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Combination of glycemic and oxidative stress in lens: Implications in augmentation of cataract formation in diabetes

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Abstract

It is well known that the incidence of cataract is higher in diabetics as compared to non-diabetics. Its rate of maturation is also faster in the diabetics. The precise mechanism of this acceleration is not clearly understood. It is hypothesized that this could be a result of the combination of the metabolic and oxidative stress induced by glycemia itself with the age-associated increase in ambient generation of oxyradical species. In the current studies, we have investigated this possibility using the galactose cataract model. Galactosemia was induced by feeding rats a 50% galactose diet. The increased susceptibility of the glycemic lenses to physiological damage by reactive oxygen species (ROS) was studied by incubating them in Tyrode in the absence and presence of menadione. The resulting physiological damage to the lens was assessed initially in terms of its ability to maintain Na^+–K^+ ATPase dependent active transport of potassium ions, as represented by the uptake of rubidium ions. Subsequently, the level of ATP, indexing the general metabolic status, and the level of glutathione (GSH), indexing the status of antioxidant reserve, were also determined. The uptake of rubidium in the normal lenses incubated in the presence of the quinone was depressed to more than 50% of the controls run in the basal medium. A similar depression existed in the galactosemic lenses in comparison to the normal lenses. However, in the presence of menadione, the inhibition of the uptake was accentuated further in the case of galactosemic lenses, the uptake here being only 20% of the normal controls. Similarly, the galactosemic lenses were also more susceptible to menadione dependent decrease in ATP and GSH.

Keywords: *Cataract, galactosemia, menadione, oxidative stress, rubidium pump, glutathione*

Introduction

Second to the cornea, the major light transmitting tissue of the eye is the ocular lens. However, its transparency continues to decline with aging, the decline becoming functionally and clinically more significant by late 50's to 60's. A significant number of people in this age group have advanced opacities in their lens, causing excessive light scattering with the consequence of the formation of distorted images on the retina. In a large number, the opacity becomes so advanced that the person becomes visually handicapped, frequently ending with blindness. The etiology of these age associated changes remains incompletely understood. A number of investigations suggest that the progressive loss of transparency associated with the increasing age is cumulative and can be caused by a number of toxic physiological and environmental factors that lead to an excessive generation of reactive oxygen species (ROS) in the intraocular chambers, particularly in the aqueous humor [1–6]. In addition, certain nutritional and genetic factors can also be involved. The main environmental factor that can lead to the generation of ROS is the continued penetration of light into the eye, especially during photopic vision [2–4]. In general, the most important physiological factor playing a salient role in ROS generation is the age related decline in the cytochrome dependent respiratory activity that normally leads to a tetravalent reduction of the respired oxygen with eventual formation of water [7,8]. The decline in this mode of oxygen utilization has the consequence of its diversion towards several auto-oxidative reactions which generate super-oxide followed by its eventual

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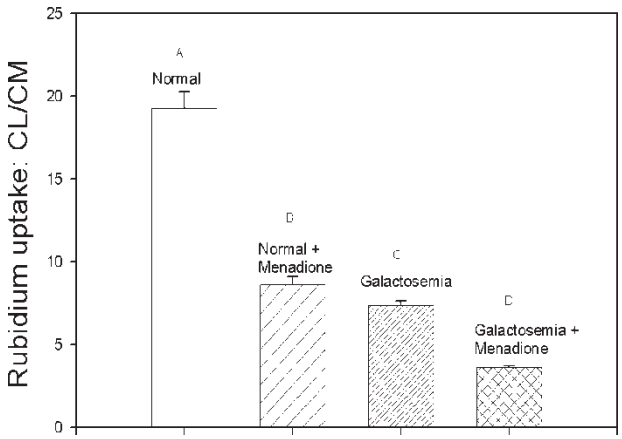

Figure 1. Rubidium uptake by normal and galactosemic lenses. The results are expressed as the distribution ratio (CL/CM) of the ion determined as described in the text. (A): Normal lenses incubated in basal medium (bm), (B): Normal lenses incubated in bm + menadione, (C): Galactosemic lenses incubated in bm, (D): Galactosemic lenses incubated in bm + menadione. The uptakes were depressed in groups B, C&D as compared to the basal controls (A). This depression was most severe in the case of D. $N > 6$ in each group. Values are expressed as Means ± S.D. P values between B&A, C&A and D&C are < 0.001.

conversion to other ROS. These species in turn can initiate a battery of unwanted reactions such as lipid peroxidation, protein carbonyl formation and a generalized oxidation of —SH bearing compounds that are essential in maintaining tissue redox status and its metabolism. The adverse effects of the ROS generated are hence very wide and highly varied.

