 3 times for 12 h at 4°C against the buffers, 50 mM Tris-HCl, 1 mM sodium salicylate and 0.005% EDTA, pH 7.8, and 50 mM Tris-HCl, pH 7.6, respectively to remove inorganic phosphate (Pi) present in the enzyme preparations. The extensive dialysis was proved to eliminate essentially all the Pi from the enzyme solutions by Pi assay. Ox brain was obtained from a slaughter house.

2. Methods:

1) Preparation of brain microsomal membrane fraction

The microsomal fraction was prepared from the gray matter of freshly obtained ox brain according to the method of Skou and Hilberg (1969). The membrane fraction obtained was suspended in 50 mM Tris-HCl, pH 7.4 to 1-2 mg protein/ml and stored at -20°C. Under this condition there was no detectable change in the ATPase activity for 2 weeks. Protein concentration was measured by the method of Lowry et al. (1951).

2) Treatment of the microsomal membrane with xanthine and xanthine oxidase

The microsomal membrane (400 μg protein/ml) was incubated with xanthine oxidase (30 munits/ml) in the reaction mixture containing 4 mM xanthine, 1 mM EDTA and 50 mM Tris-HCl, pH 7.4 at 37°C. The reaction was started by adding xanthine after 10 min preincubation. At time intervals, aliquots were removed and assayed for the ATPase, SH-groups and malondialdehydes. Constant agitation was provided during the incubation by Dunoff metabolic incubator with a rate of 120/min.

3) Treatment of the microsomal membrane with Cu^{++} and H_2O_2

The microsomal preparation (400 μg protein/ml)

was incubated with varying concentrations of Cu^{++} and H_2O_2 in the 3.0 ml reaction medium of 50 mM Tris-HCl, pH 7.4 at 37°C and the reaction was stopped with 30 μl of 50 mM EDTA and 7.5 μl of 2 mg/ml of catalase. Aliquots were also assayed for the ATPase and malondialdehyde.

4) ATPase assay

For ATPase assay, 0.2 ml aliquots taken from the previous experiments were used. When the microsomal memberane was treated with X/XO, the aliquots were mixed with 0.05 ml of 50 mM allopurinol and kept cold in an ice-bath to terminate the enzymatic action of xanthine oxidase. But with the membrane treated with Cu^{++}-H_2O_2, the aliquots were used directly since the reaction was already stopped with EDTA and catalase. The aliquots obtained were then incubated for 10 min in 1.0 ml reaction media containing 100 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 2 mM Na_2ATP and 25 mM Tris-HCl, pH 7.4. The reaction was initiated by adding 2 mM Na_2ATP after 10 min preincubation. The reaction was stopped with 0.25 ml of 15% trichloroacetic acid (TCA) and the mixure was centrifuged at 1,000 g for 15 min. 0.5 ml of supernatant was used for determination of Pi according to the method of Horwitt(1952). The ouabain-insensitive ATPase was assayed in the presence of 1 mM ouabain. Na^+-K^+-ATPase was determined as the difference between assays in the absence and the presence of ouabain. Allopurinol at the concentration used showed essentially no effect on the Na^+-K^+-ATPase.

5) Measurement of lipid peroxidation

Lipid peroxidation was followed by measuring malondialdehyde with TBA method (Bidlack and Tappel, 1973). After the microsomal membrane was treated with X/XO or $Cu^{++}H_2O_2$, 1 ml of aliquots cointaining 400 μg protein were mixed with 0.5 ml of distilled water and 0.5 ml of 30% TCA and the mixtures were centrifuged at 3,000 g for 15 min. The supernatant (1.5 ml) was added to an equal volume of aqueous 0.67% TBA and the chromophore was developed by boiling in a water bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm. The concentration of malondialdehyde was expressed as nmoles/mg protein using the molar extinction coefficient of 1.52 x 10^5/M/Cm (Placer et al., 1966).

6) SH-group assay

SH-groups of microsomal membrane were determined spectrophotometrically by Ellman's method (1959). After treatment of microsomal membrane with X/XO, 1.0 ml of aliquots containing 400 μg protein were mixed with 0.1 ml of 10% sodium dodecylsulfate (SDS). When the mixture became clear, 0.02 ml of 10 mM DTNB in 100 mM Tris-HCl, pH 7.0 was added. After incubation for 10 min at 37°C, the absorbance was measured at 412 nm. The concentration of SH-groups was estimated from the molar extinction coefficient of *p*-nitrothiophenol anion, 1.36 x 10^4/M/Cm (Ellman, 1959).

7) Preparation of phospholipid vesicles

Two milligrams of each phospholipid (L-α-phosphatidylcholine, L-α-phosphatidylserine and sphingomyelin) were dissolved in chloroform and then flushed with nitrogen gas to evaporate the chloroform. The dried phospholipids were suspended in 1.0 ml of 50 mM Tris-HCl, pH 7.4 which was previously saturated with nitrogen gas and stirred for 10 min. The suspension was sonicated for 10 min (20 runs of 30 sec with 30 sec intervals) at set 7 of a Branson sonicator. The vesicle preparations were made just before each experiment.

8) Modification of SH-groups with DTNB

The microsomal membrane (400 μg/ml) in 50 mM Tris-HCl, pH 7.4 was incubated with 0.2 mM DTNB at 37°C for 30 min and then it was washed twice with 50 mM Tris-HCl, pH 7.4 at 4°C. The DTNB-treated microsomal membrane was then treated with X/XO at 37°C as described in section 2), washed once with the same buffer containing 10 mM allopurinol and incubated with 5 mM DTT at 37°C for 30 min. The preparation obtained was washed twice with 50 mM Tris-HCl, pH 7.4 and assayed for the ATPase activity.

