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Effects of Oxygen-Derived Free Radicals on Brain Microsomal Na^+-K^+-ATPase Activity

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=국문요약=

산소유리라디칼이 뇌조직 미크로좀분획의 Na^+-K^+-ATPase 활성도에 미치는 영향

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중추신경계의 혈관폐쇄 또는 충격손상에 의한 허혈병소에서 진행되는 병리적 변화에 산소유리라디칼이 중요한 역활을 할 것으로 시사되고 있다.

저자는 산소유리라디칼이 뇌조직에 미치는 영향 중 특히 신경세포 정지막전위 유지에 중요한 Na^+-K^+-ATPase의 활성도에 미치는 영향을 관찰하기 위하여 산틴산화효소 반응계와 뇌조직미크로좀을 이용하여 본 연구를 시행하였다.

미크로좀분획(microsomal fraction)을 산틴과 산틴산화효소와 함께 반응시켰을 때, 분획의 Na^+-K^+-ATPase의 활성도는 현저한 불활성화를 보인 반면, Mg^{++}-ATPase의 활성도는 별로 영향을 받지 않았다. 이 불활성화는 산틴과 산틴산화효소 중 어느 한 물질이라도 반응계에 존재하지 않는 경우에는 나타나지 않았고, 두 물질이 같이 반응계에 존재할 때 나타났다.

산틴과 산틴산화효소의 반응에서 생성되는 산소유리라디칼들 중, 어떤 것이 Na^+-K^+-ATPase 불활성화에 관계하고 있는가를 알아보기 위하여, 산소유리라디칼 각각에 대하여 제독작용을 가진 효소나 화학물질을 사용하여 불활성화의 저해유무를 관찰하였다. O_2^-·의 제독효소인 superoxide dismutase, H_2O_2의 제독효소인 catalase와 $^1O_2$의 제독물질인 1,4-diazabicyclo(2,2,2)octane을 각각 사용하였을 때, 이들 물질들의 농도에 비례하여 Na^+-K^+-ATPase의 불활성화가 저해되었다. 그러나, OH·의 제독물질인 mannitol은 뚜렷한 효과를 보이지 못하였다.

이상의 결과는 산소유리라디칼들 중 O_2^-·, H_2O_2 및 $^1O_2$가 Na^+-K^+-ATPase의 불활성화에 관계하고 있고, OH·는 거의 관계하지 않는다는 것을 시사하여 주었다. 이로 미루어, 산소유리라디칼에 의한 뇌조직 Na^+-K^+-ATPase의 불활성화는 뇌 허혈병소에서 관찰되는 신경세포의 기능적 장해를 유발시키는 한 요인으로 사료되었다.

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 (Iyer et al., 1961), hydroxyl radical, OH· (Rosen and Klebanoff, 1979) and possibly singlet oxygen, $^1O_2$ (Krinsky, 1974).

These oxygen species are highly reactive and can alter most types of cellular macromolcules. In in vitro experiments, they have been shown to oxidize proteins (Venkatasubramanian and Joseph, 1977) and unsaturated fatty acids (Kellogg and Fridovich, 1977), damage nucleic acids (Lavelle et al., 1973) and cleave polysaccharides (McCord, 1974). These oxygen species can be formed as products of normal oxidative respiration (Doveris, 1977; Nohl and Hegner, 1978) but more importantly, as products from altered cellular metabolism in pathologic states(Weissmann et al., 1978). Consequently their destructive action to many biological compounds may play a role in tissue damage under pathologic conditions. There has accumulated evidence that implicates the reactive oxygen species as toxic intermediates in serveral pathologic processes (Demopoulos et al., 1980; Weissmann, 1979). Destruction of normal tissue in inflammatory reactions has been studied as an example where the damging process has been attributed to their degradative action.

Leukocytes, when subjected to phagocytic, immunologic or chemical stimuli, are known to exhibit a sequnence of morphological and biochemical events which lead to production of H_2O_2, $O_2^-\cdot$ and even $OH\cdot$ as well as secretion of various lysosomal lytic enzymes into the extracellular environment (Klebanoff et al., 1976). All these phenomena can be typically observed in an inflammatory situation where the oxygen species released can be cytotoxic and act as mediators resulting in damage to adjacent tissues (Weissmann, 1979).

Recently, Demopoulos et al., (1980) suggested that the reactive oxygen species can be produced in the ischemic state of brain tissue usually accompanied by cerebral vascular occlusion or impact injury and they also reported that in this pathologic state peroxidation of membrane phospholipids gave rise to irreversible damage of brain tissue resulting in functional impairment or loss of nerve cells.

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 e-transport system. When oxygen supplies are 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).

Under these circumstances, the oxygen species produced are highly suggested to cause the pathologic changes and functional loss of nerve cells observed in the ischemia of brain tissue. In view of the fact that Na^+-K^+-ATPase takes an important part in physiological functions of nerve cells (Verity, 1972), studying the effects of the oxygen species on this enzyme may contribute to elucidating their roles on the pathogenesis of brain ischemia. Moreover, the precise identity of oxygen species responsible for the deleterious action on tissues is controversial. Thus far, $O_2^-\cdot$, H_2O_2, $OH\cdot$ and $^1O_2$ have all been implicated as toxic agents when macromolecules or cells are exposed to externally generated oxygen radicals (Mc Cord, 1974; Kellogg and Fridovich, 1977; Fridovich, 1978; Hess et al., 1981; Simon et al., 1981). Therefore it is of importance for the understanding of free radical pathology to know which of the oxygen species acts as a mediator to cause pathologic changes to the brain tissue as well as others under pathologic

conditions where oxygen species are suggested to be involved in damging processes.

