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Generation of Superoxide Radical from Rat Brain Mitochondria and Mechanism of Its Toxic Action to Mitochondrial and Extra-mitochondrial Components[1]

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ABSTRACT

The generation of $O_2^{\cdot-}$ and its toxic effects were studied with rat brain mitochondria. The production of $O_2^{\cdot-}$ from mitochondria in the presence of succinate and antimycin was demonstrated by SOD-inhibitable reduction of NBT. Although succinate can support the $O_2^{\cdot-}$ formation, the highest rate needs antimycin indicating that blockade of electron flow in the respiratory chain augments the univalent reduction of molecular oxygen. Under this condition, H_2O_2 was also observed to be produced. But its formation appears to be derived from the dismutation of the primary product, $O_2^{\cdot-}$ since the rate of H_2O_2 production was markedly decreased by NBT and ferricytochrome c. The $O_2^{\cdot-}$ and H_2O_2 produced were able to cause toxic actions to mitochondrial and extra-mitochondrial components as shown by lipid peroxidation of mitochondrial membrane, and inactivation and lysis of isocitrate dehydrogenase and erythrocytes added to the medium, respectively. In all the toxic actions observed, Fe^{++} was required. It appears that in the toxic actions $OH^{\cdot}$ generated from the iron-catalyzed Haber-Weiss reaction acts as a mediator. This was supported by the finding that mitochondria in the presence of succinate and antimycin produced ethylene from methional, and Fe^{++} added increased the ethylene production. The observed toxic actions of mitochondrial $O_2^{\cdot-}$ may provide evidence supporting a potential role of mitochondria as a source of oxygen radicals to cause tissue damage.

Key Words: mitochondria, oxygen radical, lipid peroxidation
Abbreviations: SOD; superoxide dimutase, MDA; malondialdehyde, GSH; glutathione, NBT; nitroblue tetrazolium

INTRODUCTION

Univalent reduction of molecular oxygen (O_2) produces superoxide radical ($O_2^{\cdot-}$) and occurs in a wide range of biological reactions involved in the metabolism of O_2 (Fridovich, 1975). Because of its high reactivity enough to alter most types of cellular macromolecules (McCord, 1974; Kellogg & Fridovich, 1977; Lesko *et al.*, 1980), $O_2^{\cdot-}$ is expected to have deleterious effects to living cells. Its potential danger to all respiring cells can be further supported by the facts that superoxide dismutase (SOD), an enzyme which breaks down $O_2^{\cdot-}$ is an essential constituent of all organisms that utilize O_2 and it has been shown

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to inhibit a number of $O_2^{\cdot-}$ mediated adverse reactions (Fridovich, 1975).

Since Nohl and Hegner(1978), and Nohl *et al.*(1978) demonstrated the production of $O_2^{\cdot-}$ from mitochondria, O_2 metabolism in mitochondrial respiratory chain has been established as one of the important sources of $O_2^{\cdot-}$ in cellular metabolism. Detailed studies on the effects of various respiratory chain inhibitors and uncouplers of oxidative phosphorylation have shown that among the carriers, one between rotenone- and antimycin-sensitive sites, most likely ubiquinone reacts in its reduced form with O_2 to produce $O_2^{\cdot-}$ (Boveris *et al.*, 1976; Cadenas *et al.*, 1977). Under the conditions where $O_2^{\cdot-}$ is generated, H_2O_2 was also detected through dismutation of O_2 by intra-mitochondrial SOD (Boveris *et al.*, 1976; Nohl & Hegner, 1978)

Most of $O_2^{\cdot-}$ and its product, H_2O_2 can be removed by antioxidant enzymes, SOD, catalase and glutathione (GSH) peroxidase (Nohl & Jordan, 1980). But when the equilibrium between their production and removal is disturbed, the species unremoved are expected to cause cellular damage. In fact, there has been implication that destructive processes in some pathological conditions are attributable to the unbalance of $O_2^{\cdot-}$ metabolism in mitochondria, for examples, aging (Nohl & Hegner, 1978), and tissue injury in hypoxia (Demopoulos *et al.*, 1980). However, studies have not much done to show the toxic effects mediated by $O_2^{\cdot-}$ generated from mitochondria, which can serve as direct evidence supporting the role of this organelle as a source of the toxic oxygen species.

In the present study, an attempt was made to demonstrate the toxic effects of $O_2^{\cdot-}$ generated from mitochondria by observing the destruction of mitochondrial and extra-mitochondrial structures in the system where brain mitochondria were stimulated to genreate $O_2^{\cdot-}$ by succinte and antimycin. Lipid peroxidation of mitochondrial membrane was observed, and when added to the system, enzyme (isocitrate dehydrogenase) and erythrocytes were inactivated and lyzed, respectively. In the toxic effect observed, it appears that $OH^{\cdot}$ generated by iron-catalyzed Haber-Weiss reaction was responsible.

MATERIALS AND METHODS

Chemicals

Superoxide dismutase, catalase, horse-radish peroxidase, antimycin, scopoletin, rotenone, ferricytochrome c, nitroblue tetrazolium, thiobarbituric acid and Chromosorb 102 were purchased from Sigma Chemical Co.; hydrogen peroxide from Shinyo Pure Chem. Co.; ferrous sulfate ($FeSO_4 . 7H_2O$) from Ishizu Pharmaceut. Co.; carbon monoxide (2860 ppm in air) from Korea Standard Research Institute. Other chemicals were of reagent grade. Pure ethylene was a kind gift from Dr. Kyung-Hoon Chung, Korea Advanced Institute of Science and Technology.

Preparation of brain mitochondria

Male Sprague-Dawley rats, weighing 200 to 250g were used as the source of brain tissue. Mitochondria were isolated as described by Schneider and Hogeboom (1950) with a little modification. Protein content of the mitochondrial preparation was determined by the method of Lowry *et al.* (1951). The preparation was freshly made in every experiment.

Preparation of human erythrocytes

Heparinized 5.0 ml of fresh human blood was mixed with 9 volumes of 150 mM KCl and centrifuged at 3000 g for 15 min. Packed erythrocytes obtained were washed 3 times with 9 volumes of the isotonic solution with centrifugation as above, and then suspended in an equal volume of 150 mM KCl. The suspension was used in the experiments.

