Effect of Diabecon on sugar-induced lens opacity in organ culture: mechanism of action

M.S. Moghaddam a, P. Anil Kumar b, G. Bhanuprakash Reddy b, V.S. Ghole a,∗

a Biochemistry Division, Department of Chemistry, University of Pune, Pune 411007, India
b National Institute of Nutrition, Hyderabad 500007, India

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Abstract

Cataract is the leading cause of blindness worldwide. Apart from ageing, diabetes has been considered to be one of the major risk factors of cataract. The high sugar levels in diabetes may cause tissue disruption and intumescences by osmotic changes induced via aldose reductase (AR) mediated polyol pathway. Therefore, agents that can inhibit AR and prevent sorbitol accumulation may be helpful to combat sugar-induced cataract. In the present study, AR inhibitory activity of Diabecon (an herbal drug used for diabetes) was studied together with its effect against sugar-induced lens opacity in organ culture. Diabecon aqueous extract (DAE) showed potential inhibitory activity with an IC50 value of 10 μg/ml against rat lens AR. Incubation of goat lens with supraphysiological concentrations of glucose (100 mM) led to the loss of lens transparency associated with increased AR activity, decreased soluble protein and increased protein carbonyls and glycation. Addition of DAE (0.3 mg/ml) to the medium preserved transparency and ameliorated the decrease in lens soluble protein due to hyperglycemia and also prevented the formation of glycated protein. Interestingly, DAE inhibited aldose reductase activity in lens incubated with 100 mM glucose. DAE decreased protein carbonyls, prevented the loss of α-crystallin against 100 mM of glucose. We have also demonstrated here that most of these effects are mainly due to Gymnema sylvestre, one of the constituent herbs of Diabecon. These results suggest that Diabecon protect the lens against sugar-induced cataract by multiple mechanisms.

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Cataract is the opacification of eye lens, associated with the breakdown of the eye lens micro-architecture, which interferes with transmission of light onto the retina. Cataract remains the leading cause of blindness and visual impairment worldwide. It was estimated that 38 million people in the world are blind while an additional 110 million people have visual impairment (Thylefors, 1998). The age-attenuated prevalence of cataract in India is three times that of the United States (Brian and Taylor, 2001). Even though effective surgical procedures are available for treatment, the problem of post-operative complications, cost of surgery and high number of people requiring surgery pose a substantial economic burden. Apart from aging, various risk factors of cataract include: nutritional inadequacy, metabolic and inherited defects, UV radiation and smoking. Diabetes has been considered to be one of the major risk factors of cataract (Harding, 1991; Ughade et al., 1998). Many experimental studies in vivo and in vitro support the view that diabetes enhances the risk of cataract formation.

During hyperglycemia the cellular levels of glucose greatly increase in tissues where glucose entry is independent of insulin, which include lens, retina, kidney and peripheral nerves (Harding, 1991; Kador, 1998). The excess glucose levels in diabetes flux via polyol pathway (Kinoshita, 1990), which accounts for 30% of glucose utilized (Gonzalez...
et al., 1984) whereas this pathway accounts only 3% under normoglycemic conditions (Morrison et al., 1970). Aldose reductase (AR, EC 1.1.1.21), key enzyme of polyol pathway, catalyzes the reduction of glucose into the corresponding sugar alcohol, sorbitol (Kinoshita, 1990), which is subsequently metabolized into fructose by sorbitol dehydrogenase. Sorbitol, an osmolyte, accumulating in the cells due to its poor penetration through cellular membranes and slow metabolism by sorbitol dehydrogenase, leads to osmotic swelling, changes in membrane permeability, leakage of glutathione and myo-inositol and perhaps even the generation of free radicals and hydrogen peroxide (Kinoshita, 1974; Kinoshita and Nishimura, 1988; Wolff et al., 1991) leading to generation of oxidative insult. The resulting hyperosmotic stress to cells is postulated to be the primary cause for the development of diabetic complications such as cataract, retinopathy, neuropathy and nephropathy (Williamson et al., 1992). Since AR induced/mediated changes being major results in the development of diabetic cataract, the inhibition of aldose reductase is, therefore, one of the potential pharmacological approach that has been proposed to treat or ameliorate secondary complications of diabetes including cataract. It has been estimated that a delay in cataract formation by at least 10 years would reduce the cataract surgical burden perhaps by 45%.

