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Oxidative Stress to Rat Lens *In Vitro*: Protection by Taurine

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The concentration of taurine is high in the lens. However, its function therein remains unknown. Studies from other tissues suggest that in addition to several other modes of action, it acts as an antioxidant. We therefore hypothesize that taurine may be a part of the antioxidant defense mechanisms involved in protecting the lens against oxidative stress and consequent cataract formation. In these studies, the protective effect of taurine was examined using lens culture system with menadione as an oxidant. Inclusion of this compound in the incubation medium was found to have several adverse effects on the lens, such as a decrease in its ability to accumulate rubidium against a concentration gradient and fall in the levels of glutathione, ATP and an increase in water insoluble proteins. All these deleterious effects were attenuated significantly by addition of physiological amounts of taurine to the menadione-containing medium.

Keywords: Cataract, lens, oxygen radicals, menadione, taurine

INTRODUCTION

The concentration of taurine (2-aminoethanesulfonic acid) in the lens is noticeably high.[1–3] It can be derived by active transport from the aqueous humor[4] as well as by *de novo* synthesis from cysteine and other sulfur amino acids.[3] Its function therein however remains unknown. In other tissues however, it has been shown to act as an antioxidant. Most convincing evidence of such a function has come out of studies with retina. For example: in the taurine deficient cats, an extensive degeneration of the photoreceptors take place due to oxidative degradation of large amounts of polyunsaturated fatty acids present therein.[5,6] Additionally, it has been demonstrated to protect against oxy-radical induced damage to several other tissues such as the heart, lung, macrophage and erythrocytes, caused by potent oxidants like paraquat, bleomycin, adriamycin and ozone.[7–9] Mechanistically, its mode of action as an antioxidant however remains unestablished, except the demonstration that it can scavenge hydroxyl radicals and hypochlorous acid (HOCl).[10,11] Hence, in view of the above reports and the fact that it is present in the lens in very high concentration, we considered the

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possibility that it may prevent oxidative damage to lens and consequent cataract formation. Menadione, a naphthoquinone, known to generate reactive oxygen species (ROS) by its redox-cycling was used as an oxidant. The results presented herein are in accordance with this hypothesis.

MATERIALS AND METHODS

Most of the chemicals were purchased from Sigma chemical company, St. Louis, MO 63178, USA. Rubidium chloride (^{86}RbCl) was obtained from the New England Nuclear Company Boston, MA 02118. Male Sprague-Dawley rats weighing between 150 ± 20 g were used in all the experiments.

Rubidium uptake and efflux studies

An overall physiological damage to the lens tissue caused by menadione was assessed *in vitro* by measuring its ability to maintain an adequate distribution ratio of rubidium ions between the lens water and the medium of incubation, represented as CL/CM. This ion, being a surrogate of the potassium ion, is actively transported from the extra-cellular fluid into the cytosol via the ATP dependent Na^+–K^+ pump. The maintenance of this ratio therefore reflects the status of this pump and subsequent leakage of the ions out of the cell, as well as the status of the tissue metabolism involved in generating ATP required for the active transport and other energy dependent cellular functions. A direct measurement of the said distribution ratio, therefore, serves as a convenient general index of the physiological well being of the tissue.[12] The measurement of this ratio was done as follows: Freshly dissected rat lenses were incubated contralaterally in petri dishes (35×10 mm) containing 4 ml of Tyrode buffer containing ^{86}RbCl, in the absence or presence of 60 µM menadione (sodium bisulfite derivative). The dishes were incubated at 37°C in an incubator gassed with 95% : 5% air/carbon dioxide mixture for a period of 16 h. Following incubation, the lenses were removed from the medium, rinsed with normal saline and their radioactivity determined by direct gamma counting (CL). The incubation medium was also counted simultaneously (CM). The distribution ratio (CL/CM) was then calculated by dividing the counts present in lens water (60% of the wet weight of the tissue) with the counts present in an equivalent volume of the incubation medium.