In the present study, effects of the reactive oxygen species on Na^+-K^+-ATPase of brain microsomal fraction have been examined with enzymatically generated oxygen species using xanthine oxidase system (Greenwald and Moy, 1979) and to determine which of the oxygen species causes the inactivation of the microsomal Na^+K^+-ATPase, the effects of inactivators or scavengers $O_2^-\cdot$, H_2O_2, $OH\cdot$ and $^1O_2$ on the inactivation were also observed.

MATERIALS AND METHODS

Xanthine oxidase, superoxide dismutase, catalase were obtained from Sigma Chemical Co.; xanthine from Wako Pure Chem.; 1, 4-diazabicyclo(2, 2, 2)octane from Aldrich Chem. Co.; ouabain from Mann Research Lab.; allopurinol from Samil Pharm. Co. Suspensions of xanthine oxidase and superoxide dismutase were dialysed 3 times for 12 h at 4°C against the buffers, 50 mM Tris, 1 mM Na salicylate and 0.005% EDTA, pH 7.8, and 50 mM Tris, pH 7.6, respectively to remove Pi (inorganic phosphate) present in the suspending mixture. The extensive dialysis was proved to eliminate essentially all the Pi from enzyme solutions by Pi assay. Ox brain was obtained from slaughter house.

1) Preparation of brain microsomal fraction

The fraction was prepared from freshly obtained ox brain according to the method of Skou and Hilberg (1969) with a little modification. Two hundred grams of gray matter of the brain was homogenized in 2 L of ice-cold 0.25 M sucrose, 30 mM Tris and 0.1% sodium deoxycholate, pH 7.2. After centrifuging at 10,000 x g for 20 min, the sediment was discarded; the supernatant was centrifuged at 20,000 x g for 60 min and the sediment was resuspended by homogenation in 500 ml of 0.25 M sucrose, 30 mM Tris, 50 mM EDTA and 0.05% sodium deoxycholate. The suspension was centirfuged at 10,000 x g for 20 min and the supernatant was left overnight at 4°C. On the followlwing day the supernatant was centrifuged at 20,000 x g for 60 min; the sediment was discarded and the supernatant was centrifuged at 100,000 x g for 30 min. The sediment obtained was resuspended in 100 ml of 0.25 M sucrose, 30 mM Tris, pH 7.2 and centrifuged at 100,000 x g for 30 min. After washing 3 times at 100,000 x g for 30 min with 50 mM Tris, pH 7.4, the sediment was suspended in the same buffer to 1-2 mg protein/ml and stored at -20°C. Under these conditions there was no detectable change in the ATPase for 2 weeks. All the procedures were performed at 0-4°C and protein concentration was measured by the method of Lowry et al. (1951).

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

The microsomal preparations (180 μg protein /ml of reaction medium) were preincubated with 80 munits/ml of xanthine oxidase in the mixture containing 0.1 mM EDTA and 50 mM Tris, pH 7.4 for 10 min at 25°C. After preincubation, reaction was started by adding various amount of xanthine as indicated and stopped by adding with 10 mM allopurinol (0.1 ml of 50 mM allopurinol was added to 0.5 ml of reaction medium). The reaction mixtures (0.6 ml) were stored in an ice-bath and assayed for ATPase. The concentration of allopurinol used was found to suppress xanthine oxidase activity almost completely in the above experiments and constant agita-

tion was provided during the incubation by Dubnoff metablic incubator with a rate of 120/min.

3) ATPase assay

To 0.6 ml of reaction mixtures obtained from the previous experiments, 0.4 ml of the assay media for ATPase were added to give final volume of 1 ml mixture containing 100 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$ and 25 mM Tris, pH 7.4. After 10 min preincubation, reaction initiated by adding 2 mM Na_2ATP was allowed to proceed for 10 min at 37°C and stopped with 0.25 ml of 15% trichloroacetic acid. After centrifuging the mixture at 1,000 x 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 (commonly referred to as Mg^{++}-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.

4) Xanthine oxidase assay

Ten microliter of the dialysed xanthine oxidase solution was placed in a cuvette which contains 3 ml of mixture containing 0.67 mM xanthine, 0.033 mM EDTA and 100 mM Tris, pH 7.4. Spectrophotometric recording of urate production at 290 nm (the peak absorbance of uric acid) was carried out in a Unicam SP 1750 spectrophotometer for 1 min, and the slope of the initial linear portion of the curve was determined. By use of the molar extinction coefficient of uric acid at pH 7.4 (1.24×10^4), the amount of urate generated was calculated. One unit of xanthine oxidase was defined as 1 μmole urate produced per min.