RESULTS

1. Effects of xanthine and xanthine oxidase on ATPase, SH-groups and lipid peroxidation of the microsomal membrane : Fig. 1 shows that the microsomal Na^+-K^+-ATPase was inactivated by the X/XO reaction. Incubation of the membrane with 4 mM xanthine and 30 munits/ml xanthine oxidase inactivated the enzyme activity to 75, 52, 24 and 11% of zero time level (58.4 μmoles of Pi/mg protein/h) at 15, 30, 60 and 90 min, respectively. At 120 min, the activity was almost completely abolished. Mg^{++}-ATPase (12.4 μmoles of Pi/mg protein/h at zero time) was not affected sig-

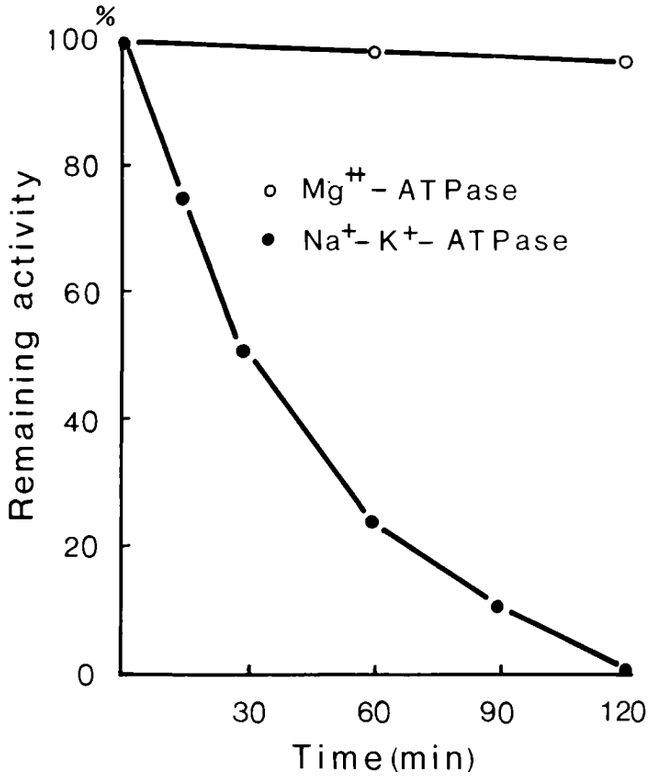

Fig. 1. Effects of xanthine oxidase system on the microsomal Mg^{++}-ATPase and Na^{+}-K^{+}-ATPase activity. Microsomal preparation (400 μg protein/ml) was incubated with 4 mM xanthine in the reaction mixture containing xanthine oxidase (30 munits/ml), 1 mM EDTA and 50 mM Tris-HCl, pH 7.4 at 37°C. Total volume was 2.0 ml. At time intervals, aliquots were removed and assayed for the ATPase activities as described in Materials and Methods. The control values (at zero time) of Mg^{++}-ATPase and Na^{+}-K^{+}-ATPase were 12.4 and 58.4 μmoles of Pi/mg protein/h, respectively.

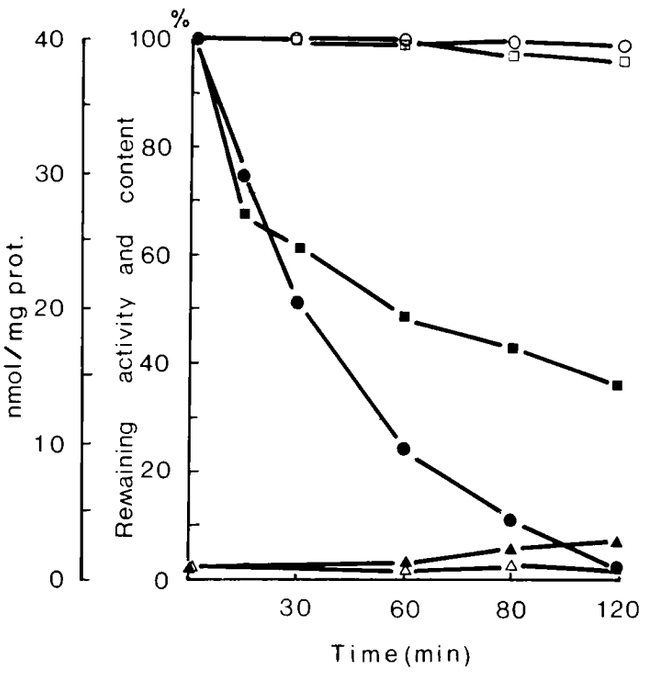

Fig. 2. Effects of xanthine oxidase system on microsomal Na^{+}-K^{+}-ATPase activity, SH-group content and malondialdehyde production. Microsomal preparation (400 μg protein/ml) were incubated with and without 3 mM xanthine in 10 ml reaction mixture containing xanthine oxidase (30 munits/ml), 1 mM EDTA and 50 mM Tris-HCl, pH 7.4 at 37°C. At time intervals, aliquots were removed and assayed for the ATPase activity (circle), SH-group content (square) and malonaldehyde (triangle). (See the Materials and Methods.) The control values (at zero time) of the ATPase activity and SH-group content were 58.4 μmoles of Pi/mg protein/h, 83.6 nmoles SH/mg protein, respectively, which were designated as 100%. ○, □, △ and ●, ■, ▲ indicate the values obtained in the absence and presence of xanthine, respectively.

nificantly by the X/XO reaction in the concentrations used in this study. Thus, only Na^{+}-K^{+}-ATPase activity was presented in this report. The X/XO-treated membrane was also analyzed for loss of SH-groups and lipid peroxidation.

As shown in Fig. 2, the SH-groups of microsomal membrane decreased steadily throughout the incubation period; 68, 61, 48, 43 and 35% of zero time level (83.6 nmoles/mg protein) were observed at the incubation period as indicated. At various time intervals, aliquots of microsomal membranes during the X/XO treatemnt were assayed for SH-group content and ATPase to explore the relation between SH-groups and the ATPase activity. Fig. 3 shows a proportionate loss of SH-groups to the inactivation of the enzyme activity (r=0.93). The line intercepted the ordinate at 37%. This indicates that a portion of SH-groups still remained when the enzyme activity was completely abolished. The unattacked portion of SH-groups did not appear to be related to the activity of the enzyme. The inactivation of ATPase and the decrease of SH-groups were found only in the presence of xanthine and xanthine oxidase. Essentially no effect was observed with either of them alone (Fig. 2).