Incubation systems

Generation of the reactive oxygen species was performed through the incubation of mitochondria in the presence of substrates and respiratory-chain inhibitors. As a typical reaction mixture, mitochondria (1.0 mg protein/ml) were mixed with 5 mM succinate and 6.7μM antimycin in O_2-saturated solution of 0.15 M KCl and 50 mM HEPES-KOH, pH 7.4. The reaction otherwise specified was started by addition of succinate. Other components were added to the reaction mixture depending upon the experimental purposes; nitroblue tetrazolium (NBT) was added for determination of $O_2^-\cdot$, scopoletin and horse-radish peroxidase for H_2O_2, and methional for $OH\cdot$. To demonstrate the molecular and cellular damaging effects of the oxygen species generated, isocitrate dehydrogenase or erythrocytes were added to the above system. When the effect of the oxygen species was observed on lipids, mitochondrial membrane was used as a target material. Fe^{++} was also added to the systems to increase the reactivity of the oxygen species.

Analytical procedures

Determination of superoxide radical: The inhibitory effect of SOD on the $O_2^-\cdot$-induced reduction of NBT to purple formazan was used to demonstrate the generation of this oxygen species (Baehner, 1975). NBT (100 μM) was incubated with mitochondria in the presence or absence of SOD (details are described in the legend of Table 1). After incubation was carried out, the reaction was stopped with the addition of 1.0 ml of 1.0 N HCl. The purple color was then solubilized with 2.0 ml of pyridine. Samples were read at 560 nm against the medium containing only mitochondria and NBT undergoing the same procedure. The absorbance values were compared to a standard curve prepared by using 20 to 400 μM of NBT solutions which were reduced with 0.2 mg ascorbic acid and then resuspended in the HCl and pyridine solution as described above. From the curve, the extinction coefficient of formazan was found to be 4.22/mM/cm. With the value, the amount of SOD-inhibitable reduction of NBT was calculated and used to estimate the rate of O_2^- production based upon the following stoichiometry (Baehner *et al.*, 1976):

$$2O_2^-\cdot + 2H^+ + NBT \longrightarrow NBTH_2(\text{formazan}) + 2O_2$$

Determination of hydrogen peroxide: Changes in the fluorescence of scopoletin oxidized by hydrogen peroxide in the presence of horse-radish peroxidase (Loschen *et al.*, 1971) were followed in a Perkin Elmer fluorescence spectrophotometer, Model 1000. The excitation wavelength was 343 nm; the emission wavelength, 460 nm. The calibration was performed with H_2O_2 solution of known concentration which was assayed by the method of Allen *et al.* (1952). The resulting decrease in fluorescence was used as a standard. Other details are given in the graph and legend of Fig. 2.

Determination of ethylene: To demonstrate the generation of OH·, production of ethylene from methional was determined by the gas chromatographic method of Beauchamp and Fridovich (1970). They showed that oxidation of methional by OH· produced ethylene gas as one of the end products. The reaction mixtures (2.0 ml) containing methional, mitochondria and other components (see the legend of Table 7 for more details) were incubated in 12.5 ml glass vials sealed with rubber caps in a water bath with vigorous shaking. Gas-tight syringes were used to sample the gas phase above the reaction mixtures. One ml of aliquots of the gas phase were analyzed for ethylene on a gas chromatograph (GC V, Pye-Unicam) equipped with a 1/8 inch × 3 meter stainless column of Chromosorb 102 and a flame ionization detector. The details of chromatographic conditions were described in the previous report (Kim *et al.*, 1984). Amounts of ethylene production was calculated from integrated areas of chromatogram of pure ethylene gas.

Determination of Malondialdehyde: Malondialdehyde (MDA) formed as a result of lipid peroxidation of mitochondrial membrane was determined by the thiobarbitureic acid method (Bidlack and Tappel, 1973). The amount of MDA produced was calculated using the molar extinction coefficient of 1.52 x

10^5/M/cm (Placer *et al.*, 1966).

Activity of isocitrate dehydrogenase: The enzyme activity was assayed by following the increase of absorbance at 340 nm which was caused by reduction of NADP to NADPH in the presence of isocitrate. Aliquots (0.1ml) of the reaction mixture (details are given in the legend of Tables 5 and 6) were taken to a cuvette containing 0.85 ml of 4.4 mM isocitrate in 50 mM NaCl and 50 mM HEPES-KOH, pH7.4. The cuvette was then transferred to the temperature controlled chamber of a Unicam SP 1750 spectrophotometer at 25°C. After 5 min incubation, reaction was started with addition of 30 μl of 30 mM NADP and 0.12 M $MnSO_4$. The enzyme activity was expressed as an increase of absorbance at 340 nm/min (A_{340}/min).

Erythrocyte hemolysis: Fresh erythrocyte suspension (20 μl) prepared as described earlier was incubated in total volume of 3.0 ml reaction mixture (see the legend of Table 8). Hemolysis was determined spectrophotometrically in two ways. One was to read the decrease in turbidity of the reaction mixture at 740 nm (Goldbery & Stern, 1977) and the other was to measure the color of hemoglobin released in the medium (Bauer, 1982). In the latter, the reaction mixture was centrifuged at 3,000 g for 10 min and the supernatant was read at 540 nm.

RESULTS

Generation of superoxide radical

Brain mitochondria were investigated for their capacity to generate $O_2^{\cdot-}$ by observing the SOD-inhibitable reduction of NBT. The results are shown in Table 1. When NBT was incubated for 30 min with mitochondria in the presence of antimycin, a respiratory chain inhibitor, and succinate as a substrate, the compound was reduced as indicated by marked increase in absorbance at 560 nm. The increase in absorbance, however, was inhibited by 100 μg/ml SOD from 0.322 to 0.267(17% inhibition) and the inhibition was dependent on the dose of SOD with labelling off at 100 μg/ml (inset of Fig. 1). In contrast, bovine serum albumin even at 250 μg/ml showed almost negligible effect on the NBT reduction (data not shown). These observations indicate that some of the electrons from the respiratory chain were transferred to molecular oxygen to form $O_2^{\cdot-}$, while most electrons reduced NBT directly. The production rate of $O_2^{\cdot-}$ was increased linearly up to 30 min and then approached the plateau level (Fig. 1).

