ULTRACYTOCHEMICAL LOCALIZATION OF SUPEROXIDE
DISMUTASE ACTIVITY IN RABBIT ALVEOLAR MACRO-
PHAGES: A COMPARATIVE STUDY USING KO_2 OR
XANTHINE-XANTHINE OXIDASE AS O_2^-
GENERATING SYSTEM

MASARU KURODA

*Department of Anatomy, Toho University School of Medicine,
Ohmorinishi, Tokyo 143*

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An attempt has been made to obtain evidence for the localization of superoxide dismutase (SOD) activity in rabbit alveolar macrophages ($\text{AM}\phi$). SOD localization was investigated with two different superoxide (O_2^-)-generating systems, the potassium superoxide (KO_2) and the xanthine-xanthine oxidase system. The hydrogen peroxide (H_2O_2) generation site, which indicates SOD activity, was electron cytochemically investigated by using the cerium method introduced by Briggs *et al.*

In the KO_2 system, the formation of cerium perhydroxide serves as an indication of SOD activity, and its presence was confirmed throughout the cytoplasm and mitochondria, but not in the nucleus and granules. Under the action of diethyldithiocarbamate and potassium cyanide, which act as Cu,Zn-SOD inhibitors, it was found that the cytoplasmic SOD activity was virtually completely suppressed, and that complete inhibition of SOD activity occurred in the presence of catalase (a scavenger of H_2O_2) or in the absence of KO_2 . In the xanthine-xanthine oxidase system, cerium deposits were observed in the cytoplasm, but rarely in the mitochondria. Occasionally some reaction product was seen in the nucleus and granules.

From the results, it is concluded that KO_2 system is an excellent method for investigation of the localization of SOD activity.

An important role played by phagocytes such as polymorphonuclear leukocytes and macrophages ($\text{M}\phi$) in the self-defense mechanism of living organisms is the production of various oxygen intermediates (O_2^- , H_2O_2 , $\cdot\text{OH}$, $^1\text{O}_2$), which are bactericidal agents, during phagocytosis and when the plasma membrane is activated by soluble agents (4, 14, 19). However, while these active oxygens exhibit a beneficial bactericidal effect, excessive generation of these active oxygen species has an injurious effect on the organism in that it may give rise to inflammation and lipid peroxidation (5, 10, 22). The defense mechanisms that protect the cell from excess production of active oxygens include SOD (21), catalase (20) and glutathione peroxidase (11, 18). SOD, in particular, is an important enzyme in the front-line of this defense.

Oxygen metabolism in the phagocytic or chemotactic stimulation process begins

with activation of NAD(P)H oxidase, which locates in the plasma membrane (3, 9, 12). With NAD(P)H as the electron donor, molecular oxygen is reduced to O_2^- by one electron reduction (3, 13), and the O_2^- is then converted to H_2O_2 by dismutation reaction. While this dismutation reaction proceeds spontaneously, its rate is accelerated by SOD about 10,000 times. It has been reported that there exist three types of SOD having Cu^{2+} - Zn^{2+} , Mn^{3+} and Fe^{3+} in each active center (7). Biochemical studies suggest that, in vertebrates, Cu, Zn-SOD is present in the cytoplasm and Mn-SOD in the mitochondria. However, a morphological technique has not been established to demonstrate SOD activity. In 1980, Hirai *et al.* tried to substantiate morphologically the localization of SOD activity in rat alveolar macrophages (AM ϕ) by using xanthine and xanthine oxidase as O_2^- -generating system (9). They demonstrated SOD activity in the cytoplasmic matrix, but not in the mitochondria. However, the author feels that the problem with this method may in part be associated with the O_2^- -generating system, and, on this assumption, the author has attempted to demonstrate the localization of SOD activity in rabbit AM ϕ by using a new O_2^- -generating system consisting of KO_2 to assure the O_2^- supply.

MATERIALS AND METHODS

1. Rabbit AM ϕ

Male New Zealand white rabbits weighing approximately 2.5 kg were killed with sodium pentobarbital (50 mg/kg) administered intravenously. Immediately after death from respiratory arrest, the trachea was clamped, and the heart and lungs were removed. The trachea was then cannulated with a polyethylene catheter and the lungs were lavaged with four 25 ml aliquots of 0.1 M phosphate-buffered saline (pH 7.4) at 4°C. The cell pellets were obtained from lung lavages by centrifugation at $250 \times g$ for 10 min at 4°C. The AM ϕ routinely constituted of more than 95% of the lavaged cells from Wright-stained differential counts of cytocentrifuged preparations. Then the AM ϕ were fixed in 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) containing 5% sucrose for 10 min at 4°C. The fixed cell materials were washed twice with 0.1 M tris-maleate buffer (pH 7.4) containing 7% sucrose at 4°C.