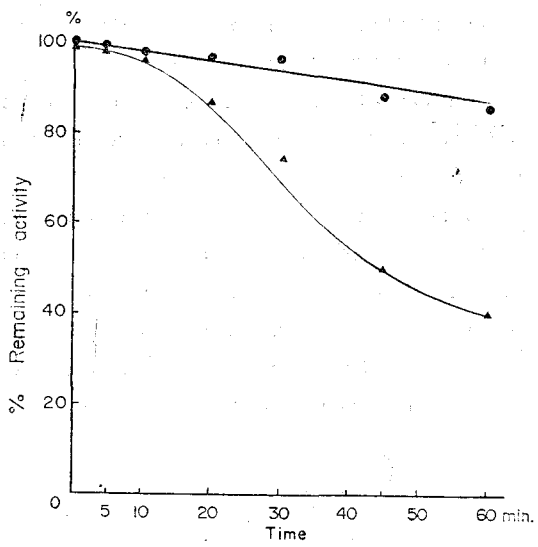

Fig. 1. Effect of xanthine oxidase system on microsomal ATPase activity. Microsomal preparations (180 μg protein/ml) were incubated with (▲) and without (●) 2 mM xanthine in 10 ml reaction medium containing xanthine oxidase (80 munits/ml), 1 ml EDTA and 50 mM Tris, pH 7.4 at 25°C. At time intervals, 0.5 ml aliquots were removed to test tubes containing 0.1 ml of 50 mM allopurinol and chilled in ice and then assayed for the ATPase activity as described under Methods and Materials. The specific activity of the control sample (at zero time) was 36.9±0.2 μmol of Pi released per mg of microsomal protein per h, which was designated as 100%.

RESULTS

1) Inactivation of ATPase activity of brain microsomal fraction by xanthine oxidase system

Fig. 1 shows that total microsomal ATPase activity was inactivated by xanthine/xanthine oxidase reaction. Incubation in the presence of xanthine oxidase (80 munits/ml) with 2 mM xanthine (▲) inactivated the enzyme activity with time to 87, 72, 46, 42% of zero time level (36.9±0.2 μmol of pi released/mg of

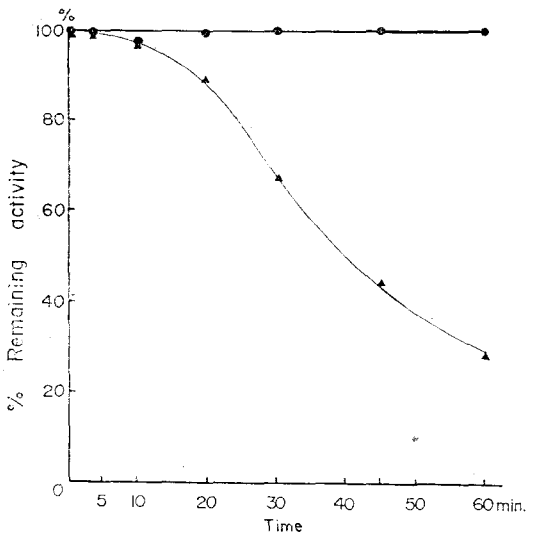

Fig. 2. Effect of xanthine oxidase system on microsomal Na^+-K^+ ATPase activity. Experimental conditions were the same as in Fig. 1. Na^+-K^+ ATPase activity was deterimined by substracting basal Mg^{++}-ATPase from total ATPase activity. ●, without xanthine; ▲, with xanthine. The specific activity at zero time was 28.0±0.21 μmol of Pi released per mg of microsomal protein per h, which was taken as 100%.

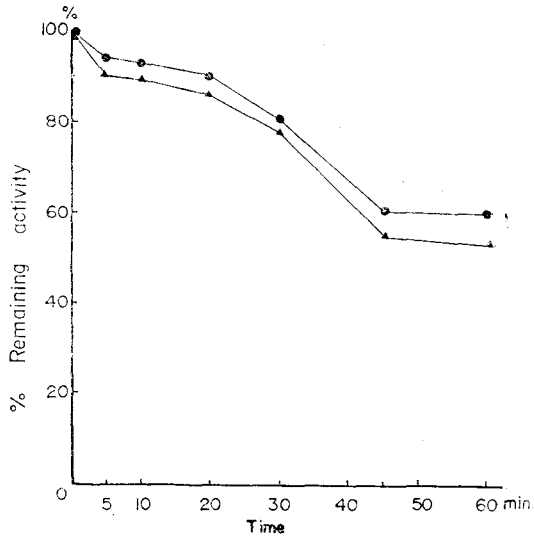

Fig. 3. Effect of xanthine oxidase system on microsomal Mg^{++}-ATPase activity. Experimental conditions were the same as in Fig. 1. Mg^{++}-ATPase activity was determined in the presence of 1 mM ouabain. ●, without xanthine; ▲, with xanthine. The specific activity at zero time was 8.9±0.3 μmol of Pi released per mg of microsomal protein per h, which was taken as 100%.

microsomal protein/h) at 20, 30, 45, 60 min, respectively, When xanthine was omitted from the reaction mixture (●), the inactivation was much slower and not significant compared to that observed in the presence of xanthine; 85% of the zero time activity remained up to even 60 min (Fig. 1). One thing to be noted here is that the ATPase activity (as well as Na^+-K^+-ATPase activity in Fig. 2) was further inactivated after 30min. At this time of incubation, the xanthine oxidase (80 munits) used was expected to convert all of the xanthine added in the reaction mixture to urate and no more oxygen species may be produced. That will be considered in Discussion. With xanthine only or without both xanthine oxidase and xanthine the degree of inactivation was almost the same as that with xanthine oxidase alone. Thus, neither xanthine nor xanthine oxidase when given alone seemed to have any effect on the ATPase activity and the diminution of the ATPase activity observed with xanthine oxidase alone was thought to be inactivation during incubation time. However, in all experiments of the present study, the activity observed with xanthine oxidase was taken as a control to exclude any possible error in the assay due to contaminated Pi in the xanthine oxidase solution although it was eliminated by extensive dialysis as mentioned in Materials and Methods. No detectable change in the activity of ATPase was also observed by the allopurinol added to stop the reaction of xanthine oxidase.