Table 1. SOD-inhibitable NBT reduction by mitochondria

Additions	NBT reduction (A_{560})		
	without SOD	with SOD	Difference
None	0.085 ± 0.0032	0.086 ± 0.0046	−0.0008 ± 0.0017
Antimycin (6.7 μM)	0.098 ± 0.0035	0.097 ± 0.0053	0.0012 ± 0.0032
Succinate (5 mM)	0.127 ± 0.0023	0.113 ± 0.0036	0.014 ± 0.0026
Antimycin (6.7 μM) + Succinate (5 mM)	0.322 ± 0.0176	0.267 ± 0.0209	0.055 ± 0.0036

The reaction mixtures containing 1.0 mg protein of mitochondria, 100 μM NBT, 150 mM KCl and 50 mM HEPES-KOH, pH 7.4 were incubated with the additions shown in the table in the presence or absence of 100 μg SOD. Total volume was 1.0 ml. The incubation was performed for 30 min with vigorous shaking in a water bath at 25 °C. Determination of NBT reduction was done as described in the Materials and Methods. The numbers indicate mean ± S.E.M. from five experiments.

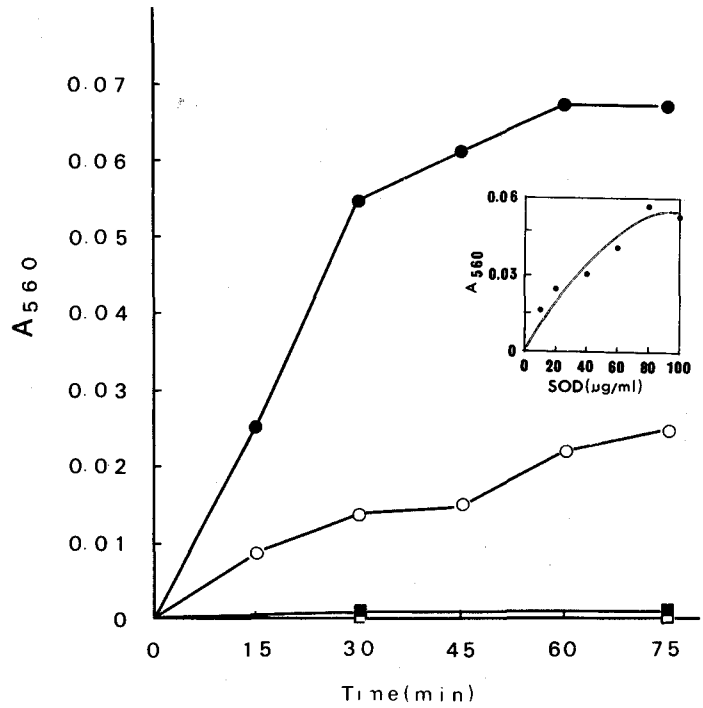

Fig. 1. SOD-inhibitable NBT reduction with time. The experiments were performed under the same conditions as in Table 1. NBT reduction inhibited by SOD was plotted against the incubation time. Inset shows effect of SOD concentrations on the NBT reduction in the presence of 5 mM succinate and 6.7 μM antimycin.
●; 5 mM succinate and 6.7 μM antimycin, ○; 5 mM succinate, ■; 6.7 μM antimycin and □; with no addition.

Using the extinction coefficient, 4.22/mM/cm of reduced NBT dissolved in pyridine (see the Materials and Methods), the production rate in the linear phase was 0.87 nmoles/mg protein/min. As shown in Table 1 and Fig. 1, the rate of $O_2^{\cdot-}$ production was highest in the presence of the substrate and inhibitor. $O_2^{\cdot-}$ was produced by succinate alone but the rate was only one fourth of the observed with both. Of no significance was the production with antimycin alone.

Generation of hydrogen peroxide

The production of H_2O_2 was followed with scopoletin method in the same system where $O_2^{\cdot-}$ production was induced. The results are shown in Fig. 2 where the traces indicate the changes in fluorescence corresponding to each experimental step in sequence.

Addition of scopoletin to the reaction mixture presented the increase in fluorescence. When addition of antimycin was followed, there was no change in fluorescence (the slight increase observed was due to intrinsic fluorescence of antimycin). But the fluorescence was rapidly decreased with addition of succinate. The same decrease was observed when the substrate was added first and then the inhibitor was followed. Although succinate itself was able to cause the decrease in fluorescence, the rate of decrease was much lower (data not shown). The observed findings demonstrate that H_2O_2 was also detected when the substrate was added to the respiratory chain-blocked mitochondria with antimycin. The production of H_2O_2 was further supported by the observation that the trace was stopped completely when catalase was added during the reaction (Fig. 2). Using the calibrated deflection in the trace, the rate of procuction was calculated to be 0.25 nmoles/mg protein/min.

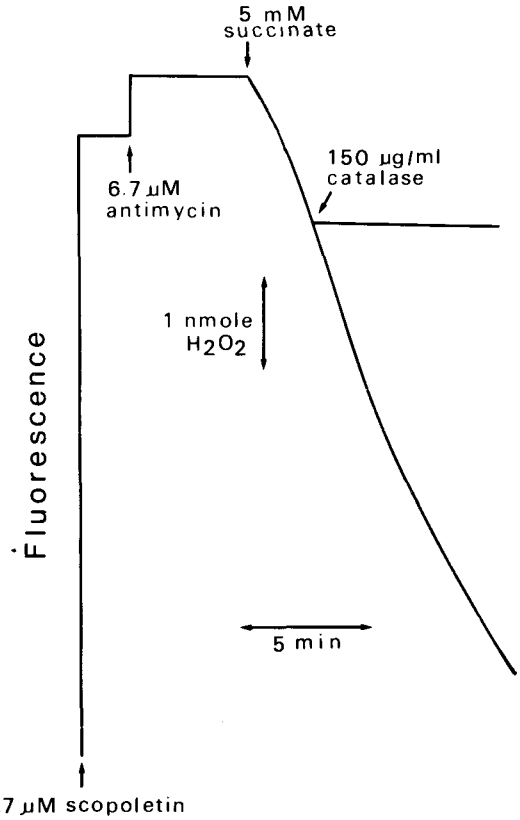

Fig. 2. Production of hydrogen peroxide from mitochondria. Mitochondria (1.0 mg/ml) were incubated in the reaction mixtures containing 5 μg/ml horse-radish peroxidase, 150 mM KCl, 50 mM HEPES-KOH, pH 7.4 at 25°C for 10 min, and then various components were added as indicated by arrows in the graph. The traces show change in fluorescence resulting from each addition at excitation 343 nm and emssion 460 nm. Total volume was 3.0 ml when all the components were added with final concentrations shown.

