SUPEROXIDE DISMUTASE OF BOVINE AND FROG ROD OUTER SEGMENTS

MICHAEL O. HALL

Jules Stein Eye Institute, The Center for the Health Sciences, University of California, Los Angeles, CA 90024.

D.O. HALL

University of London King's College, Department of Plant Sciences, 68 Half Moon Lane, London SE24 9JF.

Received August 10, 1975

SUMMARY Bovine rod outer segments (ROS) contain soluble superoxide dismutase (SOD) which from cyanide sensitivity and electrophoretic mobility appears identical to Cu-Zn SOD of erythrocytes. Enzyme activity of ROS extracts is 200-400 times as much as remainder of retina. Frog ROS also contains a cyanide-sensitive SOD which is not due to erythrocyte contamination since the retina is avascular. SOD in ROS may inhibit free radical oxidation of polyunsaturated fatty acids. In light, high oxygen concentrations in developing retina may activate lipid peroxidation leading to retrolental fibroplasia. High concentrations of ascorbic acid in the retina may act as a protective mechanism against superoxide.

INTRODUCTION Superoxide dismutase has been identified in numerous plant and animal systems(1), and it has been proposed that this enzyme constitutes the primary defense against the cytotoxic superoxide radical, produced by the univalent reduction of oxygen. It has been known for some years that retinas of premature infants are damaged by exposure to high oxygen tension, which results in the blinding condition known as retrolental fibroplasia. The inner portions of the retinas of mammals are well vascularized, while the photoreceptor cell outer segments receive nutrients by diffusion from the choriocapillaris through the pigment epithelium. Thus oxygen is present throughout this neural tissue. Recently it has been shown that exposure of frog retinas to light results in the accumulation of free radical oxidation products in the lipid phase of photoreceptor cell outer segments(2). Since the lipids of the rod outer segments (ROS) contain a high level of polyunsaturated fatty acids(3)(particularly docosahexaenoic acid) which appear to have a functional significance(4), it seemed likely that a mechanism should be

Copyright © 1975 by Academic Press, Inc.
All rights of reproduction in any form reserved.

present in the ROS to prevent free radical oxidation of these polyunsaturated fatty acids. Superoxide dismutase (SOD) would provide such a protective mechanism. In light of this, and the recent demonstration(5) that the entire retina contains SOD, we examined the ROS of cows and frogs for the presence of this enzyme.

METHODS Bovine eyes were obtained from a local slaughterhouse and were kept on ice, in the dark, until dissection, which was carried out under dim red light, until the dialysis step, at which point the preparation was exposed to light. The anterior segment of the eye was removed and the vitreous was poured out. The eyecup was rinsed with 0.6% NaCl in 0.04M potassium phosphate buffer, pH 7.8 (PBS). The retinas were dissected from the eyecup, rinsed gently in cold PBS, and dropped into a centrifuge tube containing cold PBS. After dissection was completed, PBS was added to the tube so that the volume of solution was twice the volume of the retinas. The tube was capped, gently inverted twenty times to liberate the ROS, and centrifuged in the cold for 5 min at 50g. The supernatant, which contains the ROS, was removed, two volumes of PBS were added to the pellet and the procedure was repeated. The combined supernatants were centrifuged at 50g for 10 min to remove retinal debris. The supernatant was centrifuged at 2000g for 10 min. The precipitate of ROS was washed a further three times by centrifugation in PBS to remove erythrocytes, which remain in the supernatant (J. Heller, personal communication). The pellet of ROS was diluted to 5 ml with PBS and homogenized in a glass tube with a loose fitting teflon pestle (0.5 mm clearance). This fragments the ROS while causing minimal breakage of residual erythrocytes. The ROS fragments and erythrocytes were removed by centrifugation at 35,000g for 1 hr and the supernatant was dialyzed overnight against 0.05M potassium phosphate buffer, pH 7.8 (0.5KP7.8). The retinal residue remaining after detachment of the ROS, was homogenized in PBS in a loose fitting teflon-glass homogenizer. After centrifuging at 35,000g for 1 hr, the supernatant was dialyzed against 0.5KP7.8 overnight. ROS and retinal extracts were prepared