Alternative medicine, use of herbs, dietary supplements and nutraceuticals, have become a major part in the clinical treatment of many chronic disorders including diabetes (Grover et al., 2002; Outre et al., 1997; Swanson-Flatt et al., 1991). However, relatively little work has been done on the antidiabetic agents with respect to their effect on secondary complications of diabetes. Diabecon (commercial name of a polyherbal drug) is a mixture of various extracts derived from Indian indigenous herbs commonly used against diabetes. In the present study, we have investigated the AR inhibitory potential of Diabecon and as well as protection against the effects of supraphysiological concentration of glucose in lens organ culture system.

1. Methods

1.1. Materials

Glucose, TC-199 medium (#M-3769), tris-glyceraldehyde, lithium sulfate, β-mercaptoethanol, NADPH, 2,4-dinitrophenylhydrazine (DNPH), trichloro acetic acid (TCA), phenyl boronate affinity chromatography column (8A-S530) were purchased from Sigma Chemical Co. (St. Louis, MO). Diabecon was obtained from Himalaya Drugs Co. (Bangalore, India).

1.2. Plant materials and sample preparation

Diabecon available as tablets were powdered and its constituents were extracted into water and other organic solvents. Aerial parts of some of the ingredient plants of Diabecon such as Gymnema sylvestre, Eugenia jambolana, Gymnema arborea were obtained from local market and dried, powdered and aqueous and organic extracts were prepared.

1.3. Rat lens aldose reductase

Rat lenses were used as source of AR for AR inhibitory assay as rat lens possesses maximum AR activity. Crude aldose reductase was prepared from rat lens. Eyeballs were removed from 9-week-old control WNN male rats obtained from National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad. Lenses were dissected by posterior approach and homogenized in 10 volumes of 100 mM potassium phosphate buffer pH 6.2. The homogenate was centrifuged at 15,000 × g for 30 min at 4 °C and the resulting supernatant was used as the source of AR.

1.4. Aldose reductase assay

AR activity was assayed essentially as described earlier (Suryanarayana et al., 2004). For inhibition studies concentrated stocks of various extracts of Diabecon and other ingredient plant extracts were added to assay mixture and incubated for 5 min before initiating the reaction by NADPH considering AR activity without inhibitor was 100%.

1.5. Lens organ culture

Eyes were enucleated from 6 months old male goats obtained from slaughterhouse. Lenses were dissected from the eyes by anterior approach. Each isolated lens was incubated in 5 ml of modified TC-199 medium with antibiotics according to Zigler and Hess, (1985) as described earlier (Suryanarayana et al., 2004) under conditions of 95% air and 5% CO2 at 37 °C with 100 mM glucose for a period of 10 days. Lenses incubated with 5.5 mM glucose (physiological conc.) served as control. Damaged lenses were identified by determining the protein content of an aliquot of the medium after an equilibration period of 2h and were terminated. When added to medium, a stock of aqueous extract of Diabecon or Gymnema sylvestre was prepared in water and filtered. Medium was changed every 48h and supplemented with Diabecon aqueous extract (DAE) or Gymnema aqueous extract (GAE), along with glucose 100 mM (supraphysiological concentration of glucose). All the reagents used in lens culture were filtered through 0.2 μm filter.