Studies with experimental animals have shown that a high level of sugars in the lens can also initiate several unwanted biochemical reactions such as the formation of high levels of sorbitol and fructose [9–13], glycation of lens proteins [14–17], excessive diversion of glucose in the HMP shunt, and inhibition of glycolysis [18]. Existence of oxidative stress is also indicated by decrease in glutathione (GSH) and increase in the level of malonaldehyde, the latter being an index of lipid peroxidation [17]. Cataract formation in diabetes may hence represent a combination of glycemic and oxidative stress, the former stress being absent in the case of non-diabetics. The objective of these investigations was hence to evaluate this hypothesis. We have verified this by determining the susceptibility of a glycemic lens to physiological damage, relative to a normal lens, when exposed to ROS. Lenses were made glycemic by feeding animals a high galactose diet. The extent of relative oxidative stress was determined in culture by incubating the normal and glycemic lenses with menadione. The results seem to offer a suitable explanation of why cataract formation is more frequent in diabetics. The incidence of cataracts in diabetic individuals is well known to be higher than in the non-diabetic population. It also has an earlier onset and faster maturation.

Materials and methods

Sprague Dawley rats were used in accordance with ARVO guidelines. Animals weighing 75–100 g were divided into two groups with 10 animals in each. Group 1 was maintained on standard Purina Lab Chow and water ad lib. The other group was fed 50% galactose diet. On day 8 of the diet, the animals were sacrificed by CO_2 inhalation, eyes enucleated and lenses isolated atraumatically by the posterior approach. Immediately after isolation, they were incubated in culture dishes containing 4 ml of Tyrode medium mixed with $^{86}RbCl$ as a tracer [19]. Incubation was done for a period of 5.5 h at 37 degrees in a humidified incubator. Lenses from normal as well as galactosemic groups were incubated in contralateral pairs, one lens being incubated as control and the other with 1 mM menadione dissolved as sodium bisulphite (Sigma catalogue # M5750). 1 mM NaCl was added in the controls. Following incubation, the lenses were briefly rinsed with physiological saline to get rid of the adherent radioactivity. Subsequently, they were transferred to vials and their radioactivity determined by gamma counting. They were then homogenized in 1 ml of dH_2O and centrifuged to obtain a clear supernatant. ATP was determined by mixing 50 µl of the supernatant with 200 µl of phosphate buffer (0.3 M, pH 7.4) containing 0.02 M $MgSO_4$, luciferin and luciferase (Sigma FLE-50) housed in a luminometer and noting the peak luminescence [20]. ATP standards were run simultaneously. GSH was determined in the trichloracetic acid extract prepared by adding the acid to the above homogenate to a final concentration of 10% and centrifugation. 100 µl of this extract was mixed with 0.3 ml of 0.6 M di-sodium hydrogen phosphate and 100 µl of Ellman's reagent (2,5-dithionitrobenzoic acid, DTNB) prepared by mixing 4 mg DTNB in 10 ml of 1% Trisodium citrate solution [21]. The resulting 5-thionitrobenzoate was determined spectrophotometrically at 412 nm with reference to standards prepared simultaneously.

Results

The primary aim of this study was to determine if the adverse physiological and biochemical changes induced in the lens by glycemic stress are enhanced further by oxyradical induced oxidative stress. The possibility of such an enhanced susceptibility of the glycemic lenses to damage by oxidative stress was initially assessed by determining the extent of membrane damage, as apparent by its ability to carry out active transport of Rb^+, represented by the distribution ratio of the ion between the lens water (CL) and the medium of incubation (CM). As indicated in Figure 1, the CL/CM attained in the normal controls was 19.3 ± 1. It decreased to 8.6 ± 0.5 in the presence of menadione.