Table 2. Effects of NBT and ferricytochrome c on the production of hydrogen peroxide from mitochondria

Additions	H_2O_2 produced (nmoles/mg protein/min)
None	0.21
NBT	
33 μM	0.13
133 μM	0.04
Ferricytochrome c	
13 μM	0.08
27 μM	0.03

Mitochondria were incubated with 5 mM succinate and 6.7 μM antimycin in the presence of different concentrations of NBT and ferricytochrome c. Other conditions were the same as in Fig. 2. The rate of hydrogen peroxide production was calculated as described in the Materials and Methods.

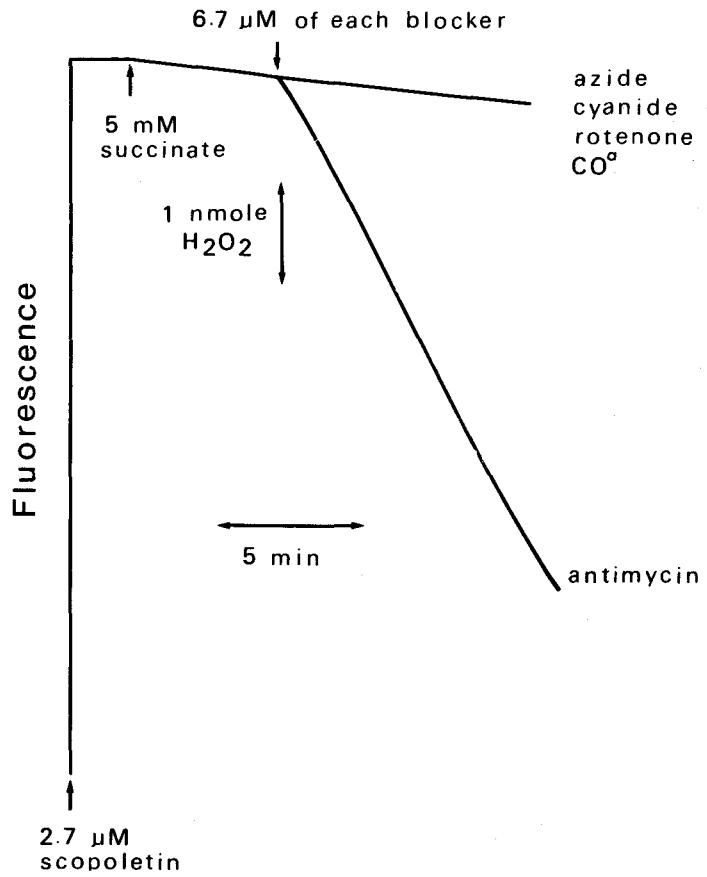

Fig. 3. Production of hydrogen peroxide from mitochondria in the presence of various inhibitors of the respiratory chain. The experimental conditions were the same as in Fig. 2 but each inhibitor was added last.
a: When CO was used as an inhibitor, mitochondrial preparation (see the Materials and Methods) was bubbled with 2860 ppm CO in air for 30 min, suspended in the reaction mixture and then reaction was started with the addition of succinate.

In the generation of the two oxygen species, the highest rate of production was observed under the same condition; presence of both the substrate and inhibitor. In additions, the rate of H_2O_2 production was inhibited by the compounds which can interact with $O_2^{\cdot-}$. As shown in table 2, NBT and ferricytochrome c inhibited the H_2O_2 production to 80%. Boveris *et al.* (1976) observed that the rate of H_2O_2 production by succinate and antimycin from unwashed SOD-containing submitochondrial particles was higher than that from washed SOD-free ones, and the rate of $O_2^{\cdot-}$ production from the SOD-free particles was decreased with addition of exogenous SOD, and concomitantly H_2O_2 production was increased to the same extent. Thus, it is suggested that the H_2O_2 produced from the whole mitochondria comes from the dismutation of $O_2^{\cdot-}$ involving intra-mitochondrial SOD.

Various combinations of substrates and inhibitors were compared for their capacity to induce the univalent reduction of O_2 from the mitochondria; succinate, α-ketoglutamate, malate and isocitrate were employed as substrates, and antimycin, rotenone, carbon monoxide (CO), cyanide, and azide as inhibitors. The highest rate of H_2O_2 production was observed with succinate and antimycin. With antimycin as a inhibitor, increasing order of potency was malate, glutamate, α-ketoglutarate and succinate (data not shown). When succinate was used as a substrate, only antimycin stimulated the H_2O_2 pro-

duction and others showed almost negligible stimulation (Fig. 3). But rotenone showed some effect when a NAD-linked intermediate, α-ketoglutarate was used as substrate; the rate was 20% of that observed with succinate and antimycin. Because of the highest activity, antimycin and succinate were used in the following experiments to demonstrate the toxic effects of $O_2^{-}\cdot$ and H_2O_2 generated from the mitochondria.

Lipid peroxidation by the oxygen species

O_2^{-} and H_2O_2 were produced from the brain mitochondria. Because of their reactivity, it is expected that the species cause the break-down of cellular macromolecules adjacent to the site of generation. The membrane lipids of mitochondria may be the components to be attacked first. As expected, lipid peroxidation was observed but Fe^{++} was required for the peroxidation (Table 3). Even in the presence of

Table 3. Production of malondialdehyde from mitochondria

Additions	MDA produced (nmoles/h)
None	2.60 ± 0.03[a](2)[b]
Antimycin (6.7 μM)	2.47 ± 0.10 (2)
Succinate (5 μM)	2.83 ± 0.53 (2)
Antimycin (6.7 μM) + Succinate (5 mM)	2.63 ± 0.23 (5)
Antimycin (6.7 μM) + Succinate (5 mM) + Fe^{++} (10 μM)	20.63 + 0.34 (8)
Fe^{++} (10 μM)	12.61 ± 0.31 (6)

The reaction mixtures (2.0 ml) containing mitochondria (1.0 mg protein/ml), 150 mM KCl, 50 mM HEPES-KOH, pH 7.4 were incubated with the components shown in the table at 37 °C. After incubation for 1 h, aliquots were assayed for MDA (see the Materials and Methods).

a: Values shown are mean ± S.E.M.

b: Figures in the parentheses indicate number of experiments.

Table 4. Effects of SOD and catalase on the production of malondialdehyde from mitochondria in the presence of ferrous ion.

