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Oxidative Inhibition of Ca^{2+}-ATPase in the Rabbit Lens

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Hydrogen peroxide inhibition of maximum Ca^{2+}-ATPase and Na^+,K^+-ATPase activity was measured in a membrane-enriched preparation of rabbit lens cortical fibers and epithelium. At 5×10^{-6} M hydrogen peroxide maximum Ca^{2+}-ATPase activity was inhibited by 39%, while maximum Na^+,K^+-ATPase activity was stimulated. Ca^{2+}-ATPase activity was almost completely inhibited at 5×10^{-4} M hydrogen peroxide, in comparison to Na^+,K^+-ATPase activity, which was only inhibited by 28% at a concentration of hydrogen peroxide an order of magnitude larger. The addition of catalase to hydrogen peroxide-pretreated samples did not reverse the inhibition of Ca^{2+}-ATPase by hydrogen peroxide. Invest Ophthalmol Vis Sci 30:1633–1637, 1989

The possible involvement of lenticular calcium metabolism in the development of experimental and human cataract has been explored in several studies.[1-3] Regulation of lens calcium content is accomplished by restricted membrane permeability and a calcium pump resident in the lens cell membrane. The activity and distribution of Ca^{2+}-ATPase in the lens has been described by Hightower et al[4] and Borchman et al.[5]

Many studies provide evidence for derangement of membrane function and compositional changes in cataractous lenses.[6] It has been suggested that oxidative mechanisms might account for many of these changes.[7] Oxidative damage to the lens during cataractogenesis is well documented and appears to be initiated at the cell membrane.[8]

Studies on lens Na^+,K^+-ATPase, the sodium pump enzyme, have demonstrated its susceptibility to inhibition by hydrogen peroxide.[9-14] However, there is no information available relating the sensitivity of lens Ca^{2+}-ATPase to oxidative insult. The purpose of this study was to evaluate the impact of hydrogen peroxide upon lens Ca^{2+}-ATPase activity in comparison to maximum Na^+,K^+-ATPase activity.

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Materials and Methods

Animal Tissues

Rabbit eyes were obtained from healthy 2 kg New Zealand strain albino rabbits, about 10 weeks old, killed painlessly by intravenous administration of T-61 euthanasia solution (American Hoechst Corp., Somerville, NJ). Lenses were dissected from the globe by a posterior approach. Separation of the lens epithelium, cortex and nucleus was the same as previously described.[5] The experiments adhered to the ARVO Resolution on the Use of Animals in Research.

Membrane-Enriched Preparation

Cortical and epithelial material was pooled separately from eight rabbit lenses. Six separate pools of tissue were used in this study. After Teflon douncer homogenization, membrane-enriched microsomal preparations were made from each pool using a differential centrifugation protocol described previously.[5] Membrane-enriched preparations, at a concentration of 2–7 mg/ml in buffer, were divided into 0.5 ml aliquots and stored frozen in liquid nitrogen for up to 1 week. All pools were prepared separately. After the assays for one pool of membrane material were completed, another pool was prepared. We have established that Ca^{2+}-ATPase activity in our preparation does not diminish over a 1 month period storage in liquid nitrogen.

ATPase Activity

Ca^{2+}-ATPase activity was measured at 37°C as described in detail previously,[5] using membrane at a

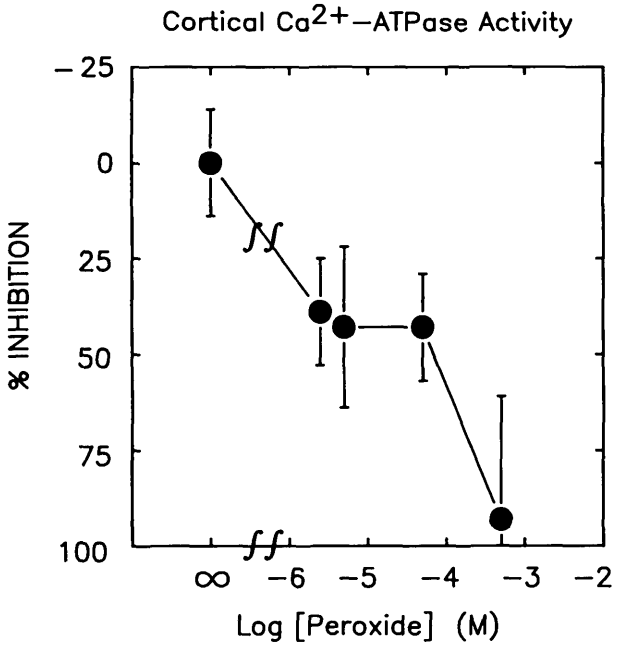

Fig. 1. Inhibition of lens Ca^{2+}-ATPase activity by hydrogen peroxide in a membrane-enriched sample from rabbit lens cortical fibers. Ca^{2+}-ATPase activity was measured at 37°C, pH 7.4 at a free calcium concentration of 1×10^{-5} M. Calcium was buffered with EGTA, K_b 1×10^6. Error bars are ± standard error, n = 5 pools.