On the other hand, lipid peroxidation occurred to a very small extent; malonaldehyde could not be detected until 60 min, and even at 120 min when the ATPase was completely inactivated, its production was only 3.2 nomles/mg protein which was much smaller than that produced from the Cu^{++}

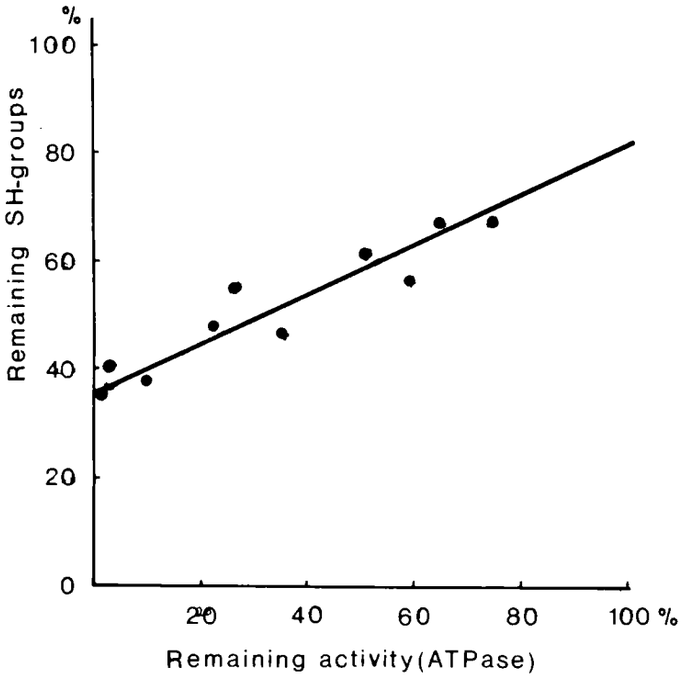

Fig. 3. Na^+-K^+-ATPase activity and SH-group content of microsomal membrane treated with xanthine oxidase system. Experimental conditions were the same as in Fig. 1. Total reaction mixture was 15 ml. At various time intervals during 2 h incubation, aliquots were removed and assayed for Na^+-K^+-ATPase and SH-group content as described in the Materials and Methods. ATPase activity and SH-group content obtained from each aliquot were expressed as % of the zero time value, and plotted.

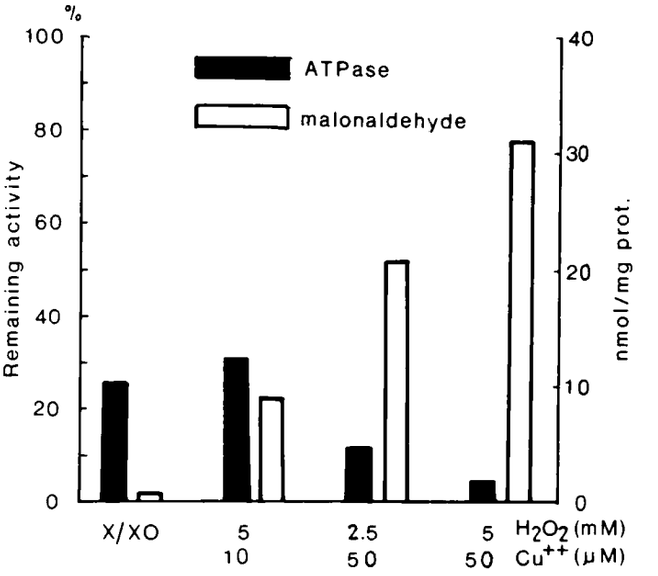

Fig. 4. Inactivation of Na^+-K^+-ATPase and the malondialdehyde production of the microsomal membrane treated with xanthine oxidase system or Cu^{++}-H_2O_2. Microsomal membrane (400 μg protein/ml) was treated with either xanthine oxidase system or Cu^{++}-H_2O_2 for 1 h, and aliquots were assayed for ATPase and malondialdehyde. Other details are described in the Methods and Materials. ATPase activities were shown as % of the zero time level.

-H_2O_2-treated microsomal membrane as shown below.

The results observed suggest that the Na^+-K^+-ATPase inactivation by the X/XO reaction under the present experimental conditions appears to be related to loss of SH-groups rather than the lipid peroxidation of microsomal membrane.

2. Effects of Cu^{++} and H_2O_2 on the ATPase and lipid peroxidation of the microsomal membrane: The observed degree of lipid peroxidation was rather an unexpected phenomenon in view of the reactivity of the reactive oxygen species generated and the susceptibility of membrane phospholipids to their oxidative attack (Kellogg and Fridovich, 1975 and 1977; Kim, 1984). In an attempt to confirm the sparing production of malondialdehyde from the microsomal membrane treated with X/XO, comparison was made between Cu^{++}-H_2O_2 and X/XO with respect to their peroxidative activities. Cu^{++}-H_2O_2, as a strong peroxidative system, have been reported to oxidize and degrade a variety of biological compounds including lipids (Chan et al., 1982) and proteins (Chung et al., 1983 and 1984).

As shown in Fig. 4, with 10 μM Cu^{++} and 5 mM H_2O_2, the amount of malondialdehyde produced was 20.9 nmoles/mg protein/h, which was much higher than that produced by X/XO(2.3 nmoles/mg protein/h), while the extent of Na^+-K^+-ATPase inactivation by both oxidative systems was similar (74 and 69% inactivation with X/XO and Cu^{++}-H_2O_2, respectively). In contrast to X/XO system showing complete abolition of the enzyme activity with negligible lipid peroxidation, increasing doses of Cu^{++} and H_2O_2 caused progressive inactivation of the enzyme that was accompanied by higher degree of lipid peroxidation (95% inactivation and 31.1 nmoles malondialdehyde of mg protein/h were observed with 50 μM Cu^{++} and 5 mM H_2O_2). Thus, the lipid peroxidation does not seem to contribute to the ATPase inactivation in the X/XO reaction.

