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IN VITRO DAMAGE TO RAT LENS BY LUMAZINE AND XANTHINE OXIDASE: PREVENTION BY SUPEROXIDE DISMUTASE

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Intact rat lenses incubated with lumazine and xanthine oxidase are physiologically damaged as evidenced by a decrease in the net accumulation of rubidium ions against a concentration gradient. Superoxide dismutase protected the tissue against this damage. These experiments, therefore, demonstrate the susceptibility of the lens tissue to O_2^- injury under ambient and nonphotochemical conditions, suggesting a possible implication of this radical in the tissue *in vivo* and eventual cataract formation. The lumazine/xanthine oxidase system which is known to cause oxygen reduction predominantly by the monovalent route, producing superoxide, appears quite suitable to evaluate the toxicity of O_2^- to the tissues *in vitro*.

KEY WORDS: Rat lens, superoxide, lumazine, xanthine oxidase, superoxide dismutase

INTRODUCTION

Oxygen has been recognized to be one of the endogenous toxicants.[1] Exposure of animals to hyperbaric oxygen has been shown to be lethal.[2] In sublethal doses, it may induce convulsions and respiratory distress. Exposure of premature human infants and certain animals to excessive oxygen may trigger the onset of retrolental fibroplasia[3] and cataracts.[4] Besides these and several other acute effects of hyperoxia, the normotensive oxygen has been implicated in the pathogenesis of many age-dependent disabling manifestations.[1,5] This effect has been attributed to the conversion of sluggishly reactive oxygen to superoxide and its derivatization to other oxidants such as hydrogen peroxide and hydroxyl radicals.[6] The eye, because of the continued light penetration during the photopic vision, provides a unique situation for an *in situ* photochemical generation of O_2^- and its derivatives. O_2^- produced in this manner has been shown to be injurious to the physiology of the lens *in vitro*.[7,8] It was, however, felt necessary to generate O_2^- under nonphotochemical conditions and examine if such generation can adversely affect the lens even in the dark. Such a study was considered important to rule out the effect of light on the tissue itself so as to make it more susceptible to damage by the active oxidants. In these experiments, O_2^- and its derivatives were generated by the xanthine oxidase/lumazine system, a system recently shown to reduce oxygen primarily by the monovalent route.[9,10]

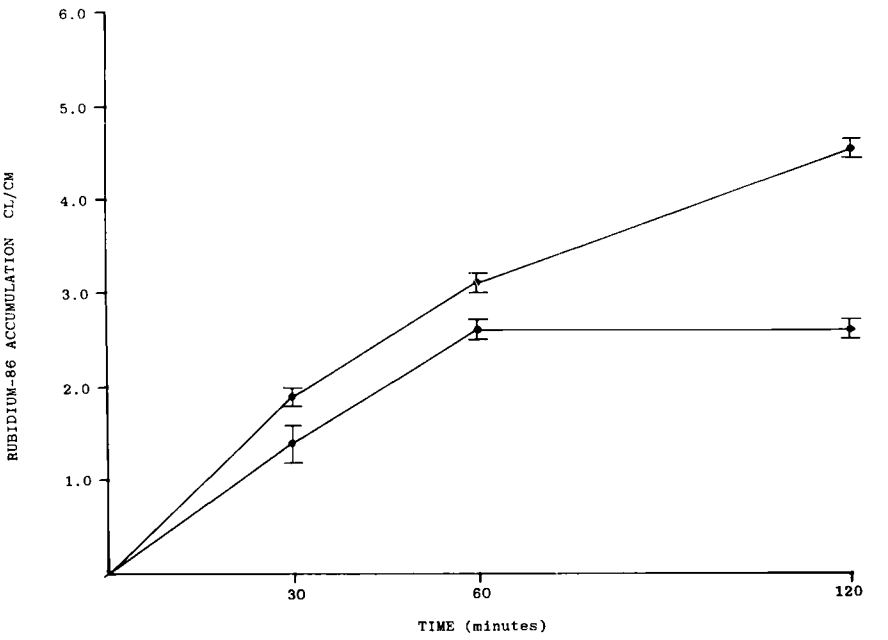

FIGURE 1 Accumulation of Rubidium by Rat Lenses. Lenses incubated by the method described in the text were taken out at indicated intervals and the distribution ratio (CL/CM) of the ion between tissue water and the medium determined. The values are described as mean ± standard deviation. The upper curve represents uptake in the presence of superoxide dismutase; the lower curve in its absence. The points on the two slopes represent contralateral lenses; two pairs in the case of the 30 and 60 minute points and three pairs at the 120 minute point.