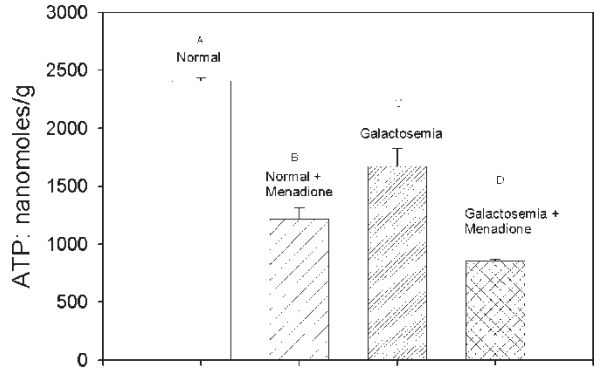

Figure 2. ATP content of the lenses. The levels in normal lenses incubated with menadione (B) and in the galactosemic lenses (C) are substantially lower than that in (A). An additional decrease was apparent when the galactosemic lenses were incubated with menadione (D), the level in this case being lower than in (B) as well as in (C). $N > 6$ in each group. Values are expressed as Means ± S.D. *P* values between B&A, C&A and D&C are < 0.001.

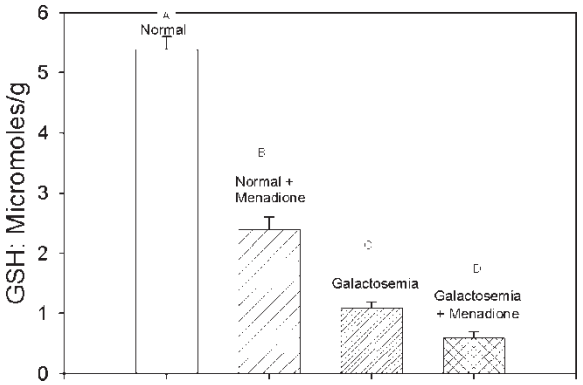

Figure 3. Level of lens glutathione. (A): Normal lenses incubated in basal medium (bm), (B): Normal lenses incubated in bm + menadione, (C): Galactosemic lenses incubated in bm, (D): Galactosemic lenses incubated in bm + menadione. The levels in (D) were most severely affected. $N > 6$ in each group. Values are expressed as Means ± S.D. *P* values are similar to that under Fig. 2.

However, the ratio in the galactosemic controls was 7.4 ± 0.2, with a further decrease to 3.6 ± 0.1 when menadione was present in the medium. It is therefore apparent that the inhibition of the active transport of rubidium caused by galactosemia alone was further aggravated in presence of ROS. If this aggravation is related merely to oxidative damage to the membrane itself or it also has a metabolic basis, was ascertained by measurements of ATP required for efficient operation of the rubidium pump. Hence, further experiments were conducted to determine the level of this compound in the lenses of various groups. As shown in Figure 2, its level in the normal controls was 2411 ± 20 nmol/g. Its level in the lenses of galactose-fed animals was depressed to 1675 ± 150 nmol/g, correlating with its lower transport activity, in comparison to the normal controls. On incubation with menadione, its level decreased in the normal as well as galactosemic groups, the corresponding values being 1215 ± 100 and 859 ± 75 nmol/g, respectively. The greater inhibition in the rubidium pump activity of the galactosemic lenses in the presence of menadione as compared to the normal, therefore, also correlates with the greater decrease in the levels of ATP.

Previous studies have demonstrated that the level of GSH, a major antioxidant reserve present normally in the lens in high concentrations, is depressed to a significant extent in hyperglycemia [17]. It also decreases in lenses exposed to oxidative stress, *in vitro* as well as *in vivo*. As anticipated, the decrease in the content of this tripeptide has also been found to be more substantial when the glycemic and oxidative stresses were combined. As shown in Figure 3, the GSH in the normal controls was 5.4 ± 0.2 μmol/g, in conformity with previous reports [3]. Addition of menadione to the medium led to its decrease to 2.4 ± 0.2 μmol/g. The GSH in the galactosemic controls without menadione was 1.1 ± 0.1 μmol/g, with a further lowering to 0.6 ± 0.1 with menadione.