3. Effects of phospholipids on the Na^+-K^+-ATPase of microsomal membrane treated with xanthine and xanthine oxidase: As an alternative to see the involvement of lipid peroxidation in the ATPase inactivation shown in the present study,

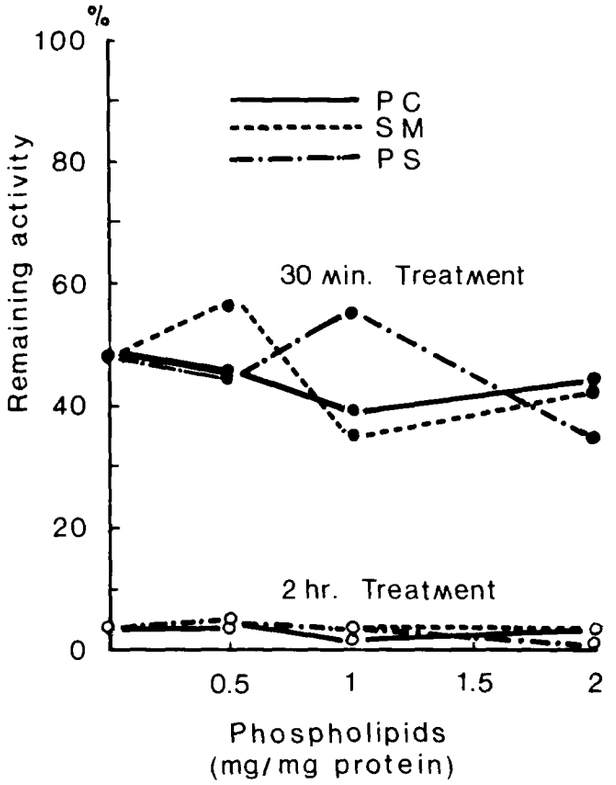

Fig. 5. Effect of phospholipids added to microsomal membrane treated with xanthine oxidase system on the recovery of Na^+-K^+-ATPase activity. Microsomal membrane (400 μg protein/ml) was treated with xanthine oxidase system in the 5 ml of reaction mixture as described in Fig. 1. 0.2 ml aliquots were taken at 30 and 120 min and then mixed with 0.05 ml of 50 ml allopurinol. The resulting mixtures were incubated for 15 min with concentrations of various phospholipids as indicated in the figure, and then assayed for ATPase. PC, SM and PS are phosphatidylcholine, spingomyelin and phosphatidylserine, respectively. ATPase activities were expressed as % of the activity obtained at zero time of xanthine/xanthine oxidase treatment. ● and ○ indicate 30 and 120 min of incubation with the X/XO reaction, respectively.

the ability of phospholids to regenerate the ATPase in the X/XO-treated preparations was tested. The microsomal fractions treated with X/XO for 30 or 120 min were incubated with phosphatidylcholine, phosphatidylserine or spingomyelin for 15 min prior to assay for the ATPase. As shown in Fig. 5, any significant restoration of the ATPase activity was not observed by any of the phospholipids employed in the X/XO-treated membrane.

4. Effects of various quenchers for reactive oxygen species on ATPase activity and SH-groups of microsmal membrane treated with xanthine and xanthine oxidase: The previous results indicate that loss of SH-groups seems to be a major cause for decrease of the ATPase activity in the X/XO-treated membrane. An indirect way to see the relation between the two phenomena is to observe the recovering effects of quenchers for the reactive oxygen species suggested to be generated in X/XO system on both the ATPase and SH-groups. The results were shown in Fig. 6. Among the quenchers incubated with the microsomal membrane during the X/XO treatment, SOD, catalase and DABCO prevented the inactivation of ATPase and loss of SH-groups against the X/XO reaction. Mannitol showed no effect on both. In other words, recovery of SH-groups was observed by only those which afforded preventive action against the enzyme inactivation or vice versa. The observed findings indicate that the quenchers which reversed the inactivation of the ATPase attributed their effect to the removal of the oxygen species participating in SH-group oxidation which seems to be a major mechanism responsible for the ATPase inactivation in the X/XO reaction.

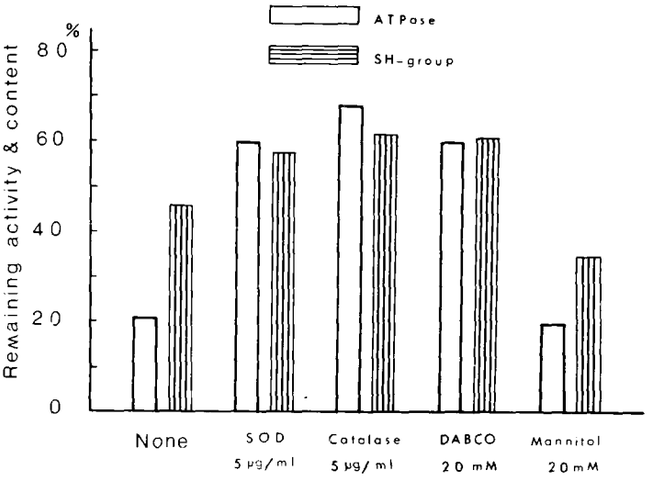

Fig. 6. Effect of various quencehers for reactive oxygen species on Na^+-K^+-ATPase activity and SH-group content of microsomal membrane treated with xanthine oxidase system. Microsomal fraction (400 μg protein/ml) was treated with xanthine and xanthine oxidase in the presence of each quencher for 1 h. Experimental conditions were the same as in Fig. 1. Aliquots were assayed for ATPase activity and SH-group content, which were expressed as % of their levels obtained at zero time of xanthine/xanthine oxidase treatment in the absence of the quenchers.