MATERIALS AND METHODS

All chemicals, including CuZn-superoxide dismutase, catalase and xanthine oxidase, were obtained from Sigma Chemical Company. Mn-superoxide dismutase was obtained from U.S. Biochemical Corporation. Rat lenses isolated from overnight fasting animals weighing 150 ± 20 grams were used. Freshly extracted lenses were incubated in an isotonic medium pulsed with Rb–86 and their physiological status assessed by the uptake of this cation. The medium was prepared by dissolving 8.8 g NaCl, 200 mg KCl, 264 mg $CaCl_2{:}2H_2O$, 1.0 g $NaHCO_3$, and 50 mg NaH_2PO_4 in a liter of water and adjusting the pH to 7.4 by gassing with an air: CO_2 mixture (95:5). The tonicity of the medium was adjusted to 290 ± 10 mOsm/kg. The concentration of lumazine was 0.25 mM. Xanthine oxidase activity was adjusted to 0.03 units/4 ml of the medium. Initial experiments demonstrated a significant difference in the amount of rubidium taken up in the presence and absence of superoxide dismutase by approximately 120 minutes. Subsequent incubations were therefore conducted for 120 minutes. The results are expressed as the distribution ratio of the cation between lens water and the medium attained at the end of the incubation period. The incubations were conducted in an atmosphere of 95% oxygen:5% CO_2, at 37°C. Hydrogen peroxide was determined iodometrically. Two ml of the incubation medium was mixed with 0.5 ml of 1.5 M H_2SO_4, 1 ml of 0.6 M potassium iodide solution, 0.5 ml of 1.7×10^{-3} M

TABLE 1
Rubidium accumulation by rat lenses incubated in the presence and absence of various additives

Group	Additions to the Medium	CL/CM	E/C × 100	n
1.	BM	6.20 ± 1.0		8
2.	BM + Lumazine	5.80 ± 0.8	98 ± 6 (2/1)	12
3.	BM + Lumazine + X.O.	2.71 ± 1.0	47 ± 14 (3/2	40
4.	BM + Lumazine + X.O. + SOD (CuZn)	4.70 ± 0.86	173 ± 20 (4/3)	17
5.	BM + Lumazine + X.O. + SOD (Mn)	4.80 ± 0.80	177 ± 20 (5/3)	5
6.	BM + Lumazine + X.O. + Catalase (unpurified)	3.46 ± 1.00	152 ± 30 (6/3)	15
7.	BM + Lumazine + X.O. + Catalase (purified)	2.40 ± 0.40	90 ± 16 (7/3)	5
8.	BM + Lumazine + X.O. + albumin	3.01 ± 0.22	110 ± 7 (8/3)	4

The results in the first column are described as means of the distribution ratios (CL/CM) attained following two hours of incubation ± standard deviation, n = number of lenses incubated in each case. E = Experimental, C = Control. E/C × 100 values have been obtained by taking into consideration the uptake data of the contralateral lenses only. BM = Basal medium. Lumazine, 0.25 mM. X.O. = Xanthine Oxidase, 0.03 units/4 ml. SOD = superoxide dismutase, 2 units/4 ml. Catalase, 22 units/4 ml. All E/C × 100 values are significant with a $p < 0.001$ except between 2 & 1, 7 & 3, and 8 & 3 where the differences are not significant. 1 unit of SOD = 0.7 ug protein, denatured human serum albumin 1.4 μg/4 ml. Numbers in parentheses indicate the group compared. For example, (2/1) indicates that E/C × 100 was determined by dividing the CL/CM in group 2 with that in group 1 and the quotient multiplied by 100.

ammonium molybdate solution, and 1 ml of freshly prepared 1% starch solution. The mixture was titrated with 5×10^{-4} M solution of sodium thiosulphate until the disappearance of the blue color of the starch-iodide complex. A blank titration of the sample was conducted after allowing it to react with 20 units of catalase. Superoxide dismutase activity was determined by monitoring spectrophotometrically (ΔOD_{550} nm) the inhibition of the O_2^- dependent reduction of ferricytochrome *c*, O_2^- being produced by the action of xanthine oxidase on xanthine.[6] Catalatic activity was determined by monitoring the ΔOD_{240} of a 40 mM solution of hydrogen peroxide prepared in 0.05 M phosphate buffer pH 7.4, in the absence and presence of the enzyme preparation. The catalatic activity was completely inhibited by sodium azide, 2 mM.