Fig. 2 shows the effect of xanthine oxidase system on the Na^+-K^+-ATPase activity. Na^+-K^+-ATPase activity was markedly diminshed

as a function of time by xanthine/xanthine oxidase reaction (▲). 88, 67, 45 and 27% of the zero time level (28.0±0.21 μmol of Pi released/mg of microsomal protein/h) were observed at 20, 30, 45, 60 min, respectively. In contract, the control (●) showed essentially no change in the activity. Since Mg^{++}-ATPase was shown to be much less susceptible as shown below (Fig. 3). The decrease of the total ATPase was mainly attributed to the inactivation of Na^+-K^+-ATPase.

Fig. 3 shows the effect of xanthine oxidase system on Mg^{++}-ATPase activity. With xanthine and xanthine oxidase (▲), marked decrease in the activity was observed with time. At 60 min, 55% of the zero time level (8.9 ±0.13 μmol of Pi released/mg of microsomal protein/h) remained. In contrast to Na^+-K^+-ATPase as shown in Fig. 2, Mg^{++}-ATPase showed marked decrease in its activity in the control study. The control activity (●) was diminished in similar manner to that of the treated one. Less than 10% of net difference between the two throughout the incubation period was observed. The results indicate that Mg^{++}-ATPase was much less susceptible to xanthine/xanthine oxidase reaction compared to Na^+-K^+-ATPase. The decrease in total ATPase activity of the control observed in Fig. 1 was mainly due to the decrease of Mg^{++}-ATPase activity.

Fig. 4 shows the effect of concentrations of xanthine on the inactivation of microsomal Na^+-K^+-ATPase. The activities of Mg^{++}-and Na^+-K^+-ATPase decreased with increasing concentration of xanthine up to 2 mM. At each concentration of xanthine, Na^+-K^+-ATPase was shown to be much more susceptible to xanthine/xanthine oxidase reaction. The above findings that inactivation after 1h incubation was observed only in the presence of both xanthine and xanthine oxidase, not either of them alone and extent of inactivation was dependent upon the concentration of xanthine suggest that the inactivation was attributed to reactive oxygen species generated by the enzymatic action of xanthine oxidase upon xanthine.

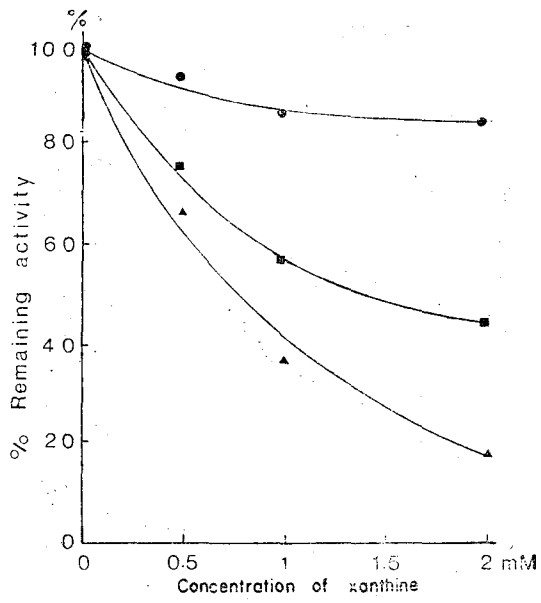

Fig. 4. Effect of xanthine concentrations on inactivation of microsomal Na^+-K^+-ATPase activity by xanthine oxidase system. Microsomal preparations (90 μg protein) were incubated for 1 h with 40 munits of xanthine oxidase and varying concentrations of xanthine in 0.5 ml of reaction mixture containing 1 mM EDTA and 50 mM Tris, pH 7.4 at 25°C and reaction was stopped in the same way as in Fig. 1. The specific activity of total, Na^+-K^+-and Mg^{++}-ATPase of the control (with no xanthine) were 33.3±0.18, 28.0±0.21 and 5.3±0.12 μmol of Pi released per mg of protein per h, respectively. which were taken as 100%. ●, Mg^{++}-ATPase; ■, total ATPase; ▲, Na^+-K^+-ATPase.

2) Effects of various scavengers on the inactivation of Na^+-K^+-ATPase activity

To determine which oxygen species caused

the inactivation of microsomal ATPase, various inactivators or scavengers of $O_2^-\cdot$, H_2O_2, OH· and $^1O_2$ were added to the mixture for 1 h where microsomal fraction was exposed to the oxygen radicals generated in the reaction. Because of its high susceptibity to the oxygen species which was observed in the previous experiment, Na^+-K^+-ATPase was used for evaluating their quenching effects in this scavenger study. Although the scavengers showed no detectable change on the ATPase activity within the concentration ranges used, control activity was measured in the presence of xanthine oxidase and each scavenger.