5. Effects of SH-group containing compounds on the Na^+-K^+-ATPase of microsomal

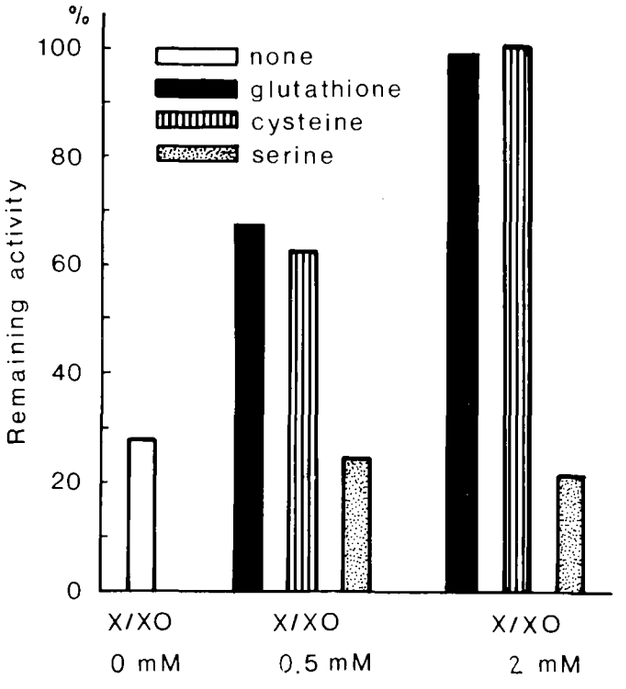

Fig. 7. Effects of SH-group containing compounds on Na^+-K^+-ATPase activity of microsomal membrane treated with xanthine oxidase system. Microsomal membrane was treated with xanthine and xanthine oxidase for 1 h in the presence of the compounds as shown in the figure. Other conditions were the same as in Fig. 1. ATPase activities were expressed as % of the activity obtained at zero time of xanthine/xanthine oxidase treatment without the added compounds.

membrane treated with xanthine and xanthine oxidase: To provide further evidence for SH-group oxdation as a mechanism of the ATPase inactivation, SH-group containg compounds were tested to see if they have the ability to protect the ATPase when incubated with the microsomal membrane during X/XO treatment. As shown in Fig. 7, glutathione and cysteine indeed protected the ATPase inactivation as a function of their concentrations. With 2 mM of both compounds, complete protection of the ATPase was observed. No effect, however, was found with the same range of concentrations of serine which has the same structure as cysteine except that SH-group is replaced by OH-group.

6. Essential requirement of free SH-group for Na^+-K^+-ATPase activity and protection of SH-groups: Evidence obtained thus far suggests that ATPase inactivation by the reactive oxygen species from X/XO reaction was mainly due to oxidative attack to SH-groups of microsomal membrane. Before reaching the above conclusion, however, essential role of SH-groups for catalytic activity of the enzyme should be demonstrated in the microsomal preparation used in the present study. DTNB was used to modify the SH-groups to mixed-disulfide groups. As shown in Table 1, when the microsomal membrane was treated with 0.2 mM DTNB, Na^+-K^+-ATPase was inactivated to 13.1% of the control (Tube No. 3). But it was restored to the control activity by the reduction of the modified groups with 5 mM DTT (Tube No. 2). Therefore, it was confirmed that free SH-groups are essentially required for the ATPase activity of the microsomal membrane.

In view of the suggested postulation that the ATPase inactivation by X/XO reaction is primarily attributable to oxidation of SH-groups of the membrane, one possibility can be considered that modification of SH-group by DTNB is expected to have protective effect against oxidation of free SH-groups by the reactive oxygen species, leading to the prevention against inactivation of the ATPase. In order to test this possibility, DTNB-treated membrane was incubated with X/XO, then washed with DTT to regenerate free SH-groups and assayed for the ATPase. The activities of the modified membranes were 34.9 and 24.3 μmoles Pi/mg protein/h with 15 and 30 min X/XO treatment (tube No. 6 and 7), respectively which were not higher than what was observed in the membrane without modification of SH-groups (tube No. 4 and 5). No protection was found with simple modification of SH-groups to mixed disulfides. In the membrane which was not treated with DTNB, DTT showed no effect on the inactivated ATPase by the X/XO reaction, either.

DISCUSSION

Ischemic conditions of brain tissue induced by various causes have been know to lead to irreversible damage of nerve cells. Free radical reactions have been suggested as an underlying mechanism responsible for the pathologic changes in the ischemic brain tissue (Demopoulos et al. 1979 and 1980). Because of their molecular characteristics, phospholipids in the nerve cell membrane have been suggested to be a major target of biomolecules to be attacked by the free radical reactions (Demopoulos 1973). The supporting evidence for this includes the appearance of increased levels of malondialdehyde (Milvy et al. 1973), destructive loss of polyunsaturated fatty

acids and extractable cholesterol (Demopoulos et al. 1979) and consumption of a major CNS antioxidant, ascorbic acid (Flamm et al. 1978) in ischemic CNS of experimental animals.

Na^+-K^+-ATPase among the cellular activities has been assayed as an index to assess the extent of functional impairment of nerve cells in ischemia of CNS because the enzyme has an important role on nerve cell function and moreover is highly dependent in its activity on phospholipid intergrity of the membrane (Sun 1974). Therefore, the decrease of Na^+-K^+-ATPase activity in the ischemic brain tissue *in vivo* may result secondarily from the membrane perturbation by lipid peroxidation (Demopoulos et al. 1979).

The results in the present study show that treatment of brain microsomal membrane with X/XO caused a total loss of the Na^+-K^+-ATPase activity with a proportionate loss of SH-group but with negligible lipid peroxidation (Fig. 2 and 3). Measurable lipid peroxidation was observed when additional xanthine was added after the ATPase was completely inactivated with extra 2 mM xanthine for additional 60 min incubation; 6.2 nmoles malonaldehyde/mg protein was produced (data not shown).