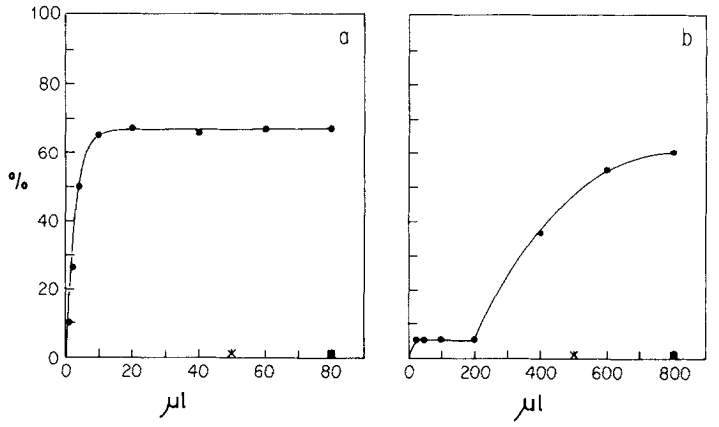

Figure 1. Inhibition of photoreduction of NBT by (a) extract of bovine rod outer segments and (b) extract of bovine retina. SOD activity was measured spectrophotometrically in the ROS and retinal extracts by the photoreduction of nitro blue tetrazolium (NBT) in a total volume of 1.0 ml(7). When the extracts were boiled (x), or when 10 µl of 0.2M KCN was added to the reaction mixture (■), no inhibition of NBT reduction occurred.

from dark-adapted frog eyes by the technique described for bovine eyes. Protein in the dialyzed ROS and retinal extracts was determined by the method of Lowry, et al.(6). SOD activity was localized on polyacrylamide disc gels(7) prepared according to Davis(8). Protein was stained by immersing the gels in 0.44% amido black, followed by electrophoretic destaining in 10% acetic acid.

RESULTS Figure 1a shows the effect of adding increasing amounts of bovine ROS extract to the NBT photoreduction system. The maximal inhibition which could be achieved with this crude extract is about 68%, indicating an oxygen independent route of electron transfer from riboflavin to NBT(7). Maximum inhibition is reached with 10-20 µl of bovine ROS extract, which is equivalent to 22.6-45.2 µg of protein. Figure 1b shows the inhibition of photoreduction of NBT with the bovine retina extract. In this case, maximal inhibition is only achieved upon adding 800 µl of the retinal extract, which is equivalent to 10.24 mg of protein. In both systems, the inhibition of photoreduction was completely released by boiling the dialyzed extract, or upon addition to the reaction mixture of 10 µl of 0.2M KCN buffered to pH 7.8.

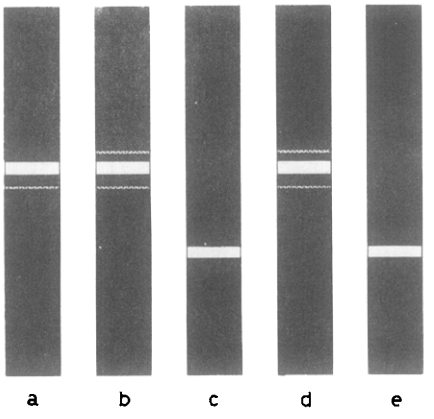

Figure 2. Polyacrylamide disc gel electrophoresis of soluble superoxide dismutases. (a) purified bovine erythrocyte SOD; (b) bovine ROS extract; (c) frog ROS extract; (d) bovine retina extract; (e) frog retina extract. Gels were stained for SOD activity.

A curious phenomenon was repeatedly observed when studying the bovine retinal extract. As shown in Fig. 1b, a small amount (5-10%) of inhibition is observed upon adding 25 µl of dialyzed retinal extract. No further inhibition can be induced until more than 200 µl of extract is added to the reaction mixture. This suggests that the inhibition observed when less than 200 µl of retinal extract is added to the reaction mixture is not due to SOD activity, but rather to some non-specific reduction of NBT.

Dialyzed ROS and retinal extracts from frogs gave spurious results in the NBT photoreduction assay. The maximum inhibition which could be achieved was between 30% and 40% of the control. Addition of increasing amounts of ROS or retinal extracts resulted in decreasing inhibition, indicating that some endogenous component of the extracts was interfering with the reduction of NBT in this system.

Figure 2 shows the polyacrylamide disc gel patterns of SOD activity from bovine and frog ROS and retinal extracts, as well as purified bovine erythrocyte SOD. Superoxide dismutase from bovine ROS and retina has the same electrophoretic mobility as pure bovine erythrocyte SOD. If the extracts are boiled prior to electrophoresis, or if the staining solutions are made 2 mM

with KCN, the SOD activity on the gels is completely abolished. Both the ROS and retinal extracts show numerous protein bands when gels are stained with amido black, but only one major band of SOD activity. A minor band of SOD activity is always seen migrating just below and, in some cases, just above the major SOD band in extracts of bovine ROS and retina.