Subsequently, we determined the effect of menadione and taurine on the efflux of $^{86}Rb^+$ from rat lenses preloaded with $^{86}Rb^+$ by incubating them for 2 h in Tyrode medium containing 0.1 µCi/^{86}RbCl per ml. The loaded lenses were rinsed with 0.5 ml of normal saline and their initial radioactivity determined in a gamma counter. They were then transferred atraumatically to petri dishes containing 4 ml of Tyrode medium containing 250 µM menadione without or with taurine (20 mM) and the efflux of $^{86}Rb^+$ determined by counting the radioactivity of the medium every hour. The medium also contained 10 mM non-radioactive RbCl to prevent the reuptake of $^{86}Rb^+$. The basal efflux rate was simultaneously determined by incubating the lenses in the normal Tyrode medium without menadione or taurine. The efflux rates were expressed as the percentage of radioactivity initially present in the lens.

The extent of damage to the lens was also determined by measuring the GSH and ATP levels,[12] as well as by following the protein oxidation and aggregation by SDS-PAGE.[13,14] Briefly, the lenses were homogenized individually in 1 ml of deionized water. The homogenate was centrifuged for 15 min at 4°C in a microcentrifuge to obtain a water-soluble fraction. This was maintained on ice. Two hunderd µl of this fraction was then mixed with 200 µl of a 10% trichloroacetic acid solution and centrifuged to get a protein free supernatant. Two hundred µl of this acid extract was then mixed with 1 ml of 0.3 M disodium hydrogen phosphate and 0.1 ml of DTNB reagent (1 mM 5,5′-diothio-bis(2-nitrobenzoic acid) in 1% trisodium citrate), and the

resulting yellow color was measured spectrophotometrically at 412 nm. A series of standards prepared from GSH and a blank were also run simultaneously.

ATP determination

Fifty µl of the fresh aqueous tissue extract prepared as above was injected into a vial placed in a luminometer and containing 200 µl of firefly lantern extract prepared by reconstituting the arsenate buffered dehydrated extract (Sigma FLE-50) with 5 ml distilled H_2O. The resulting luminescence was measured at its peak and quantitation done by reference to a series of ATP standards.

PAGE analysis of proteins

Single lenses were homogenized in 1 ml of phosphate buffered saline and soluble and insoluble proteins were separated by centrifugation. The insoluble protein fraction (precipitate) was washed three times with deionized H_2O to remove any adhering soluble proteins. This was accomplished by vortexing the pellet with 1 ml of distilled H_2O, centrifugation and aspiration of the supernatant. The washed pellet was then dissolved in 0.3 ml of 7 M urea prepared in 50 mM Tris-HCl buffer, pH 7.4 by incubating it for 30 min at 37°C. The supernatant obtained after centrifugation was designated as urea soluble proteins. Aliquots of this urea soluble proteins were mixed with equal volume of denaturing solution (sample buffer) containing 0.0625 M Tris (pH 6.8), 10% glycerol (v/v), 4% sodium dodecyl sulfate (w/v), and 0.0025% bromo phenol blue (w/v) and heated at 100°C for 3 min. The mixture was then cooled and then subjected to PAGE.[13] The concentration of polyacrylamide in the stacking and separating gels were 4 and 12% respectively. The size of the gel cast was 7.3 × 10.2 × 0.1 cms. The voltage used was 200 V. Following electrophoresis, the gels were submerged in a fixative solution containing methanol, acetic acid and water (40 : 50 : 10 v/v) for 30 min, and silver stained.[14]

Subsequent experiments were carried out to determine the protective effect of taurine on the menadione-mediated damage to the lens under culture. This was accomplished by incubation of the contralateral rat lenses in Tyrode medium containing menadione (60 µM) in the absence or presence of 10 and 20 mM taurine. The extent of protection against the menadione-induced damage to the tissue offered by taurine was then determined using the above physiological parameters. Experiments were also carried out simultaneously with β-alanine, an amino acid structurally similar to taurine, but containing a carboxyl group instead of a sulfonic group present in taurine.