Akera and Brody (1970) reported that the ATPase inhibition of brain microsomal membrane by chloropromazine free radicals was due to interaction of the SH-group with this radical. Thus, the findings observed suggest that Na^+-K^+-ATPase was inctivated by the reactive oxygen species produced from X/XO reaction mainly as a result of SH-group oxidation. Some SH-groups of cell membranes are essential for catalytc action of the enzyme (Chan and Rosenblum 1969) and in the present study their essential role was confirmed by observing the loss of the ATPase with DTNB treatment of microsomal preparations which was recovered by washing with DTT (Table. 1).

Reactive oxygen species are highly reactive and can cause oxidative degradation of most types of cellular macromolecules including lipids. Indeed, lipid peroxidation has been observed by these oxygen species using X/XO on fatty acids (Kellogg and Fridovich 1975), liposomes (Pederson and Aust 1973), erythrocytes (Kellogg and Fridovich 1977) and sarcoplasmic reticulum (Kim 1984). Thus, it was further needed to verify the findings in the present study suggesting that lipid peroxidation was not involved.

In addition to X/XO, several oxidizing agents are powerful enough to cause peroxidative destruction of lipids. These are O_3 (Chan et al. 1977), Fe^{++}-ascorbate (Bidlack and Tappel, 1973; Rehncrona et al. 1980) and Cu^{++}-H_2O_2 (Chan et al. 1982). Using Cu^{++}-H_2O_2 as an oxidizing system, lipid peroxidation was clearly shown and its extent appeared to be in parallel to that of the enzyme inactivation. Although direct causal relation ship between lipid peroxidation and loss of the ATPase activity in Cu^{++}-H_2O_2 treated preparation can not be said, the findings support at least that the X/XO reaction in the present study inactivated Na^+-K^+

Table 1. Effect of SH-group protection with DTNB on the inactivation of microsomal Na^+-K^+-ATPase by treatment with xanthine oxidase system

Tube No	DTNB treatment	X/XO treatment(min)	DTT rteatment	ATPase activity μmol Pi/mg prot/h(%)
1	−	0	−	55.8(100)
2	+	0	+	52.4(96.6)
3	+	0	−	7.7(13.1)
4	−	15	−	43.0(73.2)
5	−	30	−	29.9(50.9)
6	+	15	+	34.9(66.5)
7	+	30	+	24.3(46.4)

Microsomal fraction (400 μg/ml) was incubated with 0.2 mM DTNB for 30 min and centrifuged at 100,000 g. The resulting pellet was washed 2 times with 50 mM Tris-HCl, pH 7.4 by centrifugation at 100,000 g at 4°C, and then treated with xanthine oxidase system under the same conditions as in Fig. 1. After the reaction was stopped, the reaction mixture was further incubated with 5 mM DTT for 30 min and centrifuged at 100,000 g. The resulting pellet was washed 2 times as described above and assayed for the ATPase.

-ATPase with negligible extent of lipid peroxidation involved.

However, Schaefer et al. (1975) in their study showing lipid peroxidation of brain microsomal membrane by Fe^{++}-ascorbate reported that Na^+-K^+-ATPase inactivation was attributed to lipid peroxidation of the membrane. Chan et al. (1977) also reported that lipid peroxidation has a causative role on the ATPase inactivation in O_3-treated erythrocyte membrane. Moreover, detection of malondialdehyde as an index of lipid peroxidation was reported to be less sensitive than that of other products, i.e. dienes or lipid soluble fluorescence materials (Seligman et al. 1977). Therefore, there remains one possibility that although the extent of lipid peroxidation by X/XO reaction was not great in terms of malondialdehyde production, some change of lipid components really occurs and may influence the enzyme activity. To test the possibility, effects of several phospholipids were observed on the microsomal membranes treated with X/XO. But essentially no effect of the phospholipids was found on the inactivated ATPase. The results were in contrast to the observation that the inactivated ATPase in the membrane subjected to enzymatic lipolysis (Martonosi et al. 1968) or peroxidation by ozonolysis (Chan et al. 1977) was recovered by reconstitution with the phospholipids used in the present study.

With exclusion of lipid peroxoidation as a contributing factor, SH-group oxidation was considered to be a direct cause for the ATPase inactivation in the X/XO-treated membrane. In addition to the demonstration of inactivation of the ATPase and concurrent loss of SH-groups (Fig. 3), recovery of SH-groups was attempted by using the quenchers for the reactive oxygen species suggested to be involved in the X/XO reaction. In the previous report (Oh et al. 1982), it was observed that the inactivation of Na^+-K^+-ATPase of the microsomal membrane treated with X/XO was effectively limited by SOD, catalase and DABCO, suggesting O^-_2, H_2O_2 and $^1O_2$ as mediators. OH· was excluded since no effect of its various quenchers including mannitol was observed. In the present study the same results were observed (Fig. 6). That was also repeated in the studies observing inhibition of calcium binding activity of cardiac sarcoplasmic reticulum (Kim 1984) and peroxidation of linolenate (Kellogg and Fridovich 1975) and liposomes (Pederson and Aust 1973) by the X/XO reaction. In these reports, $^1O_2$ was involved as a final mediator. These findings were contrasted with other reports (Beauchamp and Fridovich 1970; McCord 1974; Greenwald and Moy 1979) suggesting OH· as a final mediator in the X/XO reaction as shown in the following equation.

$$O^-_2\cdot + H_2O_2 \longrightarrow OH\cdot + OH^- + {}^1O_2$$

At any rate, recovery of SH-groups was only possible by the quenchers which provided the protection against the enzyme inactivation. However, further studies may be necessary to explore the reason for the discrepancy on the findings observed in the quencher studies of many reports including the present study.