In Fig. 5 superoxide dismutase was used to evaluate the contribution of $O_2^-\cdot$ to the inactivation of Na^+-K^+-ATPase. This enzyme which greatly accelerates the spontaneous dismutation of $O_2^-\cdot$ to H_2O_2 and O_2 was capable of protecting the inactivation of Na^+-K^+-ATPase by xanthine oxidase system. The protecting effect was dose-dependent; when the microsomal Na^+-K^+-ATPase was treated with xanthine oxidase system, its residual activity was 21% of the control, which was recovered to 62, 73 and 88% with 0.5, 1, and 5 μg/ml of superoxide dismutase, respectively.

In Fig, 6 catalase was used to evaluate the effect of H_2O_2 by its ability to catalyze the conversion of H_2O_2 to O_2 and H_2O. When catalase was added to the reaction, there was also dose-dependent protection of the inactivation of Na^+-K^+-ATPase. With 50 μg/ml, almost 90% of the control activity was observed.

The role of the OH· as a mediator of the Na^+-K^+- ATPase inactivation was studied in Fig. 7. Mannitol, a scavenger of OH· (Hess et al., 1980) showed essentially no effect on the inactivation of Na^+-K^+-ATPase with concentrations used. Since ATPase assay was

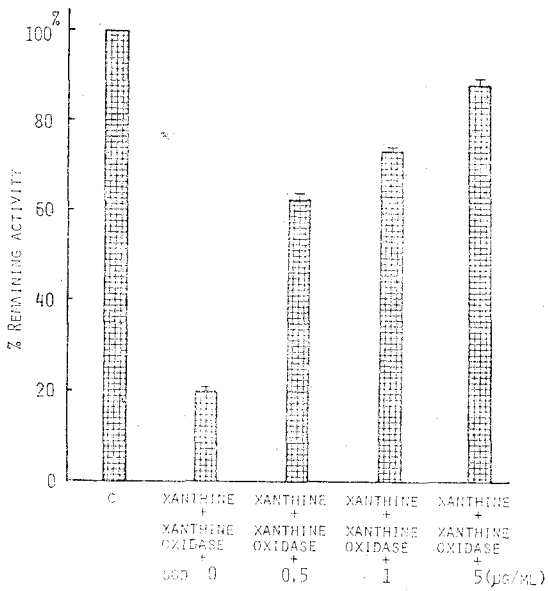

Fig. 5. Effect of superoxide on the inactivation of microsomal Na^+-K^+-ATPase activity by xanthine oxidase system. Microsomal protein (90 μg) were incubated for 1 h with 2 mM xanthine and 40 munits of xanthine oxidase in the presence of varying amount of superoxide dismutase in 0.5 ml of reaction mixture containing 1 mM EDTA and 50 mM Tris, pH 7.4 at 25°C. C, control (with no xanthne); SOD, superoxide dismutase. The specific activity of the control was 30.2±0.21 μmol of Pi released per mg protein per h which was taken as 100% Bars indicate mean+1 S.E.; five duplicates in each treatment group.

interfered with mannitol over 20 mM, higher concentration than this could not be tried. However other OH· quenchers, Na benzoate and formate (Chan and Kesner, 1980) tested showed almost negligible effect on the ATPase inactivation up to 60 mM (data not shown). The results indicate that OH· is not likely to be a mediator in the inactivation of Na^+-K^+-ATPase by xanthine oxidase system.

In Fig. 8 a $^1O_2$ quencher, 1,4-diazabicyclo (2,2,2) octane (Klebanoff et al., 1976) which does not react with $O_2^-\cdot$ and H_2O_2 was used

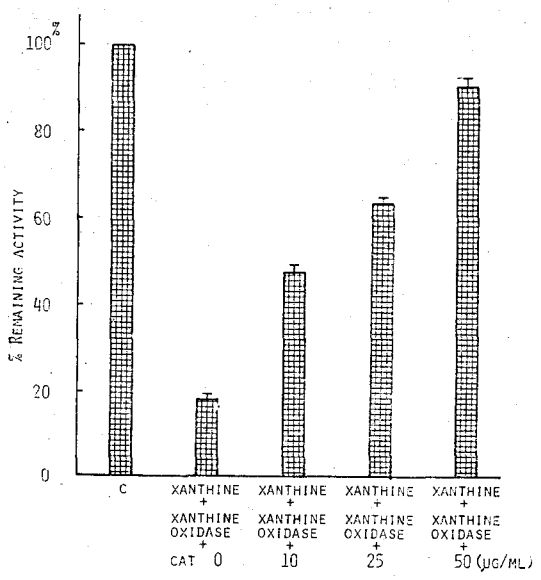

Fig. 6. Effect of catalase on the inactivation of microsomal Na^+-K^+-ATPase activity by xanthine oxidase system. The experimental conditions were the same as in Fig. 5. C, control (with no xanthine); CAT, catalase. Bars indicate mean+1 S.E.; five duplicates in each treatment group.