The presence of menadione in the lenses incubated with the quinone in the absence or presence of taurine was determined by spectroscopic measurement on the aqueous extracts prepared by homogenizing the post-incubation lenses in 1 ml of water and subsequent centrifugation.

RESULTS

The distribution ratios of the rubidium ion between the lens water and the medium of incubation under various conditions are summarized in Figure 1. The basal value was 37 ± 7. In the presence of menadione it was 6 ± 4, indicating a severe damage to the tissue. In the presence of taurine, the menadione-induced damage was minimized. The ratios with 10 and 20 µM taurine were 20 ± 6 and 25 ± 6 respectively. Interestingly, β-alanine, which differs from taurine only in having a carboxyl group instead of a sulfonate group was without any protective effect. This suggests that the effect observed is dependent on the presence of the sulfonate group. The requirement of taurine in concentrations higher than that of the menadione used might be attributable to a continuous generation of the ROS by menadione via its redox-cycling, and consequently the need for the continued availability of the protective agent.

The results of ^{86}Rb efflux from lenses incubated under various conditions are summarized in

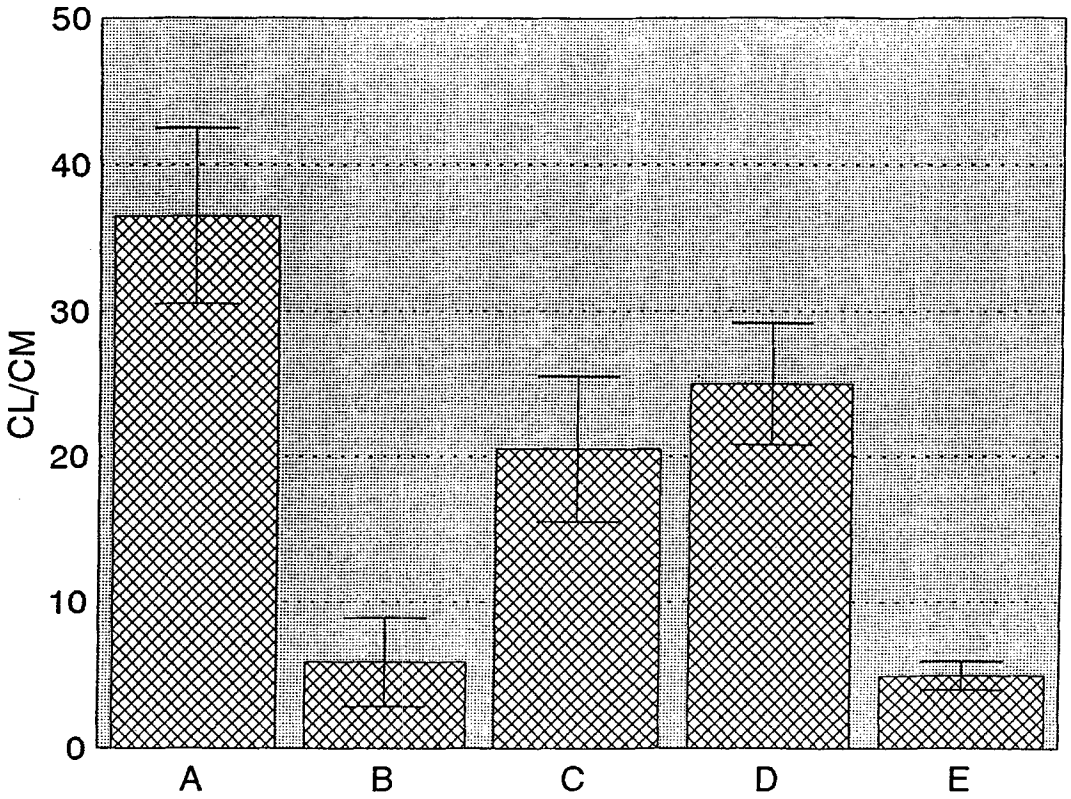

FIGURE 1 Distribution ratio of $^{86}Rb^{+}$ between lens water and medium of incubation: Freshly dissected rat lenses were incubated over night in Tyrode medium (spiked with $^{86}Rb^{+}$) containing 60 μM menadione in the absence (BAR: B) or presence of 10 and 20 mM taurine (Bars: C & D), or β-alanine (Bar: E). Bar: A = blank control. Following incubation, CL/CM ratios were determined as described in the methods. At least 6 experiments were conducted in each case. The values are mean ± standard deviation.

Figure 2. As shown therein, the efflux of ^{86}Rb was much greater in lenses incubated with menadione as compared to controls, indicating that it affects the passive efflux also. However, when taurine was added to the menadione-containing medium, the lenses were able to maintain an efflux rate similar to that of the controls.

Figure 3 shows that the absorption spectra of the aqueous extracts prepared from lenses incubated in the basal medium as well as in medium containing menadione with or without taurine. As may noted, there is a general increase in the absorption in the case of menadione incubated lenses with or without taurine, as compared to the basal controls. Also, the absorption of the extracts in the former two groups was similar. Their specific absorption at 303 nm (λ_{max} for menadione bisulfite) was also very close to each other (1.44 ± 0.033 for menadione and 1.40 ± 0.072 for menadione + taurine). Hence the preventive effect of taurine cannot be attributed to any blockage of menadione penetration into the lens.

Tissue damage by menadione and its prevention by taurine was further apparent by the levels of ATP and GSH. As summarized in Table I, addition of menadione to the incubation medium decreased the levels of both ATP and GSH significantly. Again, these levels were significantly higher in the presence of taurine. Taurine, in addition, prevented the oxidation-induced aggregation of soluble lens proteins into high

