Effect of Age on Superoxide Dismutase Activity of Human Trabecular Meshwork

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**Purpose.** It has been hypothesized that accelerated aging of the trabecular meshwork, perhaps because of oxidative damage, is involved in the pathogenesis of primary open angle glaucoma. The authors sought to evaluate the effect of donor age on the specific activity of superoxide dismutase and catalase in normal fresh human cadaver trabecular meshwork.

**Methods.** Total superoxide dismutase and catalase were assayed in tissue extracts generated from fresh human trabecular meshwork. Cadaver tissue was obtained from 19 donors (18 paired) of a wide age range (30 to 91 years). The assays were performed within 6 hours of enucleation and within 36 hours of donor death. Enzyme-specific activities were calculated using protein concentration of the extract as the denominator.

**Results.** Multiple regression analysis modeled with age and time from death until the beginning of the experiment was performed. The specific activity of superoxide dismutase declined with age ($P = 0.00022; r^2 = 0.67$). There was no effect of age on catalase specific activity ($P = 0.24; r^2 = 0.16$). The time from donor death until the beginning of the experiment was not a significant factor ($P > 0.28$).

**Conclusions.** The specific activity of superoxide dismutase, but not catalase, demonstrates an age-dependent decline in normal cadaver human trabecular meshwork. The potential role of superoxide dismutase in primary open angle glaucoma, a disorder of the aging trabecular meshwork, warrants further investigation. Invest Ophthalmol Vis Sci. 1996;37:1849–1853.
20,000/mm³, diabetes mellitus, chemotherapy within the past 6 months, prolonged intubation (greater than 24 hours), past known ocular disease, and previous eye surgery. Conneoscleral and uveoscleral trabecular meshwork tissue was dissected using a standard technique. No blood was observed in any of the trabecular meshwork specimens. The excised trabecular meshwork tissue was rinsed gently with ice-cold 0.10 M potassium phosphate buffer, pH 7. The tissue was homogenized using a glass Wheaton Micro Tissue Grinder (Wheaton Scientific, Millville, NJ) with 50 μl of phosphate buffer. After sonication, the homogenate was centrifuged at 13,000g for 5 minutes, and the tissue extract was removed for use in the assays. Soluble protein concentration of the tissue extract was measured with the Bio-Rad (Richmond, CA) protein assay using bovine serum albumin as the standard. The remainder of the tissue extract was used immediately for the SOD or catalase assay.

Because of the small amount of tissue in the trabecular meshwork, the number of assays that could be performed was limited. Catalase activity and SOD could not be measured from the trabecular meshwork of the same eye. Therefore, SOD activity was measured from one eye of each donor pair (randomly chosen as right or left), and catalase activity was assayed in tissue obtained from the fellow eye. For one donor (age, 83 years), there was tissue available only for the catalase assay. Assay of enzymes using the homogenate supernatant was chosen because the enzymes are localized primarily within the cytosol, mitochondria, or both. Using the techniques for isolation described herein, these enzymes would be expected to be released into the supernatant.

Spectrophotometric determination of enzyme activity was performed using a Shimadzu (Columbia, MD) UV-260 spectrophotometer, 3 ml quartz cuvettes, path length 10 mm. All assays were performed at 25°C. Unless otherwise specified, all reagents were obtained from Sigma Chemical (St. Louis, MO). Total intracellular SOD activity was determined based on a previously described kinetic assay. Xanthine oxidase was prepared from bovine cream. The reaction mixture consisted of 2 × 10⁻⁵ M cytochrome c, 100 μM xanthine in 0.05 M phosphate buffer, pH 7.8, containing 1 × 10⁻⁴ M ethylenediaminetraacetic acid. The amount of xanthine oxidase was sufficient to cause an increase in absorbance of 0.025/minute at 550 nm under the conditions described.