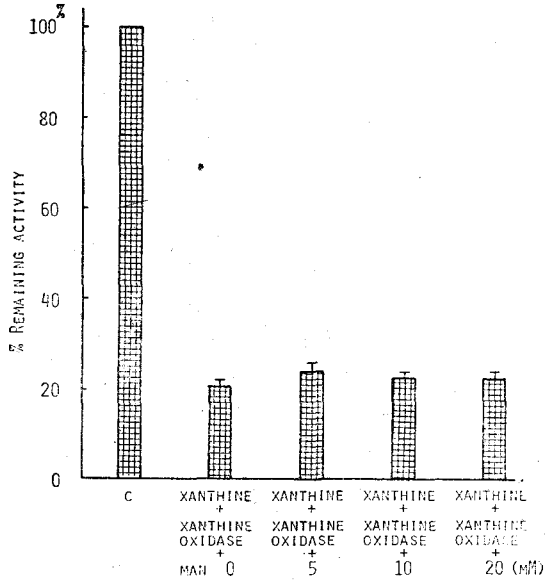

Fig. 7. Effect of mannitol on the inactivation of microsomal Na^+-K^+-ATPase activity by xanthine oxidase system. Microsomal protein was treated in the presence of mannitol under the same conditions as in Fig. 5. C, control (with xanthine); MAN, mannitol. Bars are mean+1 S.E.; five duplicates in each treatment group.

to evaluate the role of $^1O_2$. As shown in the figure, inactivation was prevented by this $^1O_2$ quencher in dose-dependent manner. Thirty mM of this agent almost completely prevented the loss of Na^+-K^+ATPase activity by xanthine oxidase system. The results suggest that $^1O_2$ may be generated secondarily from interaction of $O_2^-\cdot$ and participate in the inactivation on Na^+-K^+-ATPase.

That may be supported by the finding shown in Fig. 9. A combination of low concentration of superoxide dismutase (0.25 μg/ml) and catalase (5 μg/ml) (at the levels where 20% of remaining ac ivity of Na^+-K^+-ATPase was restored to 23 and 41%, respectively) was

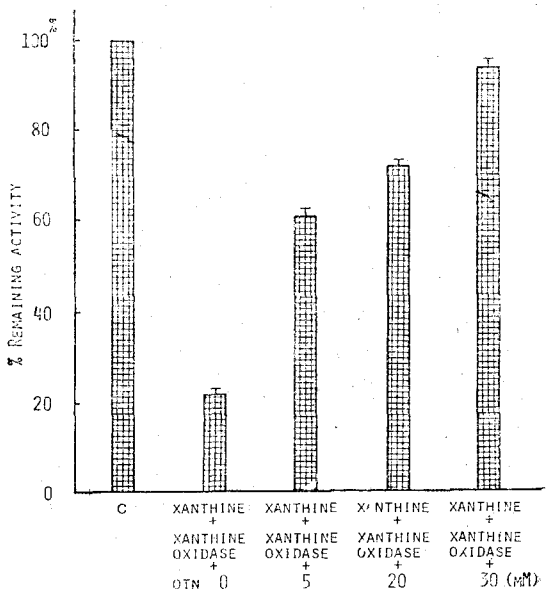

Fig. 8. Effect of $^1O_2$ quencher on the inactivation of microsomal Na^+-K^+-ATPase activity by xanthine oxidase system. The microsomal protein was treated with xanthine and xanthine oxidase in the presence of 1,4-diazabicyclo(2,2,2)octane. Other conditions were the same as in Fig. 5. C, control (with no xanthine); OTN, 1,4-diazabicyclo(2,2,2)octane. Bars are mean+1 S.E.; five duplicates in each treatment group.

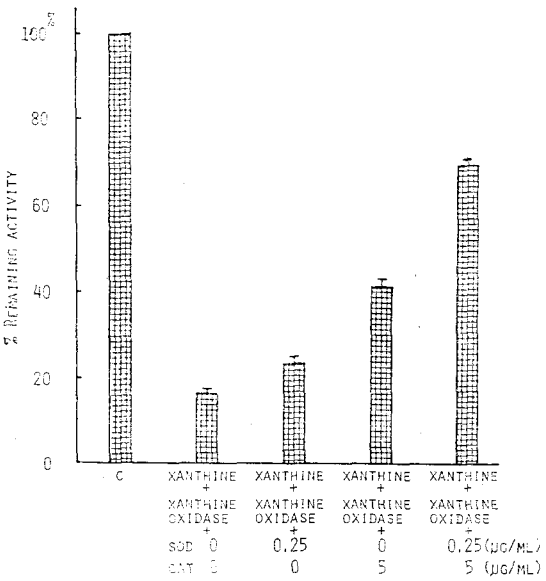

Fig. 9. Effect of catalase and superoxide dismutase on the inactivation of microsomal Na^+-K^+-ATPase activity by xanthine oxidase. The experimental conditions and abbreviations were the same as in Fig. 5. Bars are mean+1 S.E.; five duplicates in each treatment group.

shown to be additive on overcoming the inactivation of the enzyme by xanthine oxidase system (70% of the control activity was observed).

DISCUSSION

There are a number of reports that suggest that pathologic free radical reaction can take place in vivo in the CNS when the brain tissue becomes ischemic as a result of cerebral occlusion or impact injury. These include the appearance of increased levels of malonaldehyde (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. Because of their molecular characteristics, phospholipids in the brain have been suggested to be main biomolecules to be attacked by the free radical reactions (Demopoulos, 1975).