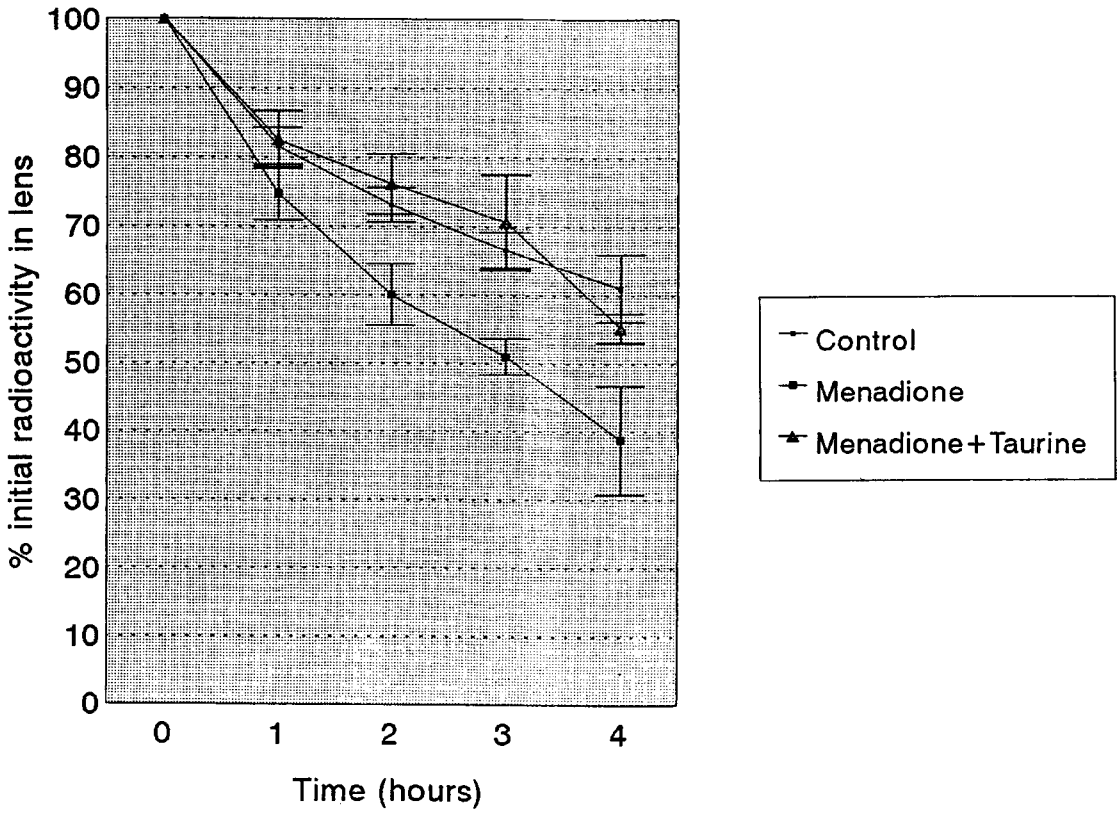

FIGURE 2 Efflux of $^{86}Rb^{+}$ from the lens: Freshly dissected rat lenses were incubated for 2 h in Tyrode medium containing $^{86}Rb^{+}$ (0.1 μCi/ml) at 37°C. Following incubation, they were rinsed with normal saline and their initial radioactivity determined by gamma counting. They were then transferred to 4 ml of Tyrode medium containing 250 μM menadione with (20 mM) or without taurine, and the efflux of $^{86}Rb^{+}$ was determined by counting an aliquot of the medium (100 μl) at indicated times. The efflux rate was expressed as the percent of initial radioactivity present in the lens.

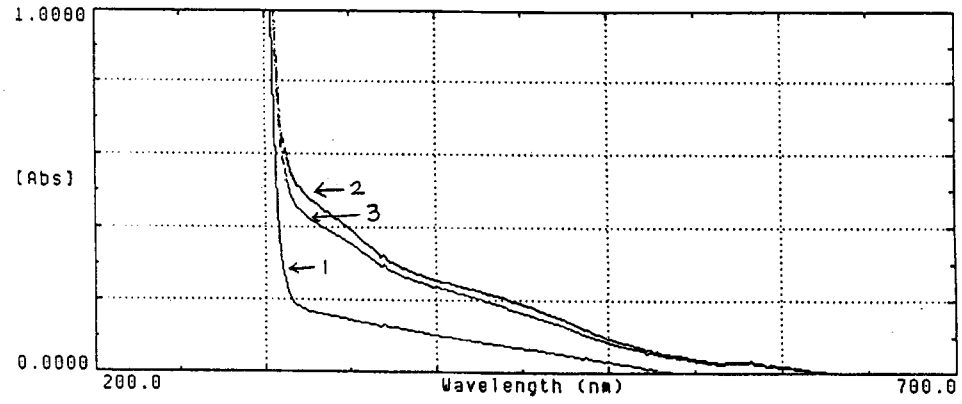

FIGURE 3 Spectral change in the lens caused by menadione: Rat lenses were incubated with menadione and taurine as described in Figure 1 without any $^{86}Rb^{+}$. Following incubation, they were rinsed with normal saline and homogenized in 1 ml of distilled water. A supernatant was obtained by centrifugation. The absorption spectrum of the supernatants was then recorded in a Beckman spectrophotometer. 1 = Basal control, 2 = menadione, 3 = menadione + taurine.

TABLE I ATP and GSH content of rat lenses: Effect of menadione and taurine

	Blank control (A)	Menadione (B)	B + Taurine (C)
ATP nmol/g lens	1534 ± 332	457 ± 238*	1048 ± 373*
GSH μmol/g lens	3.68 ± 1.0	0.86 ± 0.29*	1.60 ± 0.36**

ATP and GSH contents were determined in the incubated lenses as described in the Methods section. The values are mean ± standard deviation. $N = 6$ in each case. P values are as follows: *between A and B < 0.001, **between B and C < 0.05.