Experimental conditions	MDA produced[a] (n moles/h)
(A + S + Fe^{++}) − (Fe^{++})	7.55 + 0.30[b]
(A + S + Fe^{++} + SOD) − (Fe^{++} + SOD)	4.51 ± 0.86 (40%)[c]
(A + S + Fe^{++} + CAT) − (Fe^{++} + CAT)	0.35 + 0.25 (95%)

Mitochondria were incubated with 10 μM Fe^{++} and other components shown in the table under the same conditions as in table 3. Abbreviations: A; 6.7 μM antimycin, S; 5 mM succinate, CAT; 150 μg/ml catalase, and SOD; 100 μg/ml superoxide dismtase.

a: Differences in the amount of MDA produced from the experiments between in the presence and the absence of antimycin and succinate.

b: Values shown are mean +S.E.M. from four experiments.

c: Numbers in the parentheses indicate % inhibition by the enzymes added.

Table 5. Inhibition of isocitrate dehydrogenase activity by mitochondria

Additions	A_{340}/min
None	0
Antimycin (6.7 μM)	0
Succinate (5 mM)	0
Antimycin (6.7 μM) + Succinate (5 mM)	0
Antimycin (6.7 μM) + Succinate (5.5 mM) + Fe^{++} (10 μM)	0.52
Fe^{++} (10 μM)	0.07

Isocitrate dehydrogenase (0.2 mg/ml) was incubated for 2.5 h with mitochondria (1.0 mg protein/ml) in the presence of the components shown in the table at 37 °C. Total volume was 1.2 ml and other conditions were the same as in Table 1. The enzyme activity was measured as an increase in absorbance at 340 nm per min as described in the Meterials and Methods. The values shown are the decrease in the enzyme activities (ΔA_{340}/min) for 2.5 h incubation.

Table 6. Effects of SOD and catalase on the inactivation of isocitrate dehydrogenase by mitochondria

	A_{340}/min (% inhibition)
Control[a]	0.45(0)[b]
SOD (67 μg/ml)	0.22(51)
Catalase (160 μg/ml)	0.16(64)
Albumin (250 μg/ml)	0.45(0)

Isocitrate dehydrogenase was treated in the reaction mixtures containing mitochondria in the presence of SOD, catalase or bovine serum albumin under the same conditions as in Table 5.

a: With no addition of the enzymes or albumin.

b: Numbers in the parentheses indicate % inhibition against the control which is A_{340}/min for 2.5 h obtained in the absence of the enzymes added.

Table 7. Ethylene production from methional by mitochontria

Additions	Ethylene produced in 10 min (pmoles)			
Experiments:	1	2	3	Mean
None	136	111	108	188.3
A + S	436	417	—	426.5(238.2)[a]
A + S + Fe^{++}	809	827	591	742.3(554.0)
Fe^{++}	324	254	226	268.0(79.9)
A + S + Fe^{++} + SOD	290	321	351	320.7(132.4)
A + S + Fe^{++} + CAT	128	105	—	117.5(-70.8)

Mitochondria (1.0 mg protein/ml) were mixed with the components shown in the table in 3.0 ml buffer of 150 mM KCl and 50 mM HEPES-KOH, pH 7.4. After 10 min incubation at 37 °C, 5 μl of methional was added and the vials were sealed with rubber caps. After further 10 min incubation, 1.0 ml aliquots from the gas phase were assayed for ethylene as described in the Materials and Methods. Abbreviations were the same as in Table 4 and the concentration of Fe^{++} was 10 μM.

a: Values in the parentheses are the amount of ethylene produced obtained by substracting that observed with no additions.

succinate and antimycin, production of MDA was in the range of the basal level without the metal ion. When 10 μM Fe^{++} was added, there was a marked increase in MDA production (20.63 ± 0.34 nmoles/h).

But it was found that a considerable portion of the lipid peroxidation observed (about 60%) was not due to the action of the oxygen species but to the direct action of Fe^{++}, since the peroxidation occurred with the metal ion alone (12.61 ± 0.31 nmoles/h). Further, the Fe^{++} -induced peroxidation was not inhibited by SOD or catalase (date not shown). Therefore, it is thought that about 40% of the total peroxidation was caused by the action of the oxygen species with an aid of Fe^{++} as a catalyst. This was further supported by the results in Table 4 showing that this portaion of lipid peroxidation was indeed inhibited by SOD and catalase.

Effect of the oxygen species on the isocitrate dehydrogenase

Effect of $O_2^-\cdot$ and H_2O_2 was also tested on the extra-mitochondrial component with an enzyme, isocitrate dehydrogenase added to the medium as a target molecule. Essentially, the same characteristics was found as observed in the lipid peroxidation. As shown in Table 5, the enzyme activity was decreased only in the presence of Fe^{++}. One thing different from the lipid peroxidation is that there was no significant direct action of Fe^{++} on the enzyme activity. Therefore, this system shows more clearly the oxidative action exerted by interaction between the oxygen species and the metal ion. Table 6 shows that the decrease in the enzyme activity was also inhibited by SOD and catalase whereas no inhibition was found with albumin. All the results indicate that $O_2^-\cdot$ and H_2O_2 released in the medium attacked the enzyme leading to the inactivation with aid of Fe^{++} as a catalyst.

Evidence for OH· production

In the previous experiments, it was shown that $O_2^-\cdot$ and H_2O_2 required Fe^{++} in their oxidative actions. It has been reported that either $O_2^-\cdot$ or H_2O_2 is not so reactive, rather depends in their oxidative actions upon OH· generated from the interaction between $O_2^-\cdot$ and H_2O_2 catalyzed by metal ions, particularly Fe^{++} (Fridovich, 1978). So OH· is also expected to be generated. Thus, an attempt was made to detect this radical using the assay of ethylene, a product of methional on its oxidation by OH· (Beauchamp & Fridovich, 1970). Table 7 shows the ethylene production from methional in the various experimental conditions.

Ethylene was detected from methional even in the absence of the substrate and inhibitor. That was possibly due to self-decomposition of the compound (Weiss *et al.*, 1978). When succinate and antimycin were added, the production of ethylene was increased. As expected, further increase was observed with addition of Fe^{++}. But Fe^{++} itself did not have any significant effect on ethylene production from methional. Here again the ethylene production was also inhibited by either SOD or catalase (80% and 100% inhibition, respectively). The results shown support that OH· was produced from the interaction between $O_2^-\cdot$ and H_2O_2 which was catalyzed by Fe^{++}. Without Fe^{++} added, there was also production on ethylene (238.2 pmoles). It may be ascribed to a trace amount of Fe^{++} or other metals contaminated since the inhibition by SOD or catalase exceeded the amount stimulated by the added Fe^{++}.