2. O_2^- -generating systems

KO_2 system: KO_2 readily decomposes in a protonic solvent such as water, but is sparingly soluble in aprotic solvents. Therefore, 5.0 mM KO_2 (Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethylsulfoxide (DMSO) containing 0.5% dicyclohexyl-18-crown-6 (crown-ether; Nakarai Chemicals Ltd., Kyoto, Japan), which was employed in order to increase the solubility of KO_2 (15).

After weak fixation and wash, the AM ϕ were suspended in culture dishes at a concentration of 5×10^6 /ml-the reaction mixture consisting of 1.0 mM $CeCl_3$, 10.0 mM 3-amino-1,2,4-triazole (AT; Sigma Chemical Co.) and 7% sucrose in 0.1 M tris-maleate buffer (pH 7.4). The AT was added to suppress the endogenous catalase activity. The reaction mixture with the AM ϕ was continually stirred with a magnetic stirrer and the DMSO solution of KO_2 , adjusted as described above, was

infused at a flow rate of 20 $\mu\text{l}/12$ sec into 5 ml of the reaction mixture. The infusion was continued for 20 min at 37°C. The reaction mechanism is as follows;

Xanthine-xanthine oxidase system: The cytochemical method by Hirai *et al.* was roughly followed (9). After weak fixation and wash, the AM ϕ were incubated in the reaction mixture outlined below at a concentration of $5 \times 10^6/\text{ml}$ for 60 min at 37°C. The reaction mixture used consisted of 0.1 M Tris-maleate buffer (pH 7.4) containing 0.1 mM sodium xanthine (Sigma Chemical Co.), 0.0025 U/ml xanthine oxidase (grade I; Sigma Chemical Co.), 1.0 mM CeCl_3 , 10.0 mM AT and 7% sucrose. The reaction mechanism is as follows;

3. Control and other experiments

As a control for the KO_2 system, an experiment was attempted without adding KO_2 . For this purpose, DMSO containing 0.5% crown-ether, without KO_2 , was infused into the reaction mixture. For the xanthine-xanthine oxidase system, an experiment was conducted without any addition of xanthine or of xanthine oxidase to the reaction mixture.

For both systems, the reaction was attempted after fixing the AM ϕ for 30 min with 2% glutaraldehyde or heating them for 10 min at 100°C.

2.0 mM potassium cyanide (KCN) or 10.0 mM diethyldithiocarbamate (DDC; Sigma Chemical Co.), both fairly specific Cu,Zn-SOD inhibitors, were introduced into the reaction mixture of both systems to study their effects.

Catalase (Sigma Chemical Co.), which might scavenge H_2O_2 , was added at a dose of 1.0 mg/ml into the reaction mixture for both systems to study its effect.

4. Preparations for electron microscopy

To investigate the localization of SOD activity, the cerium method, which is a modified Briggs' cytochemical method (2), was used.

For both KO_2 and xanthine-xanthine oxidase system, the AM ϕ , after the reaction had reached completion, were washed twice with 0.1 M Tris-maleate buffer (pH 7.4) at 4°C, with subsequent fixation with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 60 min at 4°C. After subsequent washing with 0.1 M sodium cacodylate buffer (pH 6.0) at 4°C, the AM ϕ were postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.3) for 60 min at 4°C.

The following steps were carried out according to routine procedures. The

samples (fixed AM ϕ) were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were prepared with a Porter-Blum MT-2 type ultramicrotome with glass knives. Sections were routinely stained with uranyl acetate and lead citrate and examined under a Hitachi H-600 electron microscope operated at 75 kV.

5. Chemiluminescence in the KO₂ and xanthine-xanthine oxidase system

In this part of the experiment, the intensity of chemiluminescence was measured on a time basis for the KO₂ and xanthine-xanthine oxidase system used as the O₂⁻ suppliers, with a TD-4000 model Lumiphotometer (Labo Science Co., Ltd., Tokyo, Japan). To amplify the chemiluminescence signals, 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) was used.