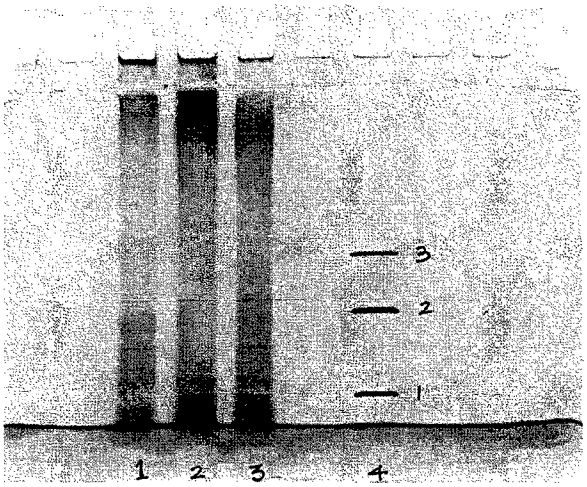

FIGURE 4 SDS-PAGE profile of the water insoluble proteins run without the use of β-mercaptoethanol in the denaturing buffer. One μg protein was loaded in each case. Lane 1 = lens incubated in basal medium, Lane 2 = lens incubated in menadione containing medium, Lane 3 = lens incubated in menadione + taurine containing medium, Lane 4 = molecular weight standards: 1 = 18 kDa, 2 = 29 kDa, 3 = 48 kda.

molecular weight species as apparent from the SDS-PAGE of the water insoluble (urea soluble) proteins. As shown in Figure 4, the stain intensities in the stacking gel, as well as at the top of the resolving gel were much greater in Lane 2, representing proteins from lenses incubated with menadione, as compared to basal (Lane 1), and the menadione + taurine treated lenses (Lane 3). The protein pattern of the taurine treated lenses (Lane 3) resembles that of the blank controls or even slightly better, suggesting the effectiveness of taurine in preventing protein oxidation as well.

DISCUSSION

Cataract is one of the major causes of visual impairment and blindness throughout the world. Several previous studies have demonstrated that an intra-ocular generation of ROS is one of the significant risk factors involved in the genesis of most cataracts, including the senile cataracts.[12,15–17] In this regard, nature has provided the lens with several enzymatic (catalase, superoxide dismutase, glutathione peroxidase) and non-enzymatic (ascorbate, glutathione) antioxidants to protect it against ROS mediated damage. The present studies demonstrate that taurine could be another such substance working in a concerted manner with other enzymatic and non-enzymatic antioxidants involved in delaying the cataractogenic process. This could be attributed to its ability to directly scavenge the highly reactive $OH^{\bullet}$[11] generated as a result of menadione redox-cycling. The results described herein are, therefore, in accordance with the above hypothesis. This has been physiologically demonstrated by the organ culture experiments. The presence of menadione in micromolar amounts in the medium induced significant physiological stress to the tissue as evidenced by the inhibition of the active transport mechanism, enhanced cation efflux rate, decrease in the contents of GSH and ATP, and an increase in the formation of high molecular weight proteins, as quantitatively apparent by SDS-PAGE. These menadione-induced changes were prevented significantly from taking place in the presence of taurine at levels normally present in the lens. The mechanism of taurine effect in this and other situations against oxidative stress however still remains unsettled. It should also be pointed out that the physiological effects of taurine under non-oxidative conditions remain more difficult to understand and can vary depending upon the nature of the physiological situation as well as the tissue involved. For example: recent studies from our laboratory have demonstrated that it can also competitively

inhibit glycation related protein modifications,[18] known to be associated with cataract formation in diabetes. Additionally, studies in other tissues have suggested that it is involved in maintaining cellular calcium levels.[19,20] Hence, further studies on the significance of taurine in lens physiology and cataract formation are desirable.

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