sample protein concentration of 0.1 mg/ml protein for cortical samples, and 0.03 to 0.07 mg/ml for epithelial samples (because of a limited amount of membrane epithelium). The reaction mixture contained either 5 mM EGTA (buffered with tris pH 7.4), or $CaCl_2$-EGTA buffer to give a final free calcium concentration of 1×10^{-5} M, previously determined to yield maximal lens Ca^{2+}-ATPase activity.[5] After a 5 min temperature equilibration the ATPase reaction was started by adding labeled (gamma-^{32}P)ATP (4×10^4 cpm/μmol). The reaction was terminated at 10, 20 and 30 min by taking aliquots of 100 μl of sample and placing the aliquot into 50 μl ice-cold trichloroacetic acid (5% final concentration). Samples were assayed in duplicate. ^{32}P liberation was determined by a method based on the extraction of a phosphomolybdate complex in isobutyl-alcohol. Ca^{2+}-activated ATPase activity was defined as the difference between the rate of P_i liberation measured in the presence and absence of calcium.

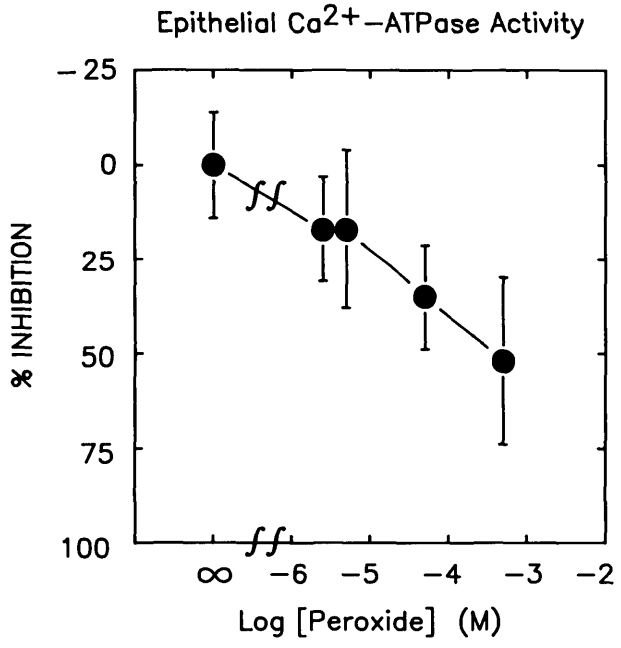

Fig. 2. Inhibition of lens Ca^{2+}-ATPase activity by hydrogen peroxide in a membrane-enriched sample from rabbit lens epithelial fibers. Ca^{2+}-ATPase activity was measured at 37°C, pH 7.4 at a free calcium concentration of 1×10^{-5} M. Calcium was buffered with EGTA, K_b 1×10^6. Error bars are ± standard error, n = 5 pools.

To determine the effect of hydrogen peroxide on Ca^{2+}-ATPase activity, hydrogen peroxide at specified concentration, or water for the controls, was added prior to temperature equilibration at 37°C. The hydrogen peroxide concentration in the reaction mixture was determined[15] before and after the calcium ATPase determinations and found to be unchanged.

To determine whether the effect of hydrogen peroxide on Ca^{2+}-ATPase activity was reversible, membrane samples were treated for 5 min with 5×10^{-4} M hydrogen peroxide and then catalase was added 5 min prior to starting the Ca^{2+}-ATPase reaction. A catalase concentration of 0.9 μg/ml was determined to completely remove hydrogen peroxide from our sample in 1 min; we used a concentration of catalase ten times that level and allowed 5-fold additional time, to ensure complete removal of hydrogen peroxide from our samples. Catalase did not interfere with Ca^{2+}-ATPase activity. Na^+,K^+-ATPase activity was measured in the same samples, as detailed previously.[5] The methodology is similar to that described for Ca^{2+}-ATPase except that Na^+,K^+-ATPase activity was defined as the difference in the rate P_i liberation measured in the presence or absence of ouabain.