Hemolysis of human erythrocytes

The toxic effect of oxygen radicals was extended to cellular damage. Hemolysis was observed as an index of cytotoxicity. On exposure to the oxygen species, erythrocytes were lysed as indicated by both the decrease of light scattering on the reaction mixture (30% hemolysis) and the release of hemoglobin in the mixture (20% hemolysis) (Table 8). The lysis also required Fe^{++}. In the absence of succinate and antimycin, Fe^{++} showed essentially no effect. The lower extent of hemolysis was observed with the assay of released hemoglobin. Possibly, the former method may give an overestimate since lysis of

Table 8. Hemolysis of human erythrocytes by mitochondria

Additions	A_{740} (whole medium)	A_{540} (supernatant)
None	1.824	0.073
A + S	1.882	0.068
A + S + Fe^{++}	1.278(29.9%)[a]	0.389(19.9%)[b]
Fe^{++}	1.872	0.100

Mitochondria (1.0 mg protein/ml) and 20 μl of human erythrocyte suspension (see the Materials and Methods) were incubated at 37 °C with components added as shown in the table with constant stirring. The abbreviations were the same as in Table 4. Total volume was 3.0 ml. After incubation for 16 h, either the reaction mixture was read at 740 nm or supernatant obtained after centrifuging at 3,000 g for 15 min was read at 540 nm. % hemolysis was caiculated against A_{740} of the medium with no additions[a] or against A_{540} of the solution of the completely lysed erythrocytes in 150 mM KCl and 50 mM HEPES-KOH, pH 7.4[b].

mitochomdria that may occur as well during the incubation will be an additional contribution to the decrease in light scattering at 740 nm.

DISCUSSION

In the present study, the respiratory chain-linked production of the reactive oxygen species was also confirmed in the isolated brain mitochondria. $O_2^{\cdot-}$ was produced when electron flow was supported by succinate, but with blockade of the flow by antimycin, its production of $O_2^{\cdot-}$ was markedly stimulated (Table 1 and Fig. 1). Under the same condition, H_2O_2 was also produced and again its rate of production was maximal when the inhibitor was added (Fig. 2). The H_2O_2 production was suppressed to 80% by cytochrome c and NBT which can interact with $O_2^{\cdot-}$ (Table. 2), indicating that $O_2^{\cdot-}$ was formed first and acted as a precursor for H_2O_2 via intra-mitochondrial SOD reaction. Among inhibitors used, only antimycin showed increased production of H_2O_2. Essentially no stimulation was observed with other inhibitors (Fig. 3). The rates of $O_2^{\cdot-}$ and H_2O_2 production were 0.87 and 0.25 nmoles/mg protein/min, respectively, which are comparable to those observed in rat heart mitochondria (Nohl *et al.*, 1978). Actual rate of the production of each species, however, would be much higher than the value detected in the medium. Nohl and Hegner (1978) reported that in rat heart mitochondria, 20% of $O_2^{\cdot-}$ produced escaped quenching by intra-mitochondrial SOD and the remainder was decomposed into H_2O_2 by the SOD. It was also estimated that of the H_2O_2 produced, only 20% escaped catalase and GSH peroxidase of the mitochondrial matrix and was released into the medium (Nohl & Jordan, 1980).

The oxyen species that survive the quenching were shown to attack mitochondrial and extra-mitochondrial structures as demonstrated by lipid peroxidation of mitochondrial membrane and inactivation of isocitrated dehydrogenase present in the medium, respectively (Tables 3 and 5). The toxic actions observed were maximal when both succinate and antimycin were present. In the damage observed, both $O_2^{\cdot-}$ and H_2O_2 are considered to be involved since the lipid peroxidation and enzyme inactivation were prevented by SOD or catalase (Table 4 and 6). But in their toxic effects, Fe^{++} was essentially required. Under the same condition, hemolysis of erythrocyte was also demonstrated indicating that the oxygen species are capable of causing cytotoxicity (Table 8).

It has been shown that by itself $O_2^{\cdot-}$ or H_2O_2 is not so reactive, rather they act as precursors more reactive species, most likely OH· (Fridovich, 1975; Kellogg & Fridovich, 1977; Weiss *et al.*, *1978*). In the presence of traces of iron or its salt, $O_2^{\cdot-}$ and H_2O_2 can react together *in vitro* to form OH·, which can attack and destroy almost all known biomolecules (Halliwell, *1978a, b;* McCord & Day, *1978*; Gutteridge

et al., 1970). It has been suggested that most, if not all, of toxic effects of $O_2^{-}\cdot$ are due to the formation of OH· from it *in vivo* (Fridovich, 1978; Halliwell 1978a, b; Deguiseppi & Fridovich, 1980). Consequently, the toxic effects observed in the present study are highly suggested to be mediated by OH· generated by so called iron-catalyzed Haber-Weiss reaction:

$$O_2^{-}\cdot + H_2O_2 \xrightarrow{Fe^{++}} OH\cdot + OH^{-} + O_2$$

In support of this, stimulation of ethylene production from methional was observed by Fe^{++} added into the medium (Table 7).

But Fe^{++} by itself caused lipid peroxidation of mitochondrial membranes even with no addition of succinate and antimycin (Table 3). It seems that this action of Fe^{++} was not related to the reactive oxygen species. Peroxidation of lipids of microsomes and other subcellular organelles including mitochondria was also observed to be promoted by Fe^{++} alone (Beloff-Chain *et al.*, 1965; Ottohenghi, 1959; McKnight *et al.*, 1965). In this case, the peroxidation was not inhibited by scavengers of $O_2^{-}\cdot$, H_2O_2 or OH·. Pederson and Aust (1975) have suggested that Fe^{++} catalyzes the lipid peroxidation by decomposing the pre-existing hydroperoxides to generate alkoxyl radicals. In the present study, MDA was also detected from freshly prepared mitochondria indicating the presence of hydroperoxides. Possibly, the observed peroxidation may be due to the interaction of Fe^{++} and the hydroperoxides.

The destructive actions of $O_2^{-}\cdot$ and H_2O_2 through the Haber-Weiss reaction have already been accepted as one of the mechanisms to explain the oxygen-induced tissue damage. Free Fe^{++} concentration of body fluids was found to be in micromolar range sufficient to promote the OH· formation from $O_2^{-}\cdot$ and H_2O_2 (Gutteridge *et at.*, 1981), and the role of Fe^{++} as a catalyst was also proved in the peroxidative processes *in vivo* (Rowley *et al.*, 1984). Therefore, once $O_2^{-}\cdot$ and subsequently H_2O_2 are formed, they are expected to generate OH·. Stimulated neutrophils produce $O_2^{-}\cdot$ and H_2O_2 at inflamed sites and the resulting OH· attacks the tissue components (Greenwald & Moy, 1980). This has been known as one of mechanisms of tissue damage mediated by neutrophils in inflammation (Fridovich, 1978; Fantone & Ward, 1982). In this respect, mitochondrial generation of $O_2^{-}\cdot$ and its toxic actions are expected to have a potential role as a mechanism of tissue damage.