Discussion

Cataract formation in diabetes involves multiple factors such as osmotic hydration of the lens due to sorbitol accumulation, protein glycation and inhibition of glycolysis. In addition to these direct biochemical changes that are induced by high levels of sugars, the diabetic lens is also considered to be under oxidative stress due to oxyradicals generated by auto-oxidation of sugars such as glucose and fructose known to be present at high concentrations in this disease. The presence of oxidative stress in these lenses was evident by the decrease in GSH level and an increase in malonaldehyde level. Such lenses therefore become deficient in their antioxidant reserve, making them more susceptible to oxidative damage by ROS normally generated in the mitochondria and cytosol. These ROS are normally scavenged by the tissue enzymatic and non-enzymatic defense mechanisms. However, the activity of such enzymes decrease with age, allowing the oxyradicals to remain relatively unscavenged and thereby making them freely available to induce oxidative modifications of proteins, lipids and nucleic acid and other susceptible components of the tissue. Such oxidative changes are now thought to be involved in the genesis of many age-related manifestations, including that of senile cataract. It is therefore possible that the acceleration of senile cataractogenesis in diabetics is due to the additive effects of hyperglycemia and age-associated oxidative stress, the oxidative stress inherent in the hyperglycemic state being aggravated further by the superimposition of age-related oxidative stress.

The possible enhancement of lens damage by a combination of these two stresses has been investigated in the present study using the rat galactose cataract model. As hypothesized, the lenses of galactose-fed rats were found to be more susceptible

to oxyradical induced damage, as compared to the normal lenses. This was first apparent by comparing the Na^+–K^+ ATPase dependent transport function of the lenses, represented by the distribution ratio of Rb^+ ions as described above. The ratio in the galactose fed rat lenses (glycemic stress) was about 30% of that in the normal controls. However, the depression of this ratio in the galactosemic lenses when subjected to oxidative stress was significantly greater as compared to that attained in the normal lenses exposed to a similar stress. This could be attributed to a decrease in the activity of Na^+–K^+ ATPase because of its glycation [22] as well as oxidation [23] coupled with the decreased availability of ATP [17].

The above enhancing effect of reactive oxygen was also reflected in the ATP content of the lenses. Overall, its level was significantly lower in the galactosemic rat lenses as compared to the normal lenses in the absence of menadione. Although, exposure of lenses in both the groups to oxidative stress by addition of menadione to the incubation medium resulted in a decrease in the ATP levels, the decrease was more substantial in the galactosemic lenses.

A similar decreasing trend was also noted with the levels of GSH, the decrease in the glycemic lenses being significantly greater than that in the normal lenses on incubation with menadione. This decrease is attributable to its oxidation by ROS generated by the quinone redox cycling, as well as to its conjugation (alkylation) with the quinone itself [24–26]. Since GSH constitutes a major antioxidant reserve of the lens, its depletion by either mechanism makes the tissue increasingly susceptible to oxidative damage. In the mode of the action of quinone as a redox cycler, leading to a continued production of ROS, GSH loss is attributed to GSSG formation, accompanied with H_2O_2 generation. The latter is neutralized by catalase and GSH peroxidase. The formation of GSSG is followed by its reduction to GSH by NADPH. This reaction is catalyzed by GSH reductase, producing NADP. This in turn stimulates the hexose monophosphate shunt, useful in the maintenance of NADPH level. The antioxidant effect of GSH is hence largely attributable to its direct action as a ROS scavenger as well as to the maintenance of the shunt via GSSG formation. Additionally, it is well known to maintain —SH groups of structural and enzymatic proteins.

Overall, the findings suggests that the glycemic lens when subjected to oxidative stress consequent to ROS generation is potentially more susceptible to a pathophysiological damage leading to cataract formation. Physiologically, this is evident by a greater inhibition of the rubidium transport in the galactosemic lenses when incubated with menadione, as compared to the normal lenses. The maintenance of the membrane transport function in the lens is well known to be crucial for appropriate maintenance of tissue bioenergetics as well as electrolyte and osmotic balances. Such an enhancement of physiological damage in the galactosemic lenses caused by ROS is also apparent by simultaneous decreases in the levels of ATP and GSH. The results therefore strongly suggest that the imposition of oxidative stress on a diabetic lens or imposition of glycemic stress on a lens already undergoing oxidative stress could be intimately involved in the commonly observed accelerated rate of cataract formation and associated loss of visual acuity in the diabetics. Such additive factors can also explain its greater prevalence in the diabetics than that encountered in the non-diabetic population.

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