For the chemiluminescence measurements of the KO₂ system, a KO₂ solution was injected into 0.1 M Tris-maleate buffer (500 μ l; pH 7.4) containing 0.2 mM luminol (Sigma Chemical Co.) under the same conditions as above at a flow rate of 2 μ l/12 sec with the concentration being 5.0 mM KO₂. The chemiluminescence measurements were then made at intervals of 10 sec each. Also measured was the luminol-mediated chemiluminescence, with 1 mg/ml catalase being added to 0.1 M Tris-maleate buffer.

Next, the chemiluminescence intensity was measured on a time basis for the xanthine-xanthine oxidase system under the same experimental conditions as above, that is, by the addition of 0.2 mM luminol to 0.1 M tris-maleate buffer (500 μ l; pH 7.4) containing 0.1 mM xanthine and 0.0025 U/ml xanthine oxidase, respectively. Also measured was the luminol-mediated chemiluminescence, with the addition of 1 mg/ml catalase.

The chemiluminescence measurements were conducted at a temperature of 37°C.

RESULTS

1. SOD activity in AM ϕ

KO₂ system: Deposits of cerium perhydroxide, a compound indicating the localization of SOD, were observed to be scattered throughout the cytoplasm. Though the reaction product was in part difficult to recognize due to its overlapping with the ribosomes, it was possible to distinguish it by virtue of its clear difference in density and size. The reaction product was also found in the mitochondria (Figs.

FIG. 1. Rabbit alveolar macrophage for the KO₂ system. Deposits of cerium perhydroxide are scattered throughout the cytoplasm. The cristae mitochondriales appear damaged, and some are difficult to identify as mitochondria. However, reaction products can be seen in those that are identifiable as mitochondria (arrowheads). There are no cerium deposits in the granules. $\times 8,900$

FIG. 2. Magnified image of Fig. 1. Cerium deposits are seen in the cytoplasm and mitochondria. $\times 19,000$

FIG. 3. A higher magnification of rabbit alveolar macrophage for the KO₂ system. Deposits of cerium perhydroxide can be observed in the cytoplasm and mitochondrion. They are not detectable in the nucleus. In the mitochondrion, the deposits of reaction product are located in the matrix, intracristal and intermembrane spaces and on the inner membrane. $\times 52,000$

1, 2). SOD activity was detected only in the cytoplasm and in some mitochondria. However, there was no evidence of SOD activity in the nucleus or in the organelles others than the mitochondria. Detailed observation of the mitochondrial SOD activity indicated that cerium deposits were present in the matrix, intracristal and intermembrane spaces, and on the inner membrane (Fig. 3).

Xanthine-xanthine oxidase system: SOD activity was found in the cytoplasm, but rarely in the mitochondria (Fig. 4). However, occasionally some reaction

product was observed to be deposited in the nucleus and granules, and, only in these cases, it would often be possible to observe the reaction product also in the mitochondria (Figs. 5, 6). It was also frequently possible to observe clusters of cerium deposits outside the cells.

2. Control and other experiments

In the KO_2 system, it was not possible to detect any reaction product whatever in the $\text{AM}\phi$ after infusing DMSO containing 0.5% crown-ether without the addition of KO_2 (Fig. 7). There was no morphological change in the $\text{AM}\phi$. Thus, it may be concluded that crown-ether and DMSO have no effect on the $\text{AM}\phi$ in this experimental conditions. Similarly, in the xanthine-xanthine oxidase system, there was no evidence of a reaction product in the $\text{AM}\phi$ without the addition of xanthine or xanthine oxidase.

In both systems, there was no evidence of any cerium deposits in the $\text{AM}\phi$ in the reaction after fixing the $\text{AM}\phi$ with 2% glutaraldehyde (Fig. 8) or heating for 10 min at 100°C .

In the KO_2 system, cytoplasmic SOD activity was virtually completely suppressed by the addition of 2.0 mM KCN or 10.0 mM DDC. It was, however, not totally suppressed. There was almost no evidence of any effect on the mitochondrial cerium perhydroxide formation (Fig. 9). In the xanthine-xanthine oxidase system, the reaction product in the cytoplasm was completely suppressed by the addition of 2.0 mM KCN or 10.0 mM DDC. However, occasionally cerium deposits were found in the nucleus.