The toxic effects demonstrated in this study may provide an explanation at molecular level for tissue damage in ischemic brain for which primarily disturbance in O_2 metabolism in mitochondria has been implicated to be responsible (Demopoulos *et al.*, 1979). In brain ischemia, evidence has been obtained that lipids of nerve cell membranes undergo degradation by free radical reactions presumably initiated by $O_2^{-}\cdot$ from mitochondria. It includes the appearance of increased level of MDA (Milvy *et al.*, 1973), destructive loss of polyunsaturated fatty acids (Demopoulos *et al.*, 1979) and consumption of a major CNS antioxidant, ascorbic acid (Flamm *et al.*, 1978). The enhanced production of $O_2^{-}\cdot$ by antimycin may be background supporting the increased generation of $O_2^{-}\cdot$ from mitochondria in hypoxic condition. According to this hypothesis (Demopoulos *et al.*, 1979 & 1980), in the presence of adequate O_2, elections are transferred enzymatically to O_2, reduce it into H_2O. But when O_2 is diminished abruptly as in ischemia, the capacity to accept the ongoing flow of electrons is decreased and electrons are piled up resulting in the reduction of all the factors of the chain. In this situation, electrons leak through possibly CoQ and reduce O_2 univalently to form $O_2^{-}\cdot$ (Boveris *et al.*, 1976; Cadenas *et al.*, 1977).

But one finding observed in this study does not seem to be consistent with the above explanation. As showing Fig. 3, any increase in H_2O_2 production was not demonstrated with the inhibitors tested except antimycin. If this hypothesis is true, blockade of any sites to O_2 side after CoQ should increase the univalent reduction of O_2. But it was not the case even in the O_2 -saturated medium. Thus, the results make it hard to expect that simple hypoxia *in vivo* could lead to increase the univalent reduction of O_2. An alternative mechanism may also exist as a source of $O_2^{-}\cdot$ to initiate the free radical damage of membrane lipids in this pathological condition. In hypoxic condition, xanthine oxidase was converted from pre-ex-

isting D type (NAD-reducing dehydrogenase) to O type (McCord & Roy, 1982). Both types oxidize hypoxanthine to uric acid but only latter can produce $O_2^{\cdot-}$. The substrate, hypoxanthine also accumulates during ischemia as a result of ATP catabolism (Granger & Parks, 1983). This "D-to-O conversion hypothesis" was supported in ischemic injuries of intestine (Parks *et al.*, 1982) and heart (Chambers *et al.*, 1983) since ischemic changes in the organs were limited by allopurinal and SOD. But the role of xanthine oxidase in brain ischemia is not studied and remains to be explored.

In the present study, $O_2^{\cdot-}$ production from mitochondria was carried in the O_2 -saturated medium. With replacement of air, the rate was decreased to 30% (data not shown). Thus, the toxic actions of $O_2^{\cdot-}$ observed may also provide a biochemical mechanism of tissue injury in O_2 poisoning.

The result observed in this study may also support degenerative changes that lead to cellular aging processes. Antimycin was used to demonstrate the capacity of mitochondria to generate $O_2^{\cdot-}$. But univalent reduction of O_2 can occur during the normal electron flow through the respiratory chain. It was estimated that about 5% of O_2 consumed in mitochondria was linked with the reduction to $O_2^{\cdot-}$ (Fridovich, 1979). Although most of O_2 and H_2O_2 produced *in vivo* is removed by scavenging enzymes, the mitochondrial membrane as well as other extra-mitochondrial structures are constantly subjected to the action of the oxygen species and may undergo progressive destructive changes. Increasing degree of peroxidative damage in mitochondrial membranes were observed with a function of age (Nohl and Hegner, 1978). The structural of features of mitochondrial membranes were closely related to decreased functional activities of many of respiratory enzymes (Nohl *et al.*, 1978). The involvement of mitochondria in these aging processes may be further supported by higher rate of $O_2^{\cdot-}$ and H_2O_2 production from mitochondria of aged rat than young rat (Nohl & Hegner, 1978; Nohl *et al.*, 1978).

REFERENCES

Allen AO, Hochanadel CJ, Ghormley JA and Davis TW: Decomposition of water and aqueous solutions under mixed fast neutron and gamma radiation. J Phys Chem 56:575-586, 1952

Baehner R: Subcellular distribution of nitroblue tetrazolium reductase (NBT-R) in human polymorphonuclear leukocytes (PMN). J Lab Clin Med 86: 785-792. 1975

Baehner RL, Boxer LA and Davis J: The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes. Blood 48:309-313, 1976

Baner JD (ed): Clinical Laboratory Methods. The CV Mosby Company, St. Louis Toronto London, p 219, 1982

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

Beloff-Chain A, Serlupi-Crescenzi G, Catanzaro R, Venettacci D and Belliano M: Influence of iron on oxidation of reduced nicotinamide-adeninedinucleotide phosphate in rat liver microsomes. Biochim Biophys Acta 97: 416-421, 1965

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

Boveris A and Chance B: The mitochondrial generation of hydrogen peroxide. Biochem J 134:707-176, 1973

Boreris A, Cadenas E and Stoppani AOM: Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochem J 156:436-444, 4976

Boveris A, Oshino N and Chance B: The cellular production of hydrogen peroxide. Biochem J 128: 617-630, 1972

Cadenas E, Boveris A, Ragan CI and Stoppani, AOM: Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. Arch Biochem Biophys 180: 248-257, 1977

Chambers DE, Parks DA, Patterson G, Yoshida S, Burton K, Parmley LF, McCord JM and Downey J

M: Role of oxygen-derived radicals is myocardial ischemia. Fed Proc 42: 1093, 1983

Demopoulos HB, Flamm ES, Seligman ML, Mitamura JA and Ransohoff J: Membrane pertubation in central nervous system injury: Theoretical basis for free radical damge and a review of the experimental data. *In Neural Trauma* (eds. J Popp, RS Bourke, LR Nelson and HK Kimelberg) Raven Press New York, pp 63-79, 1979