One major consequence of damage to membrane lipids would be functional impairment or loss of nerve cells. A number of major proteins in the membrane assume their active configurational shape as a result of intimate interactions between the hydrophobic portions of the proteins and the fatty acid tails of specific membrane phospholipids (Marchesi, 1975). Such proteins might be viewed as having a liability in depending on phospholipid integrity of the membrane. One of these enzymes has been known to be Na^+-K^+-ATPase. Although activity of ATPase can be affected secondarily to membrane pertubation by free radical reaction, direct observation of effect of the reactive oxygen species on the enzyme could be more pertinent to assessing detrimental effect of the oxygen species on nerve cell function, because the ATPase is an important neuronal enzyme which is largely responsible for the maintenance of the neuronal membrane potential.

However, the effect of the reactive oxygen species on the Na^+-K^+-ATPase of nerve cells has not been much studied despite succinct background explaining the mechanism of production of these species in the ischemia of CNS (Demopoulos et al., 1979; Fridovich, 1979).

In normal e-transport, four electrons must be transfered to molecular oxygen enzymatically in order to reduce molecular oxygen to water without producing any intermediary oxygen species (Fridovich, 1978). The bioenergetically linked transport of electrons to

molecular oxygen involves multiple steps through factors that assume free radical configurations; FAD and CoQ are key substances that normally have a free radical form (FAD·, CoQ·) (Ruzica, 1975) and present in relatively large amounts in the mitochondria-rich neurons. In the presence of adequate O_2, these e-transport factors remain tightly associated and there is little opportunity for FAD or CoQ to initate free radical reactions. However, when O_2 is diminished abruptly, as from the ischemia that is usually accompanied by cerebral vascular occlusion or impact injury, a certain proportion of the mitochondrial e-transport chains will not be provided with sufficient concentration of O_2 to accept the ongoing flow of electrons. Eventually, FAD and CoQ, along with all the other e-transport substances become reduced. The reduced FAD and CoQ can be then oxidized in a cyclical manner even in the presence of small amount of oxygen to produce two radicals as follows.

(reduced) $CoQ + O_2 \rightarrow CoQ\cdot + O_2^-\cdot$

The CoQ accept electrons from reducing species that are still being produced and becomes reduced again; in this way the CoQ cycles and keeps producing $O_2^-\cdot$. $O_2^-\cdot$ spontaneously disporprotionates to form H_2O_2.

$O_2^-\cdot$ as well as other reactive oxygen species can be generated in vitro by enzymatic action of xanthine oxidase in converting xanthine to uric acid (Fridovich, 1970; Greenwald and Moy, 1976). For in vitro experiment, the use of xanthine oxidase has the advantage of convenience, rapidity and easy control of the oxygen radical flux. It has been shown that, in this reaction, $O_2^-\cdot$ and H_2O_2 are generated by following reactions.

$$\text{xanthine} + O_2 \rightarrow \text{urate} + O_2^-\cdot$$

$$O_2^-\cdot + O_2^-\cdot + 2H^+ \rightarrow H_2O_2 + O_2$$

In the present study, the inactivation of Na^+-K^+-ATPase activity was observed in the presence of both xanthine and xanthine oxidase, not either of them alone and the extent of inactivation increased with increasing concentration of xanthine (Fig. 2 and 4). It is interesting to note that there was a large difference in susceptibility between Mg^{++}- and Na^+-K^+-ATPase. That may be due to the high dependence of Na^+-K^+-ATPase on the membrane integrity (Sun, 1974) and also indicates that the oxygen species generated gave damaging effects to the membrane. It was reported that activity of Na^+-K^+-ATPase was shown to be markedly decreased following experimental impact injury to the spinal cord (Clendenon et al., 1978) and during ischemia of cerebral cortex (Schwartz et al., 1976), while under these conditions there was no appreciable change in the activity of Mg^{++}-ATPase. Kindya and Chan (1976) also reported that the treatment of erythrocyte membrane with O_3 caused marked decrease of Na^+-K^+-ATPase activity with no significant change in Mg^{++}-ATPase activity.

In scavenger studies (Fig. 5, 6 and 9), superoxide dismutase and catalase showed their ability to limit the inactivation of Na^+-K^+-ATPase by xanthine oxidase. Thus, it is concluded that $O_2^-\cdot$ and H_2O_2 are responsible for the enzyme inactivation. However, the inactivation were also prevented by 1, 4-diazabicyclo (2, 2, 2) octane (Fig. 9) which is known as $^1O_2$ quencher (Klevanoff et al., 1976) and not to scavenge $O_2^-\cdot$ or H_2O_2. The result suggests that $O_2^-\cdot$ or H_2O_2 gives rise to more reactive species, $^1O_2$ which may be a final mediator causing the inactivation of Na^+-K^+-ATPase activity.

Many studies have suggested that by themselves $O_2^-\cdot$ and H_2O_2 are not so reactive compared to other more reactive species such

as OH· and $^1O_2$, instead serve as precursors for them (Fong et al., 1976; Kellogg and Fridovich, 1977; Kameda et al., 1979). But the mechanism in which O_2^-· and H_2O_2 interact to yield the reactive products is still controversial. In many in vitro phenomena where O_2^-· has been implicated to be a causative factor, the process has been partially inhibited by added superoxide dismutase. However, in most cases, catalase also acts as a partial inhibitor (Fee, 1980). In the present study, the abilities of superoxide dismutase and catalase to prevent the inactivation of Na^+-K^+-ATPase were also shown to be additive (Fig. 9). Beauchamp and Fridovich (1970) were the first to recognize the interdependence of O_2^-· and H_2O_2 in the production of a extremely reactive oxidizing species, OH· capable of initiating degradative processes. $^1O_2$ has been also suggested to be a product in xanthine oxidase system.