Lenses were observed for development of generalized haziness or opacities, intumescences, disruption and other morphological changes. After 10 days of culture the lenses were homogenized in buffer containing 25 mM Tris, 100 mM NaCl, 0.5 mM EDTA and 0.01% NaN3, pH 8.0. The soluble fraction of homogenate (10,000 × g for 30 min at 4 °C) was used for further analysis. Protein concentration was determined by method of Lowry et al.
1.6. Assay of carbonyl groups

Protein carbonyl groups were estimated by the method of Uchida et al., (1998). In brief, 0.5 ml (0.5 mg) protein samples were incubated with an equal volume of 0.1% of 2,4-DNPH in 2N HCl and kept at room temperature for 1 h. After incubation, protein was precipitated by 20% TCA (0.5 ml) and washed three times with 1 ml of ethanol/ethyl acetate (1:1) mixture. Finally, precipitate was solubilized in 133 mM Tris, 13 mM EDTA buffer, pH 7.4 containing 8 M urea and absorbance was read at 365 nm. Concentration of protein carbonyls was calculated by using molar extinction coefficient \( \varepsilon_{365\text{ nm}} = 21 \text{ mM}^{-1} \text{ cm}^{-1} \).

1.7. Crystallin distribution profiles by FPLC

Crystallin distribution in the soluble protein fraction was performed by gel filtration chromatography on Superose 6 HR 10/30 column using FPLC system (AKTA-purifier, Amersham Biosciences). The column was equilibrated with buffer containing 0.05 M sodium phosphate and 0.15 M sodium chloride, pH 7.2 at a flow rate of 0.2 ml/min. Soluble protein samples (100 μl of a 1 mg/ml solution) were loaded on to the column and protein peaks were detected at 280 nm.

1.8. Fluorescence measurements

Intrinsic tryptophan fluorescence was recorded using a Cary Eclipse spectrofluorometer by exciting at 280 nm and following the emission between 300 to 400 nm. For all measurements, 0.15 mg/ml protein in 20 mM sodium phosphate buffer, pH 7.2 was used.

1.9. Estimation of glycated proteins by Boronate affinity column

Soluble fraction of lens homogenate was applied to phenyl boronate affinity column (1 cm × 10 cm) that had been equilibrated with 0.25 M ammonium acetate buffer, pH 8.8. Column was washed with same buffer used for equilibration. 0.1 M Tris-HCl, pH 7.5 containing 0.2 M sorbitol was used to elute bound glycated protein.

2. Results

Diabecon, a polyherbal drug formulation, contains extracts of plants available in India and some other Asian countries which are known for antidiabetic/ hypoglycemic (Ganguly et al., 1995; Mitra et al., 1996a), hypolipidemic properties (Mitra et al., 1996b) and are effective against diabetic retinopathy (Kant et al., 2002). In the present study, the formulation was selected to evaluate its aldose reductase inhibitory potential and protective effects against glucose induced lens opacity and associated changes in organ culture.
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Total protein (mg/g)</th>
<th>Soluble protein (mg/g)</th>
<th>Soluble protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>178 ± 6.5</td>
<td>152 ± 5.5</td>
<td>85</td>
</tr>
<tr>
<td>Glucose 100 mM</td>
<td>113 ± 4.7*</td>
<td>77 ± 5.9*</td>
<td>68</td>
</tr>
<tr>
<td>Glucose 100 mM + 0.3 mg/ml DAE</td>
<td>172 ± 11.1</td>
<td>155 ± 7.1</td>
<td>90</td>
</tr>
<tr>
<td>Glucose 100 mM + 0.3 mg/ml GAE</td>
<td>176 ± 9.6</td>
<td>153 ± 6.8</td>
<td>87</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.; n=4.

* Statistically significant from control (analyzed by ANOVA; p<0.01).

Fig. 2. Effect of Diabecon and Gymnema on carbonyl content in glucose incubated lens. (A) Control, (B) 100 mM glucose, (C) 100 mM glucose + 0.3 mg/ml DAE and (D) 100 mM glucose + 0.3 mg/ml GAE. Data are mean ± S.E. (n=4).