Diguiseppi J and Fridovich I: Ethylene from 2-keto-4-thiomethyl butyric acid: The Haber-Weiss reaction. Arch Biochem Biophys 205:323-329, 1980

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

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

Fridovich I: Superoxide dismutase. Ann Rev Biochem 44:147-159, 1975

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

Fridovidch I: Hypoxia and oxygen toxicity. *In Advences in Neurology* (ed. S Fahn). Raven Press New York, pp 255-259, 1979

Goldberg B and Stern A: The role of the superoxide anion as a toxic species in the erythrocyte. Arch Biochem Biophys 178:218-225, 1977

Granger DN and Parks DA: Role of oxygen radicals in the pathogenesis of intestinal ischemia *In A Symposium on Oxygen Radicals and The Microcirculation.* The Physiologist 26:159-165, 1983

Greenwald RA and Moy WW: Effect of oxygen-derived free radicals on hyaluronic acid. Arthritis Rheumat 23:455-463, 1980

Gutteridge MC, Richmond R and Halliwell B: Inhibition of the iron-catalyzed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferroxamine. Biochem J 184:469-472, 1979

Gutteridge JMC, Rowley DA and Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts: Detection of free iron in biological systems by using bleomycin-dependent degradation of DNA. Biochem J 199:263-265, 1981

Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates: Is it a mechanism for hydroxyl radical production in biological system? FEBS Lett 92:321-326, 1978a

Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. FEBS Lett 96:238-242, 1978b

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

Kim HW, Kim MS, Chung MH and Park CW: Evidence for hydroxyl radical and singlet oxygen on calcium binding inhibition of sarcoplasmic reticulum. Environ Mutagens and Carcinogens 4:1-12, 1984

Lesko SA, Lorentzem RJ and Ts'o POP: Role of superoxide in deoxyribonucleic acid strand scission. Biochemistry 19:3023-3028, 1980

Loschen G, Flohe L and Chance B: Respiratory chain-linked H_2O_2 production in pigeon heart mitochondria. FEBS Lett 18:261-264, 1971

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

McCord JM and Day ED Jr: Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. FEBS Lett 86:139-142, 1978

McCord JM and Roy RS: The pathophysiology of superoxide: role in inflammation and ischemia. Can J Physiol 60:1346-1347, 1982

Mcknight RC, Hunter Jr FE and Oehlert WH: Mitochondrial membrane ghosts produced by lipid peroxidation induced by ferrous ion. J Biol Chem 240:3439-3446, 1965

Milvy P, Kakari S, Campbell JB and Demopoulos HB: Paramagnetic species and radical products in cat spinal cord. Ann NY Acad Sci 222:1102-1111, 1973

Monboisse JC, Braquet P, Randoux A and Borel JP: Nonenzymatic degradation of acid-soluble calf skin col-

lagen by superoxide ion: protective effect of flavonoids. Biochem Pharmacol 32:53-58, 1983
Nohl H, Breuninger V and Hegner D: Influence of mitochondrial radical formation on energy-linked respiration. Eur J Biochem 90:385-390, 1978
Nohl H and Jordan W: The metabolic fate of mitochondrial hydrogen peroxide. Eur J Bichem 111:203-210, 1980
Nohl H and Hegner D: Do mitochondria produce oxygen radical *in vivo*? Eur J Biochem 82:563-567, 1978
Ottohenghi A: Interaction of ascorbic acid and mitochondrial lipid. Arch Biochem Biophys 79:355-363, 1959
Parks DA, Bulkbey GB, Granger DN, Hamilton SR and McCord JM: Ischemic injury in the cat small intestine: role of superoxide radicals. Gastroenterology 82:9-15, 1982
Placer IA, Cushman LL and Johnson BC: Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal Biochem 16:359-364, 1966
Rosen H and Klebanoff SJ: Role of iron and ethylenediamine-tetraacetic acid in the bacterial activity of a superoxide anion-generationg system. Arch Biochem Biophys 208:512-519, 1981
Rowley D, Gutteridge JMC, Blake D, Farr M and Halliwell B: Lipid peroxidation in rheumatoid arthritis: thiobarbituric acid-reactive material and catalytic iron salts in synovial fluid from rheumatoid patients. Clinical Science 66:691-695, 1984
Schneider WC and Hogeboom GH: Intracellular distribution of enzymes: V. Further studies on the disbtribution of cytochrome C in rat liver. J Biol Chem 183:123-128, 1950
Weiss SJ, Rustagi PK and LoBuglio AF: Human granulocyte generation of hydroxyl radical. J Exp Med 147:316-323, 1978

=국문초록=

흰쥐 뇌 미토콘드리아에 의한 superoxide radical의 생성과 이 radical이 미토콘드리아 및 미토콘드리아 외 물질에 대한 독작용과 그 기전에 관한 연구

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흰쥐 뇌 미토콘드리아에 의한 $O_2^-\cdot$의 생성과 이 radical의 유해작용 및 그 작용기전을 알아보기 위하여 본실험을 수행하였다.

Succinate와 antimycin존재하에서 미토콘드리아는 $O_2^-\cdot$을 생성하였으며 이는 SOD-inhibitable NBT환원으로 확인되었다. 동일 조건에서 H_2O_2는 일차생성물인 $O_2^-\cdot$의 dismutation으로 생성됨을 알수 있었다.

상기조건에서 미토콘드리아의 막지질이 파괴되었고 반응액에 첨가된 isocitrate dehydrogenase와 적혈구에 각각 불활성화와 용혈이 초래되었다. 이같은 작용은 Fe^{++}이 있을때만 관찰 되었다. 그리고 독작용은 superoxide dismutase 혹은 castalase에 의해서 억제되었다. 또한 methional을 첨가하였을 때 ethylene이 생성되었으며 그 생성은 Fe^{++}에 의하여 현저히 증가하였다. Ethylene 생성 역시 상기 효소에 의하여 억제되었다.

따라서 미토콘드리아에서 발생된 $O_2^-\cdot$은 거대분자 및 세포에 독성을 나타낼수 있으며 이같은 작용은 Fe^{++}의 촉매작용에 의한 $O_2^-\cdot$와 H_2O_2의 상호작용으로 발생되는 $OH\cdot$에 의한것으로 추측되었다.

이상의 결과는 미토콘드리아가 유독성 산소 radical을 발생하므로 조직손상을 시킬 수 있다는 가능성을 시사하는 증거라고 생각되었다.