Protein was determined by the Peterson[16] modification of the Lowry assay.[17]

Results

The basal cortical ATPase activity, in the absence of stimulation by calcium or sodium, was 340 ± 15 nmol/mg/hr. Maximal cortical calcium ATPase activity, in the presence of 10^{-5} M calcium, was 88 ± 30 nmol/mg/hr, matching the value of 78 ± 12 nmol/mg/hr previously measured for pooled rabbit cortex.[5] The effect of hydrogen peroxide on cortical lens membrane calcium ATPase activity is shown in Fig-

ure 1. At low concentrations of hydrogen peroxide (5×10^{-6} M), calcium ATPase activity was inhibited by 43%. Calcium ATPase activity was almost completely inhibited at 5×10^{-4} M hydrogen peroxide. The decrease in calcium ATPase activity in the presence of hydrogen peroxide concentrations ranging from 5×10^{-6} to 10^{-4} M was found to be statistically significant with a P value of 0.018 (paired variants t-test). The degree of variability in the sensitivity of different pools of membrane material to hydrogen peroxide is reflected in the standard error.

Calcium ATPase activity in preparations of lens epithelium was 250 ± 150 nmol/mg/hr. The effect of hydrogen peroxide on lens epithelial membrane calcium ATPase activity is shown in Figure 2. The linear concentration-dependent inhibition of calcium ATPase by hydrogen peroxide was found to be significant with a P value equal to 0.005.

The inhibition of calcium ATPase by hydrogen peroxide was not reversed when hydrogen peroxide was removed by adding catalase to the reaction mixture (Fig. 3). In the presence of 5×10^{-4} M hydrogen peroxide, the inhibition of cortical membrane calcium ATPase activity was 47 ± 10% in the sample pool tested. When the concentration of hydrogen peroxide was reduced to zero by adding catalase, the inhibition of calcium ATPase activity was unchanged. The addition of catalase alone to a control ATPase reaction mixture had no effect upon the calcium ATPase activity.

The Na^+,K^+-ATPase activity observed in preparations of lens cortex was 550 ± 90 nmol/mg/hr, in agreement with previous studies.[5] The ouabain sensitive component of ATPase activity was 26%, which is in agreement with our previous measurements,[5] and those measured in the lens from other species.[18] The effect of hydrogen peroxide on cortical lens membrane ATPase activity is shown in Figure 4. At a low concentration of hydrogen peroxide (5×10^{-6} M), Na^+,K^+-ATPase activity is stimulated slightly; at this same concentration of hydrogen peroxide, the calcium ATPase activity in the lens cortex was inhibited. At the highest concentration of hydrogen peroxide examined, (5×10^{-3} M) Na^+,K^+-ATPase activity was inhibited by only 28%.

Discussion

In this study we show that at low levels of hydrogen peroxide, Ca^{2+}-ATPase activity can be inhibited substantially, while maximum Na^+,K^+-ATPase activity is not diminished. This inhibition of Ca^{2+}-ATPase activity by hydrogen peroxide occurs at a concentration of the oxidant (5×10^{-6} M) an order of magnitude lower than that thought to be present in the aqueous of some cataractous lens patients.[19]

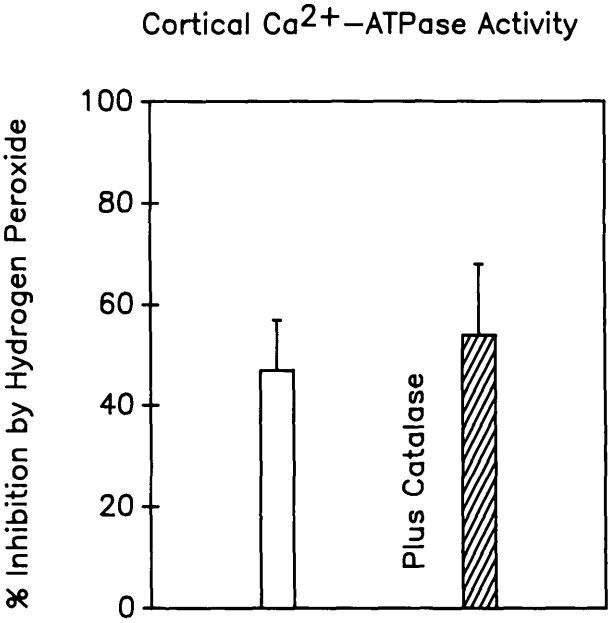

Fig. 3. Inhibition of lens Ca^{2+}-ATPase activity by 5×10^{-4} M hydrogen peroxide with and without catalase added to remove hydrogen peroxide prior to measuring Ca^{2+}-ATPase activity. Ca^{2+}-ATPase activity was measured at 37°C, pH 7.4 at a free calcium concentration of 1×10^{-5} M. Calcium was buffered with EGTA, K_b 1×10^6. Error bars are ± standard error, n = 5 pools.