The assay is based on the inhibition of cytochrome c reduction by 20 μl of the SOD-containing extract. The unit of enzyme activity was defined as the quantity of SOD required to inhibit the rate of reduction of cytochrome c by 50% under the specified conditions. Reduction of cytochrome c by endogenous tissue sources was controlled by measuring the reaction rate before the addition of xanthine oxidase. Any measurable activity was subtracted from the final reaction rate measured in the presence of xanthine oxidase. Specific activity was measured as units of enzyme activity per milligram of protein in the tissue extract.

Previously, we assayed purified SOD from bovine erythrocytes using the protocol described in this article to demonstrate the usefulness of the assay in the measurement of SOD activity. When the ratio of slope change at 550 nm in the absence of SOD to that in the presence of SOD was plotted against the concentration of SOD, a linear relation was found. Fifty percent inhibition was achieved at a SOD concentration of 0.07 μg/ml.

Assay of catalase was determined by measuring the decrease in ultraviolet absorption of hydrogen peroxide measured as a function of time. The reaction mixture consisted on 9.20 mM hydrogen peroxide in 0.05 M phosphate buffer, pH 7. The molar extinction coefficient (230 nm) used in the determination of hydrogen peroxide concentration was 71 M⁻¹ cm⁻¹. A decrease in absorbance over a 3-minute interval occurred after the addition of 10 to 20 μl of tissue extract. Care was taken to check for gas formation in the cuvette during the reaction; however, this did not occur during the reaction with the amount of sample used. Larger sample volumes were avoided because these frequently result in excess gas generation in the cuvette, which limits the accuracy of slope measurements. The slope was determined, and enzyme-specific activity was measured as μmol hydrogen peroxide consumed/mg protein · minute. There was a tendency for absorbance at 230 nm to decrease slightly before the addition of tissue extract, indicative of spontaneous nonenzymatic breakdown of hydrogen peroxide. This small rate change was subtracted from the final rate, which represented the change in absorbance caused by enzyme activity.

RESULTS

Multiple regression analysis modeled with age and time interval (from donor death until the use of tissue in the experimental protocol) was performed to evaluate statistically the effect of donor age on enzyme-specific activity. In this age range of 30 to 91 years, SOD activity declined with increasing donor age (Fig. 1; P = 0.00022; r² = 0.67; slope = -0.96/year; intercept = 104). In contrast, there was no effect of age on catalase-specific activity (Fig. 2; P = 0.24, r² = 0.16). Because enzyme activity can decline with time, we chose to limit to less than 36 hours the time interval between donor death and use of tissue in the experimental protocol. There was no significant effect of this time interval on enzyme activity for either enzyme (P > 0.28).
Superoxide Dismutase Activity of Trabecular Meshwork

FIGURE 1. Specific activity of superoxide dismutase (SOD) of normal fresh cadaver human trabecular meshwork declines with donor age ($P = 0.00022; r^2 = 0.67$).

DISCUSSION

Trabecular meshwork is exposed to chronic oxidative stress over the course of a lifetime. Endogenous sources of this stress include toxic oxygen byproducts that can form as a result of normal aerobic cellular metabolism. In addition, exogenous sources of oxidative stress include the presence of high physiological concentrations of hydrogen peroxide in the aqueous humor and induced photochemical reactions after light exposure of the anterior segment. Therefore, it is not surprising that the trabecular meshwork has a sophisticated defense system against oxidative stress. This system includes the presence of antioxidant enzymes, such as SOD, catalase, and glutathione peroxidase.

Age-related changes are known to occur in the trabecular meshwork. These include a progressive decline in cellularity. Alvarado et al estimated that the rate of loss of cells of the trabecular meshwork is linear and approximately 0.58% per year from birth through 81 years of age. An age-related protein aggregation in the trabecular meshwork has been described, and there is evidence that this involves collagen and actin. Oxidative stress has been proposed to be involved in this process. Millard et al found evidence for a decrease in actin and an increase in collagen polypeptide levels in aging human trabecular meshwork. However, no difference in the collagen content of trabecular meshwork between normal and glaucomatous eyes has been found. In addition, an electron-dense material has been shown to accumulate over time in the juxtacanalicular tissue of normal eyes. The exact nature of this material remains to be elucidated fully.