Hyperglycemia may induce oxidative stress. Supplementing with DAE or GAE decreased the carbonyls (Figs. 2 and 3). When soluble protein of lens incubated with 100 mM glucose subjected to phenyl boronate column which has affinity for glycated protein 18.6% of the protein was retained by the gel and was subsequently eluted by 0.1 M Tris–HCl, pH 7.5 containing 0.2 M sorbitol (Fig. 4). There is a decrease in the retention of glycated protein by the boronate column with soluble protein on DAE treatment (Fig. 4) showing significant antiglycating potential of this agent that needs to be investigated in detailed manner. The specific activity of AR was significantly higher in lens incubated with 100 mM glucose than control (Table 2) while AR activity was decreased significantly in lens supplemented with DAE or GAE (Table 2). Tryptophan fluorescence, a measure of protein tertiary structure and conformational changes of soluble protein of lenses incubated with 100 mM glucose found to be decreased and this decrease was prevented in the presence of DAE or GAE (Fig. 5). To investigate possible alterations in crystallin profiles due to supraphysiological concentrations of glucose and the influence of DAE, soluble protein were analyzed by gel filtration. The distribution profile by FPLC has shown a decrease in β, increase in β and a shift in α-crystallin peak in 100 mM glucose incubated lens compared to control. Addition of DAE to the medium during the lens culture with high glucose normalized the alterations in crystalline profile (Fig. 6).

3. Discussion

Since antiquity, diabetes has been treated with plant medicines and herbal drugs and some of which are remarkably effective. In recent times, some medicinal herbs are investigated for their potential against secondary complications of diabetes such as cataract (Halder et al., 2003; Suryanarayana et al., 2004). Sugar cataract formation associated with diabetes has been linked to the aldose reductase catalyzed production of sugar alcohols (polyols) (Kinoshita, 1990). We have selected Diabecon based on its hypoglycemic/antidiabetic activity and evaluated its AR inhibitory activity. Further, we have also assessed its anti-cataract potential in lens organ culture being a model frequently used for studying the multifactorial process of cataractogenesis (Xu et al., 1992; Spector et al., 1993). In the present study, AR activity was significantly increased in hyperglycemic lens, whereas in lens incubated with DAE or GAE, AR activity was decreased substantiating the in vitro inhibitory potential of DAE or GAE against rat lens AR. Further, DAE or GAE also prevented the alterations due to osmotic stress in cultured lenses. The total and soluble protein content in lenses incubated with supraphysiological concentrations of glucose is decreased (Table 1) when compared with control lens. It was known that during cataractogenesis large amounts of insoluble protein derived from the soluble protein gets accumulated resulting in decreased total and soluble protein. However, decrease in soluble protein was signif-
Fig. 4. Phenyl boronate affinity chromatogram. Soluble protein from the lens cultured with glucose applied to column. After washing the unbound fraction with 0.25 mM ammonium acetate, the bound fraction was eluted with 0.1 M Tris–HCl, pH 7.5 containing 0.2 M sorbitol and expressed as percentage of total protein loaded onto the column. Control (trace 1), 100 mM glucose (trace 2) and 100 mM glucose + 0.3 mg/ml DAE (trace 3). Inset: bar diagram represent the percentage of glycated protein; (A) control, (B) 100 mM glucose and (C) 100 mM glucose + 0.3 mg/ml DAE.

Out of three constituent herbs of Diabecon tested, only Gymnema sylvestre showed potential AR inhibition. It should be noted that most of the health benefits of Diabecon have been attributed mainly to Gymnema sylvestre and moreover Gymnema has been shown to have many other beneficial effects (Porchezhian and Dobriyal, 2003; Shanmugasundaram et al., 1990). Gymnema was shown to control blood glucose levels and exert antidiabetic activity (Grover et al., 2002; Porchezhian and Dobriyal, 2003; Shanmugasundaram et al., 1990; Yeh et al., 2003). Therefore, we have carried parallel studies with DAE and GAE. GAE was found to be more effective than DAE in terms of not only inhibitory effect against AR in vitro but other hyperglycemia induced changes in lens.
Thus, it appears that Gymnema suggest the usefulness of Diabecon and lens culture model in the present study. Finally, these results suggest the usefulness of Diabecon and Gymnema sylvestre particularly against the treatment of diabetic complications such as cataract.

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References