For both systems, there was no observational evidence of any cerium deposits in the $\text{AM}\phi$ following the addition of 1 mg/ml catalase to the reaction mixture (Fig. 10). The results are presented in Table 1.

3. Chemiluminescence for the KO_2 and xanthine-xanthine oxidase system

Immediately after injecting the KO_2 solution, a resultant increase in the intensity of chemiluminescence was detected at each injection point, with a fairly stable chemiluminescence emission being obtained within the measurement time. However, the chemiluminescence intensity by the addition of catalase tended to show a moderate attenuation, but the effect observed was not very great (Fig. 11).

For the xanthine-xanthine oxidase system, it was discovered that the light intensity would suddenly weaken very sharply at about 30 sec after the start of the measurement, with the chemiluminescence signal remaining stable for some 10 min

FIG. 4. Rabbit alveolar macrophage for the xanthine-xanthine oxidase system. Cerium deposits can be observed in the cytoplasm, but not in the mitochondrion. There are no cerium deposits in the nucleus. $\times 25,000$

FIG. 5. Rabbit alveolar macrophage for the xanthine-xanthine oxidase system. Cerium deposits can be seen throughout the cytoplasm, on the nuclear membrane, in the nucleus and the granules. $\times 14,000$

FIG. 6. Rabbit alveolar macrophage for the xanthine-xanthine oxidase system. Cerium deposits are seen in the cytoplasm and mitochondrion. They are also detectable in the nucleus. $\times 22,000$

FIG. 7. Rabbit alveolar macrophage with infusing of DMSO containing 0.5% crown-ether in the absence of KO_2 . No reaction product is observed in the cell. $\times 9,200$

FIG. 8. Rabbit alveolar macrophage for the KO_2 system with the reaction performed after fixation with 2% glutaraldehyde. Cerium deposits due to the spontaneous dismutation reaction can be seen partly outside the cell, but are not detectable in the cell. $\times 7,700$

FIG. 9. Rabbit alveolar macrophage for the KO_2 system with the addition of 10.0 mM DDC to the reaction mixture. It can be clearly recognized that the cerium deposits in the cytoplasm have been eliminated without any observable effect for cerium deposits in the mitochondria (arrowheads). $\times 36,000$

FIG. 10. Rabbit alveolar macrophage for the KO_2 system with the addition of 1 mg/ml catalase to the reaction mixture. There is no evidence of cerium deposits in the cytoplasm and mitochondria. $\times 8,100$

TABLE 1. Comparison of cerium perhydroxide formation sites for rabbit alveolar macrophages in the KO_2 and xanthine-xanthine oxidase system under various conditions

	Cytoplasm	Mitochondria	Nucleus	Diffusion outside AM ϕ
KO_2 system				
Ce+ KO_2	+	$\pm \sim +$	—	+
10 mM DDC	$\pm$	$\pm \sim +$	—	+
2 mM KCN	$\pm$	$\pm \sim +$	—	+
Catalase	—	—	—	—
Fixed 2% Glu	—	—	—	+
Heated at 100°C	—	—	—	+
Ce only (KO_2 free)	—	—	—	—
Xan-XOD system				
Ce+Xan+XOD	+	$\pm$	$\pm \sim +$	++
10 mM DDC	—	$\pm$	$\pm \sim +$	++
2 mM KCN	—	$\pm$	$\pm \sim +$	++
Catalase	—	—	—	—
Fixed 2% Glu	—	—	—	++
Heated at 100°C	—	—	—	++
Ce+Xan	—	—	—	—
Ce+XOD	—	—	—	—

Ce, cerium chloride; Xan, sodium xanthine; XOD, xanthine oxidase; Glu, glutaraldehyde

—, $\pm$, +, ++ denote negative, negligible, moderate and strong intensities of SOD activity, respectively.

FIG. 11. Chemiluminescence response in the KO_2 system. Chemiluminescence intensity was measured every 10 seconds by injecting, at 12-second intervals, a 5.0 mM KO_2 solution (2 μ l) to 0.1 M Tris-maleate buffer (500 μ l; pH 7.4, 37°C) containing 0.2 mM luminol (●—●). The light intensity by the addition of 1 mg/ml catalase to the buffer was also measured (○—○). $\blacktriangle$ indicates each injection point of a KO_2 solution.