The findings of the present quencher studies suggest that $O^-_2\cdot$ generated in the X/XO reaction dismutes to H_2O_2 and that they interact to produce $^1O_2$ which is the proximate cause of SH-group oxidation in the membrane, resulting in the inactivation of the ATPase. Under these circumstances, if SH-groups are present in the medium, SH-groups of the membranes compete with those in the medum for $^1O_2$. Thus, SH-groups present in excess in the medium are expected to afford the protection over the ones of the membranes. That was demanstrated by the findings that the ATPase was not affected by X/XO reaction in the presence of glutathione or cysteine whereas serine showed no effect in this respect (Fig. 7).

The results in the present study suggest that the reactive oxygen species in X/XO reaction, probably $^1O_2$ is highly reactive toward microsomal SH-groups. It was also demonstrated that the SH-groups are essential for the ATPase activity by observing the complete abolition of the enzyme activity with DTNB treatment and subsequent restoration with DTT. This procedure was used to test whether DTNB treatment of the membrane may protect the ATPase from the attack by the reactive oxygen species. But DTT could not regenerate any ATPase activity of DTNB-treated membrane. Neither did it in the membrane which was not treated with DTNB. It suggests that SH-groups may be oxidized to a higher oxidative state that could not be reversed by DTT.

Na^+-K^+-ATPase can be affected as a result of several mechanisms when the cell membranes are subjected to peroxidative damage by various causes. They may be SH-group oxidation, lipid peroxidation or destruction of other essential components of the enzyme complex. In the present study, oxidation of SH-groups seems to be a major

mechanism responsible for the enzyme inactivation when brain microsomal membrane was treated with the X/XO reaction as a reactive oxygen species generating system. If, in the ischemic conditions of brain tissue, the reactive oxygen species trigger pathologic free radical reactions on the membranes, it is suggested that they initially interact with and oxidize SH-groups, which appear to be more susceptible than the membrane phosphilopid components to oxidative attack by the reactive oxygen species.

REFERENCES

Akera T, Brody TM. Inhibitory sites on sodium-and potassium-activated adenosine triphosphatase for chloropromazine free radical and ouabain. Mol. Pharmacol. 1970, 6: 557-566

Ames BN. Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative disease. Science 1983, 22: 1256-1264

Beauchamp C, Fridovich I. A mechanism for the production of ethylene from methional. J. Biol. Chem. 1970, 245: 4641-4646

Bidlack WR, Tappel AL. Damage to microsomal membrane by lipid peroxidation. Lipids 1973, 8: 177-182

Chan PC, Kinda RJ, Kesner L. Studies on the mechanism of ozone inactivation of erythrocyte membrane Na^+-K^+-activated ATPase. J. Biol. Chem. 1977, 252: 8537-8541

Chan PC, Peller OG, Kesner L. Copper (II)-catalyzed lipid peroxidation in liposomes and erythrocyte membranes. Lipids 1982, 17: 331-337

Chan PC, Rosenblum MS. Effect of sulfhydryl group modification on erythrocyte membrane adenosine triphosphatase. Proc. Soc. Exp. Biol. Med. 1969, 130: 143-145

Chung MH, Kesner L, Chan PC. Degradation of lysozyme by Cu(II) and H_2O_2. Fed. Proc. 1981, 40: 1613

Chung MH, Kesner L, Chan PC. Degradation of articular cartilage by copper and hydrogen peroxide, Agents Actions 1984, 15: 328-335

Demopoulos, HB. The free radical pathology. Fed. Proc. 1973, 32:1859-1961

Demopouls HB, Flamm ES, Seligman ML, Mitamura JA, Ransohoff J. Membrane pertubation in central nervous system injury: theoretical basis for free radical damage and a review of the experimental data. In Neural Trauma. A.J. Popp, R.S. Burke, L.R. Nelson and HK. Kimelberg, Eds., 1979, pp. 63-79. New York: Raven Press

Demopoulos, HB, Flamm FS, Pietronigro DD, Seligman, ML. (1980), The free radical pathology and the micro-circulation in the major central nervous system disorders. Acta Physiol. Scand. Suppl. 1980, 492: 91-119

Ellman, GL. Tissue suifhydryl groups. Arch. Biochem. Biophys. 1959, 82: 70-77

Fontone JC, Ward PA. Role of oxygen-derived free radical and metabolites in leukocyte-dependent inflammatory reactions. J. Am. Path. 1982, 107: 397-418

Flamm ES, Demopoulos HB, Seligman, ML., Poser, RG, Ransoohoff J. Free radicals in cerebral ischemia. Stroke 1978, 9: 445-446

Fridovidh I. The biology of oxygen radicals. Science 1978, 201: 875-880

Fridovich I. Hypoxia and oxygen toxicity. In *Advances in Neurology,* S. Fahn, Ed., 1979, pp. 255-259. New York: Raven Press

Greenwald RA, Moy WW. Inhibition of collagen gelation by action of the superoxide radical. Arthritis Rheumat. 1979, 22: 251-259

Horwitt BN. Determination of inorganic serum phosphate by means of stannous chloride. J. Biol. Chem. 1952, 199: 537-541

Kellogg III EW, Fridovich I. Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J. Biol. chem. 1975, 250: 8812-8817

Kellogg III EW, Fridovich I. Liposome oxidation and erythrocyte lysis by enzymically generated superoxide and hydrogen peroxide. J. Biol. Chem. 1977, 252: 6721-6728

Kim HW. Effects of oxygen free redicals on calcium binding of cardiac sarcoplasmic reticulum. Dissertation for Ph.D. 1984

Klebanoff SJ. Oxygen metabolism and toxic properties of phagocytes. Ann. Int. Med. 1980, 93: 480-489

Levelle F, Michelson AM, Dimitrijevic L. Biological protection by superoxide dismutase. Biochem. Biophys. Res. Commun. 1973, 55: 350-357