Superoxide dismutase from frog ROS and retina has the same sensitivity to boiling and to KCN as does bovine SOD. However, the enzyme has a different electrophoretic mobility on the gel. No minor bands of SOD activity are seen. The localization of SOD activity on disc gels is not affected by the contaminant which intereferes with the spectrophotometric assay, which must therefore be removed during electrophoresis.

Since erythrocytes are a rich source of SOD, and could contaminate the bovine ROS preparation, the hemoglobin content of the ROS extract was determined as cyanohemiglobin, using a solution of cyanohemiglobin prepared from fresh blood as a standard(9) (the frog retina is avascular, and thus the frog ROS preparation has no erythrocyte contamination). By the cyanohemiglobin assay, the erythrocyte contamination was calculated to be about 2×10^6 red blood cells per ml of ROS preparation. When 2×10^4 lysed erythrocytes (equivalent to 10 µl bovine ROS extract) were tested in the spectrophotometric assay, no inhibition of NBT photoreduction was seen. Thus the SOD activity present in the bovine ROS extract was not due to erythrocyte contamination.

DISCUSSION Both bovine and frog ROS contain a soluble superoxide dismutase. From its cyanide sensitivity, and its electrophoretic mobility, the enzyme from bovine ROS appears identical to the Cu-Zn containing SOD isolated from bovine erythrocytes(1). On the basis of enzyme activity per microgram of protein, bovine ROS extracts contain 200 to 400 times as much SOD as does the remainder of the retina. No quantitative estimate could be made of the amount of SOD in frog retinas, due to the interference by endogenous substances with the spectrophotometric assay. However, since there is no contamination of

frog ROS by erythrocytes, the band of activity seen on polyacrylamide gels is due solely to SOD present in the ROS.

The reason for this high concentration of SOD in ROS can only be speculative. Free radical oxidation reactions induced by light have been shown to occur in frog ROS(2). The action spectrum of light-induced lipid peroxidation in frog ROS is similar to the absorption spectrum of rhodopsin. Thus SOD may be present to inhibit this free radical oxidation of the functionally significant polyunsaturated fatty acids which are abundant in the phospholipids of all vertebrate ROS studied. It has been suggested(10,11) that lipid peroxidation in the lipoprotein membranes of the developing retina in the presence of a high oxygen concentration, and light, may be sufficient to start the vaso-obliterative phase of retrolental fibroplasia. Of interest is the high ascorbic acid content of the retinas of a number of mammals(12). Reduced ascorbate can react with the superoxide radical(13,14), producing H_2O_2, which can subsequently be decomposed by catalase; this enzyme has not been demonstrated in ROS, but is present in microperoxisomes in the adjacent retinal pigment epithelium(15).

Acknowledgements We thank Ms. G. Otova for technical assistance. This work was supported by a grant from the National Institutes of Health to M.O.H.

REFERENCES

1. Fridovich, I. (1974) Advances in Enzymology, 41, 35-96.
2. Kagan, V.E., Shvedova, A.A., Novikoff, K.N. and Koslov, Yu.P. (1973) Biochim. Biophys. Acta, 330, 76-79.
3. Anderson, R.E. and Maude, M.B. (1970) Biochemistry, 9, 3624-3628.
4. Wheeler, T.G., Benolken, R.M. and Anderson, R.E. (1975) Science, 188, 1312-1314.
5. Fried, R. and Mandel, P. (1975) J. Neurochem., 24, 433-438.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
7. Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem., 44, 276-287.
8. Davis, B.J. (1964) Ann. N.Y. Acad. Sci., 121, 404-427.
9. van Assendelft, O.W. (1970) Spectrophotometry of Haemoglobin derivatives. Royal Van Gorcum Ltd., Assen, The Netherlands.
10. Riley, P.A. and Slater, T.F. (1969) Lancet, ii. 265.
11. Slater, T.F. and Riley, P.A. (1970) Lancet, ii, 467.
12. Heath, N., Beck, T.C. and Rutter, A.C. (1961). Vision Res., 1. 274-286.
13. Epel, B.L. and Neumann, J. (1973) Biochim. Biophys. Acta, 325, 520-529.
14. Allen, J.F. and Hall, D.O. (1973) Biochem. Biophys. Res. Commun., 52, 856-862.
15. Leuenberger, P.F., and Novikoff, A.B. (1975) J. Cell Biol., 65, 324-334.