RESULTS AND DISCUSSION

Superoxide and its derivatives generated under photochemical culture conditions have previously been shown to decrease the ability of the lens *in vitro* to accumulate rubidium against a concentration gradient.[7,8] This decrease may be attributable to a loss of cell membrane integrity, oxidative degradation of the elements of the Na–K pump, and/or a generalized toxicity to the tissue. In addition, the light–irradiated tissue may become especially sensitive to attack by the O_2^- and its derivatives because of the inherent photochemical effect on the tissue due to endogenous photosensitizers. The primary objective of this investigation was, thus, to examine the susceptibility of the lens to O_2^- generated under nonphotochemical incubation conditions. This was achieved by conducting incubations in the medium with lumazine and xanthine

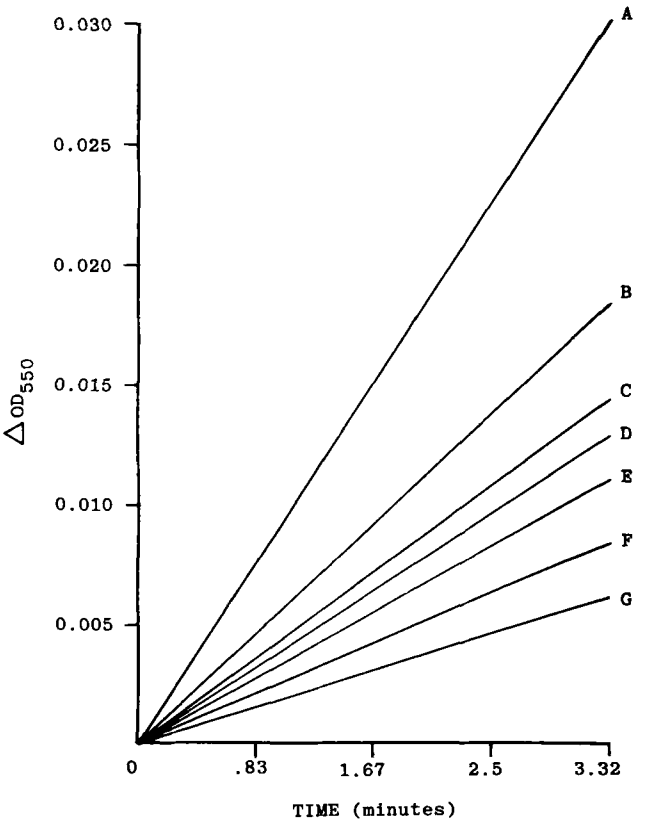

FIGURE 2 Superoxide Dismutase Activity in the Commercial Catalase. The graph indicates the O_2^- dependent reduction of ferricytochrome C monitored spectro-photometrically (ΔOD_{550}) at room temperature. The reaction mixture contained 0.1 mM EDTA Na_2, 50 μM sodium xanthine, 10 μM horse cytochrome C (Fe^{3+}) in 50 μM potassium phosphate buffer pH 7.8. The volume of the reaction mixture was 3.0 ml. Catalase units: 0, 55, 110, 165, 220, 330 and 400, respectively from A to G.

oxidase. Figure 1 describes the initial studies on the time course of the accumulation of rubidium from the medium containing lumazine and xanthine oxidase, in the presence and absence of superoxide dismutase. A two-hour period of incubation appeared suitable for eliciting the effect of superoxide dismutase and other protective agents. The detailed results are summarized in Table 1. Lumazine by itself, when added to the basal medium, had no effect on the uptake of rubidium by the rat lens. The same was the case with SOD and catalase in the absence and presence of lumazine. However, incorporation of xanthine oxidase in lumazine-containing medium, in the absence of SOD or catalase, led to a significant decrease in the rubidium accumulation. Thus, the product of xanthine oxidase activity on lumazine and oxygen is toxic to the lens. This toxicity was attenuated by superoxide dismutase. The accumulation of rubidium by the lenses incubated in the medium containing superoxide dismutase, along with lumazine and xanthine oxidase, was approximately 70% greater than the contralateral lens incubated with lumazine and xanthine oxidase in the absence of superoxide dismutase. The effect was discernible with CuZn