FIG. 12. Time course of light emission in the xanthine-xanthine oxidase system. Light intensity measurement obtained with 0.1 M Tris-maleate buffer (pH 7.4, 37°C) containing 0.1 mM sodium xanthine, 0.0025 U/ml xanthine oxidase and 0.2 mM luminol (●—●), and with the addition of 1 mg/ml catalase to this buffer (○—○).

after this. This signal would then subside some 30 min later to a very faint intensity of chemiluminescence emission. Comparison between the chemiluminescence intensity with and without catalase addition revealed that the light intensity would drop at any measurement time, with no chemiluminescence signal being detectable after 30 min (Fig. 12).

DISCUSSION

In this study, the author morphologically demonstrated that SOD activity was localized in the cytoplasm and mitochondria of rabbit AM ϕ by using KO_2 as O_2^- supplier. These results are in perfect agreement with biochemical investigation of the SOD localization (17). Detailed investigation of the localization of mitochondrial SOD has shown that cerium deposits are detectable in the matrix, intracristal and intermembrane spaces and on the inner membrane. Peeters-Joris *et al.* have investigated the intracellular distribution of SOD in the rat liver (16), and their results concerning the localization of SOD activity in the mitochondria are virtually identical with those obtained by the author.

On the basis of the following observations:

- 1) No evidence of reaction products forming in the AM ϕ without the addition of KO_2 ;
- 2) Virtually complete suppression of cytoplasmic SOD activity by the addition of KCN or DDC to the reaction mixture;
- 3) No cerium deposits detectable when making addition of catalase to the reaction mixture;
- 4) No evidence of cerium precipitation in the reaction after a fixation or heating of the AM ϕ ;

it can be concluded that the cerium deposits observed in the cytoplasm and mitochondria are not artifacts but reaction products resulting from intracellular metabolism.

SOD localization has been studied with two O_2^- -generating systems, the KO_2 system and the xanthine-xanthine oxidase system. The scope of this study was extended to include also the question of which of these supply systems is more effective. With the KO_2 system, SOD activity was established in the mitochondria, though this does not imply that it was found in all mitochondria. In this study, it was not possible to give a clear-cut account as to the reason why reaction products were not found in all mitochondria. In the xanthine-xanthine oxidase system, cerium deposits were observed in the mitochondria, but this was invariably accompanied by cerium deposits in the nucleus and granules. And it was also frequently possible to observe deposits of cerium perhydroxide outside the cell. Thus, the experimental results for the two O_2^- -generating systems indicate that there is a significant difference between them. To elucidate the reasons for this difference, the luminol-mediated chemiluminescence intensities associated with the two O_2^- -generating systems were measured, and it was found that, for the KO_2 system, a relatively constant chemiluminescence emission was obtained. Though there was weak light emission due to H_2O_2 believed to form as a result of the spontaneous dismutation of O_2^- , it is concluded that this is a stable O_2^- supplier. However, with the xanthine-xanthine oxidase system, it was found that the light intensity would fade dramatically 30 min after the start of measurement and that the chemiluminescence would decline at any point in time following the addition of catalase. This suggests that, while the oxygen intermediates are scavenged by uric acid (1), formed as a result of the xanthine and xanthine oxidase reaction, and that O_2^- is also formed with the O_2^- supplier, it may also be possible for H_2O_2 to form at the same time (6, 8). It is thus inferred that the xanthine-xanthine oxidase system is not a pure O_2^- -generating system. Moreover, with the H_2O_2 formed by the spontaneous dismutation reaction, this O_2^- -generating system represents a low-price experimental system. The uniform and constant supply of O_2^- during the reaction time is considered of the greatest importance in investigation of the localization of SOD activity. It has, thus, been established that the KO_2 system is a very practical and useful O_2^- -generating system that has attracted considerable attention in this respect.

CONCLUSION

Investigations of the localization of SOD activity in rabbit AM ϕ using KO_2 for the O_2^- supply have indicated that SOD activity can be detected in the cytoplasm and mitochondria. This has been the first clear morphological evidence of mitochondrial SOD.

SOD activity was also studied by using two O_2^- -generating systems, that is KO_2 and xanthine-xanthine oxidase system. The results suggest that KO_2 is the superior of the two methods in that it represents a more stable supply source for O_2^- . However, there were also some mitochondria without any detectable SOD activity, and it would seem a desirable future objective to perfect the morphological method for substantiating SOD activity by further improving the amount and method of KO_2 supply.

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