But various quencher studies did not show consistent evidence for the production of both $^1O_2$ and OH· in the system where evident involvement of O_2^-· and H_2O_2 as primary reactants have been suggested. In this study, mannitol, a OH· scavenger did not prevent the inactivation of Na^+-K^+-ATPase (Fig. 7), while 1,4-diazabicyclo(2,2,2)octane was shown to be effective in limiting the inactivation. Other OH· scavengers such as sodium benzoate and formate showed essentially no effect on Na^+-K^+-ATPase inactivation by xanthine oxidase reaction (data not shown). Thus, OH· was excluded as a mediator causing the inactivation of Na^+-K^+-ATPase activity. However, in view of the fact that OH· was shown to be most reactive among the oxygen species, the possibility can not be eliminated that even if generated, it, because of its high reactivity, attacked the membrane first before being scavenged by quenchers tested.

Simon et al. (1981) have recently reported that in their study observing cytotoxic effect of reactive oxygen species on fibroblast, H_2O_2, not other species such as O_2^-·, OH· and $^1O_2$ was found to be a causative factor responsible for the cell death. In the present study, Na^+-K^+-ATPase was still further inactivated even after 30 min of incubation when all of the xanthine added was used and no more oxygen species was expected to be produced. That may be also attributed partly to H_2O_2 present in the reaction mixture which was formed from dismutation of O_2^-· that was produced on conversion of xanthine to urate. H_2O_2, because of its high stability compared to other oxygen species, can exist for a long time in the aqueous solution. Thus, while it may be possible that O_2^-· or H_2O_2 can attack the membrane structure directly rather than serve as a precursor for the more reactive species, the results obtained in the present study suggest that O_2^-· and H_2O_2 are formed primarily and they interact to produce $^1O_2$ which attacks membrane structures as a final mediator to cause the inactivation of Na^+-K^+-ATPase.

Several mechanisms with which the reactive oxygen species cause the inactivation of Na^+-K^+-ATPase activity may be possible. As mentioned before, the inactivation can result secondarily to the membrane pertubation by lipid peroxidation because the activity of the enzyme has been known to be highly dependent on the integrity of the membrane (Sun, 1974). Several workers have shown that Na^+-K^+-ATPase activity in rat brain microsomal fraction can be inhibited when the membrane lipids undergo free radical damage (Schaeffer et al., 1975). However, protein components including Na^+-K^+-ATPase in the membrane can

be attacked directly by the oxygen species. Chan et al. (1977) reported that O_3 caused the inactivation of Na^+-K^+-ATPase activity of erythrocyte membrane and the extent of the inactivation was parallel in some degree to that of polymerization of membrane proteins. It has been also observed that amount of SH groups which is known to be an active group at the catalytic site of Na^+-K^+-ATPase (Chan and Rosenblum, 1969) was reduced along with inactivation of the enzyme in the erythrocyte membrane by O_3 (Chan et al., 1977) and brain microsomal fraction by chloropromazine radicals (Akera and Brody, 1970). Further study will be needed to explore the possible mechanism of the inactivation of Na^+-K^+-ATPase activity by reactive oxygen species observed in the present study in terms of those respects mentioned above.

In the present study, the oxygen species were generated from in vitro system using xanthine and xanthine oxidase. However, based upon the evidence that the oxygen species are formed in ischemic state of CNS accompanied by cerebral vascular occlusion or impact injury, the inactivation of Na^+-K^+-ATPase activity by the reactive oxygen species might be a possible evidence demonstrating that they are involved in the functional derangement of nerve cells in this pathology.

SUMMARY AND CONCLUSION

The effects of xanthine-xanthine oxidase reaction on brain microsomal Na^+-K^+-ATPase activity were studied to see possible involvement of oxygen free radicals in pathologic change occurring in ischemic state of CNS accompanied by cerebral vascular occlusion or impact injury.

When microsomal fraction was incubated with xanthine and xanthine oxidase, Na^+-K^+-ATPase activity of the fraction was markedly inactivated (80% inactivation) whereas basal Mg^{++}-ATPase was much less sensitive (less than 10% inactivation) compared to that of Na^+-K^+-ATPase. The inactivation was observed only in the presence of both xanthine and xanthine oxidase, not either of them alone, and the extent of inactivation was dependent on the concentration of xanthine.

In an attempt to determine which of the oxygen species was responsible for the inactivation, the ability of various scavengers to overcome the inactivation was tested. Superoxide dismutase, catalase and 1, 4-diazabicyclo(2, 2, 2)octane were shown to reverse the inactivation of the ATPase in dose-dependent manner. In contrast, mannitol as well as other OH· quenchers were ineffective in limiting oxygen radical-induced inactivation. Thus O_2^-·, H_2O_2 anb $^1O_2$ were implicated to be mediators involved in the inactivation.

Since oxygen radicals are suspected as being a cause of the peroxidative damaging process in brain ischemia, the ATPase inactivation by oxygen radicals may be a possible contributing factor which gives rise to functional derangement of nerve cells observed in the pathologic process.

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