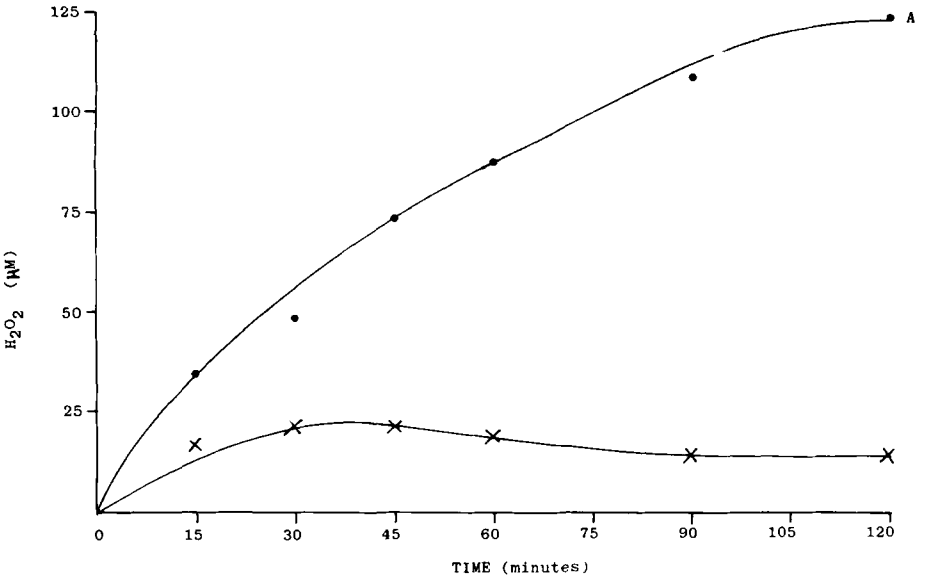

FIGURE 3 Hydrogen Peroxide Formation in the Medium of Incubation. Two 20 ml fractions of the medium containing 0.25 mM lumazine were warmed to 37°C for 20 minutes. Catalase (5.5 units/ml) was added to one of the fractions. After the initial warming, 0.15 unit of xanthine oxidase was added to both the solutions and 2 ml fractions taken out at indicated time intervals and hydrogen peroxide determined iodometrically. ●– –● without catalase. × · · × with catalase.

superoxide dismutase as well as Mn superoxide dismutase. Alkali denatured human serum albumin (Sigma #2386) in an equivalent amount (weight basis) had no protective effect. This indicates that the effect of superoxide dismutase is enzymatic and cannot be attributed simply to its proteinous nature. Thus, O_2^- is one of the important toxicants for the lens even when the latter is exposed to it in the dark. The addition of commercial catalase also protected the lens, as evidenced by the greater accumulation of rubidium in the presence of this enzyme.

However, it has been pointed out that catalase contains superoxide dismutase as a normal contaminant and that may contribute to its protective effect.[11] This was found to be true. Figure 2 describes the course of O_2^- dependent reduction of ferricytochrome C in the presence of xanthine and xanthine oxidase. The addition of the commercial catalase inhibited this reaction in a manner similar to that of superoxide dismutase. Thus a part of the protective effect of catalase was considered to be due to its O_2^- dismutating activity. Commercial catalase was, therefore, purified by passing the enzyme preparation through Sephadex G–200 column (12 cm × 1 cm) equilibrated with 50 mM phosphate buffer, pH 7.6. Three to four such filtration steps were found necessary to eliminate superoxide dismutase activity. Purification of catalase led to the disappearance of its protective effect. That is not to say that H_2O_2 is not harmful, but it does not appear to be so in this lumazine system; the effect of superoxide dismutase was substantial even while the H_2O_2 level kept rising over the period of incubation (Figure 3). Additionally, superoxide dismutase was found not to contain any catalatic activity.

Although the toxicity of O_2^- has been the subject of some controversy, present studies with the lens lend support to the superoxide theory of tissue toxicity. The

mechanism involved in the O_2^- dependent toxicity to the lens is not clear at the present time. Several oxidative/reductive reactions incompatible with the normal biochemistry and physiology of the tissue are possible. The primary objective of the experiments described in this communication was to determine if O_2^-, in a rather pure form and under ambient nonphotochemical conditions, can be toxic to the lens and that seems to have been demonstrated. These studies also demonstrate the usefulness of lumazine/xanthine oxidase combination for evaluating the role of O_2^- in other biochemical and physiological systems. Systems like xanthine/xanthine oxidase produce a mixture of active species of oxygen directly[6] and hence evaluation of O_2^- toxicity becomes difficult.[6] Under the usual incubation conditions, the predominant product of oxygen reduction is H_2O_2 with little O_2^- generation, whereas when xanthine is replaced by lumazine, O_2^- is the predominant reduction product of oxygen.

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