Evidence suggests that trabecular meshwork cells can survive under harvesting conditions similar to those used in this study. In a study to evaluate outflow facility in human tissue, eyes enucleated within 3 hours of death and stored under conditions similar to those used in our assay for 12 hours showed no significant postmortem change in trabecular meshwork on histologic analysis. When the tissue was perfused for 2 to 6 hours after harvesting under these conditions, it showed great similarity to the human outflow system in vivo with regard to morphology and several functional parameters. Studies of primary cultures of human trabecular meshwork cells have shown a satisfactory rate of establishment of primary culture for tissue obtained up to 5 days postmortem. Failure to produce primary outgrowths did not increase until more than 5 days postmortem.

Before the effect of SOD activity in the trabecular meshwork in glaucoma can be addressed fully, it is important to understand the effect of aging on the activity of this enzyme. For this reason, we chose to study the normal trabecular meshwork. Superoxide dismutase has been identified in trabecular meshwork tissue. Levels of enzyme-specific activity identified in the calf by Freedman are analogous to those measured in this study.

The exact reason for the age-dependent decline in SOD, which we identified in normal tissue, is unknown. An age-dependent decline in specific activity of this enzyme is uncommon in ocular tissues. Re-
cently, we found that SOD activity of the peripheral human retina demonstrates a decline in activity between the ages of 30 and 75 years. This decline was approximately 8-fold in magnitude. In addition, an approximately 10-fold decline in SOD-specific activity in the normal lens equator and nucleus has been described between ages 6 and 62 years. This decline in lens SOD activity may be related to the accumulation of defective SOD molecules.

There may be age-related loss of a particular cell type that contains relatively high levels of SOD. Immunohistochemical studies of SOD subtype distribution with age would be useful to evaluate this possibility. Alternatively, there may be decline in activity of one subtype of SOD with age that is related to decreased expression or to expression of an abnormal protein rather than to cellular loss. Another possible explanation for the decline in SOD activity with donor age includes an increase in soluble protein with increasing age. This could result in a measured decline in enzyme-specific activity if enzyme content is unchanged. In view of the finding that catalase-specific activity did not demonstrate an age-dependent change, this possibility seems unlikely.

The aqueous outflow pathway has great capacity to detoxify the hydrogen peroxide present in physiological concentrations in the aqueous humor. Catalase and glutathione peroxidase are involved directly in protecting against the toxicity of hydrogen peroxide. The presence of a constant specific activity of catalase may be an indicator of the importance of this protective mechanism against hydrogen peroxide toxicity in the anterior segment.

Unaltered states of catalase do not negate the hypothesis that SOD is involved in the pathogenesis of glaucoma. Superoxide radical can cause direct damage to tissue in the absence of hydrogen peroxide. It has been shown that superoxide radical can inactivate mammalian aconitase by reacting the Fe-S cluster in the active site to cause cluster degeneration. It has been proposed that this inactivation is a manifestation of the toxic effect of oxygen on cells. Thus, the lack of change in catalase-specific activity with age does not negate involvement of antioxidant enzymes in the pathogenesis of glaucoma.

The physiological significance of the differential change in SOD compared to catalase with age remains to be elucidated. Clearly, primary open angle glaucoma is a disorder of aging. It may be that persons with glaucoma are more vulnerable to the potentially damaging effect of declining SOD-specific activity. Separation of SOD activity into copper–zinc-dependent and manganese-dependent activities is indicated to evaluate whether the decline involves one or both subtypes. In addition, it is possible that there may be age-related changes in the activity of other antioxidant enzymes, such as glutathione peroxidase. Additional research to evaluate the antioxidant enzyme-specific activity in glaucomatous eyes may provide further insight into the significance of these findings.

Key Words
catalase, superoxide dismutase, trabecular meshwork

Acknowledgment
The authors thank Dr. Terry Cox for statistical assistance.

References