Leibovitz BE, Siegel BV. Aspects of free radical reactions in biological systems: ageing. J. Gerontol. 1980, 35: 45-56

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951, 193: 265-275

Martonosi A, Donley J, Halpin RA. Sarcoplasmic reticulum: III. The role of phosphoslipids in the adenosine triphosphatase activity and Ca^{++}-transport. J. Biol. Chem. 1968, 243: 61-70

McCord JM. Free radical and inflammation: protection of synovial fluid by superoxide dismutase. Science 1974, 185: 529-531

Milvy P, Karari S, Campbell JB, Demopoulos HB. Paramagnetic species and radical products in cat spinal cord. Ann. N.Y. Ascad. Sci. 1973, 222: 1102-1111

Oh SM, Son YS, Choi KS, Lim JK, Chung MH. Effect of oxygen-derived free radicals on brain microsomal Na^+-K^+-ATPase activity. Korean J. Pharmacol. 1982, 18:

1-14

Pederson TC, Aust SD. The role of superoxide and singlet oxygen in lipid peroxidation promoted by xanthine oxidase. Biochem. Biophys. Res. Commun. 1973, 52: 1071-1078

Placer ZA, Cushman LL, Johnson BC. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biological systems. Anal. Biochem. 1966, 16: 359-364

Rehncrona S, Smith DS, Akesson B, Westerberg E, Siesjo BK. Peroxidative changes in brain cortical fatty acids and phospholipids as characterized during Fe^{++}-and ascrobic acid stimulated lipid peroxidation in vitro, J. Neurochem. 1980, 34: 1630-1638

Ruzicka FJ, Beinert H, Schepler KL, Dunham WR, Sands RH. Interaction of ubisemiquinone with a paramagnetic component in heart tissue. Proc. Nat. Acad. Sci. 1975, 72: 2886-2890

Schaefer A, Komlos M, Seregi A. Lipid peroxidation as the cause of the ascorbic acid induced decrease of ATPase acitivities of rat brain microsomes and its inhibition by biogenic amines and psychotropic drugs. Biochem. Pharmacol. 1975, 24:1781-1786

Seligman ML, Flamm ES, Goldstein BD, Poser RG, Demoppulos HB, Ransohoff J. Spectrofluorescent dectection of malonaldehyde as a measure of lipid free radical damage in response to ethanol potentiation of spinal cord trauma. Lipids 1977, 12: 945-950

Skou JC, Hilberg C. The effect of cations, g-strophanthin and oligomycin on the labelling from (^{32}P) ATP of the (Na^+-K^+)-activated enzyme system and the effect of cations and g-strophanthin on the labelling from (^{32}P) ITP and ^{32}Pi. Biochim. Biophys. Acta 1969, 185: 198-219

Sun AY. The effect of phospholipases on the active uptake of norepinephrine by synaptosomes isolated from the cerebral cortex of guinea pig. J. Neurochem. 1974, 22: 551-556

Taylor AE. Oxygen radicals and the microcirculation. The Physiologists 1983, 26: 151-181

Trush MA, Mimnaugh EG, Gram TE. Activation of pharmacologic agents to radical intermediates: implications for the role of free radicals in drug action and toxicity. Biochem. Pharmacol. 1982, 31: 3335-3346

Venkatasubramanian K, Joseph KT. Action of singlet oxygen on collagen. Indian J. Biochem. Biophys. 1977, 14: 217-220

= 국문초록 =

유독성 산소 대사물에 의한 뇌조직 마이크로좀 Na^+-K^+-ATPase 활성억제 기전에 관한 연구

서울대학교 의과대학 약리학교실 및 *신경외과학교실

정명희 · 김정식* · 손영숙 · 최길수* · 임정규 · 박찬웅

뇌마이크로좀의 Na^+-K^+-ATPase 활성도는 유독성 산소 대사물에 의하여 현저하게 억제 받는다는 사실이 밝혀졌으나 그 작용 기전에 대하여는아직 알려진 바가 거의 없다.

저자는 유독성 산소 대사물이 뇌마이크로좀의 Na^+-K^+-ATPase 활성도를 억제하는작용 기전의 일단을 규명할 목적으로 소의 뇌조직에서 마이크로좀 분획을 추출하여 산틴산화효소계와 반응시킨 다음 ATPase 활성도, SH기의 산화 및 지질과산화의 변동을 관찰하였다. 아울러 이 반응계에 의한 ATPase 활성억제에 phospholipids 및 superoxide dismutase를 포함한 수종 oxygen radical scavengers의 영향을 관찰하여 아래와 같은 결과를 얻었다.

1. 뇌조직 마이크로좀을 산틴산화효소계로 처리하면 Na^+-K^+-ATPase 활성도와 SH기는 시간경과에 따라 상호비례하여 감소하였으며 지질과산화현상은 관찰되지 아니하였다.

2. 뇌조직 마이크로좀을 Cu^{++}-H_2O_2로 처리하면 ATPase 활성억제와 동시에 현저한 지질과산화현상이 관찰되었다.

3. 산틴산화효소계에 의하여 이미 억제된 뇌조직 마이크로좀의 ATPase 활성도는 여러 phospholipids를 첨가하더라도 전혀 회복되지 아니하였다.

4. Superoxide dismutase, catalase 및 1, 4-diazabicyvlo(2,2,2) octane을 첨가하면 산틴산화효소계에 의한 ATPase 활성도와 SH기의 감소는 일어나지 않았다. 그러나 mannitol을 첨가하면 ATPase 활성억제와 SH기 감소에 아무런 영향도 미치지 않았다.

5. Glutathione 혹은 cysteine 존재하에서는 산틴효소계에 의한 ATPase 활성도 억제가 관찰되지 않았다.

이상의 결과로 미루어 유독성 산소 대사물이 뇌조직 마이크로좀의 Na^+-K^+-ATPase 활성도를 억제하는 기전은 지질과산화보다는 주로 SH기 산화를 통해서 이루어짐을 알 수 있었다.