Since only a few studies have focused on the Ca^{2+}-ATPase activity of the lens, one must look to studies on other tissues to explore the phenomenon of the

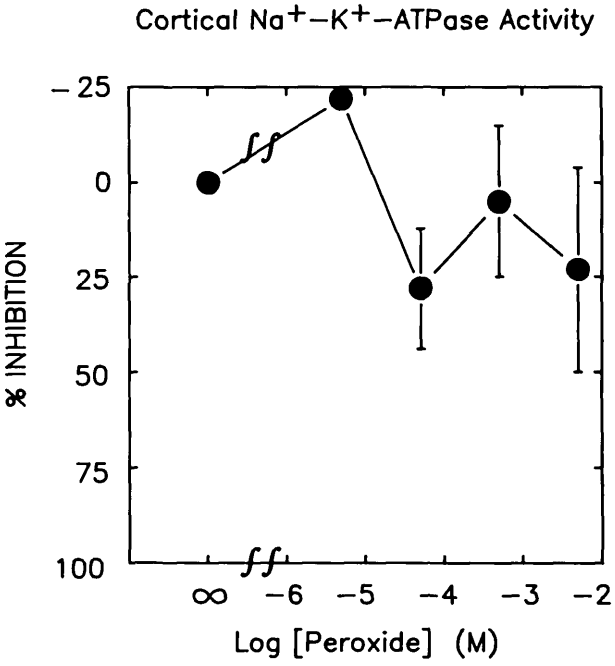

Fig. 4. Inhibition of lens Na^+,K^+-ATPase activity by hydrogen peroxide in a membrane-enriched sample from rabbit lens cortical fibers. Na^+,K^+-ATPase activity was measured at 37°C, pH 7.4. Error bars are ± standard error, n = 5 pools.

oxidative inhibition of Ca^{2+}-ATPase. The inhibition of Na^+,K^+-ATPase and Ca^{2+}-ATPase by oxidation has been explored by other investigators, but seldom concurrently in the same tissue. Kako et al[20] propose three mechanisms that together or independently contribute toward decreased ATPase activity by oxidation: (1) alterations in phospholipid environment; (2) alterations in the conformation of membrane proteins resulting in membrane dysfunction; and (3) accumulation of degradation products in the membrane.

It has been proposed that Ca^{2+}-ATPase is more susceptible to alterations in lipid environment[21-23] than the Na^+,K^+-ATPase.[24] It is perhaps because hydrogen peroxide increases the microviscosity (order) of the membrane lipid by oxidizing lipid acyl-chain double bonds that Ca^{2+}-ATPase activity is inhibited more effectively than Na^+,K^+-ATPase, as we observed. While oxidative inhibition of Ca^{2+}-ATPase in several tissues has been explained by a lipid peroxidation mechanism,[25-28] a reversible thiol oxidation reaction involving just the enzyme has been suggested for erythrocyte Ca^{2+}-ATPase in addition to an irreversible component involving lipid peroxidation.[29]

It is also possible that oxidative degradation products could bind to the ATPase pumps and selectively inhibit ATPase activity. Malondialdehyde and 4-hydroxynonenal are both released during lipid peroxidation; malondialdehyde and 4-hydroxynonenal has been shown to inhibit Ca-uptake in liver microsomes.[30] It is beyond the scope of this study to determine the precise mechanism behind the inhibition of lens ATPase activity by hydrogen peroxide. It is important to note that the active transport of ions could be more sensitive to oxidative damage than ATPase activity since oxidative damage could uncouple ion transport from ATPase activity.

It should be stressed that the experiments described in this study are performed with an isolated membrane preparation; no antioxidant enzymes, reduced glutathione or ascorbate are present. Thus, the level of hydrogen peroxide required to inhibit ATPase activity may be higher in the intact lens, where protective antioxidants are present. In the intact lens, the response of Ca^{2+}-ATPase to hydrogen peroxide may be considerably different; for example, we show here that inhibition of Ca^{2+}-ATPase by hydrogen peroxide is not reversible while an apparently normal enzyme activity has been measured in membranes from intact lenses treated with hydrogen peroxide and allowed to recover.

The current study suggests that low levels of hydrogen peroxide that do not inhibit the Na^+,K^+-ATPase pump might inhibit the Ca^{2+}-ATPase. A consequence of such inhibition would be accumulation of extracellular calcium. A build-up of intracellular lens calcium is undoubtedly deleterious to lens function.[31] Elevated levels of intracellular calcium may cause lens opacity by interacting with cytoplasmic proteins,[32-34] activating proteases[35-37] or lipases,[38] or calmodulin-activated pathways.[39]

Key words: calcium-ATPase, sodium-potassium-ATPase, lens, membrane, cortex, epithelium, hydrogen peroxide

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