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Strong inhibition of the polyol pathway diverts glucose flux to protein glycation leading to rapid establishment of secondary complications in diabetes mellitus

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ABSTRACT

Background: Polyol pathway and protein glycation are implicated in establishing secondary complications in diabetes. Their relative contribution to the process needs to be evaluated. It is essential to understand why some aldose reductase inhibitors (ARIs) trials are successful while some have failed and to study their effect on protein glycation.
Methods: Aldose reductase (AR) was assayed using xylose as substrate; protein glycation was evaluated using total and specific fluorescence, fructoseamine and protein bound carbonyl content (PCO) measurements. Long term studies were carried out on streptozotocin induced diabetic rats for evaluation of urine parameters, tissue fluorescence. Anti-cataract action was studied by lens culture studies.
Results: Epalrestat, a commercial ARI was also found to possess potent glycation inhibitory action. Long term experiments revealed strong protein glycation with higher concentration of citronellol (ARI) demonstrating shift in glucose flux. Treatment with epalrestat and limonene revealed improved urine parameters and tissue fluorescence. Lens culture studies revealed cataract formation at higher inhibition of AR while no lens opacity was observed at lower citronellol concentration and with limonene and epalrestat.
Conclusion: Strong inhibition of AR shifts the glucose flux to protein glycation causing damage. ARIs possessing protein glycation inhibition are more useful in amelioration of secondary complications.

1. Introduction

Aldose reductase (AR) has been implicated in exacerbation of diabetes through several mechanisms such as increase in sorbitol content (Toth et al., 2007), decrease in reduced glutathione (Brownlee 2001), enhanced NADH/NAD ratio (Nyengaard, Ido, Kilo, & Williamson, 2004) and enhanced oxidative stress. The polyol pathway is activated in hyperglycemia leading to these changes. Several studies using aldose reductase inhibitors ARI have reported alleviation of symptoms of neuropathy, retinopathy and nephropathy (Dunlop, 2000; Hotta et al., 2006; Sun, Oates, Coutcher, Gerhardinger, & Lorenzi, 2006). However, several studies have also reported failure of use of such inhibitors raising doubts on the efficacy of such inhibitors in the long term usage (Sima, Nathanial, Prashar, Bril, & Greene, 1991). Equally intriguing is the fact that the role of aldose reductase in clearance of 4-hydroxy trans-2-nonenal (HNE), a lipid peroxidation derived aldehyde is vital in prevention of cytotoxic injury to the cell. Further, AR inhibition has been shown to cause a significant increase in HNE in the arterial wall leading to a threefold increase in cell apoptosis (Rittner et al., 1999). Up-regulation of aldose reductase is suggested to play a major role in initiating oxidative stress induced signaling leading to activation of Protein Kinase C (PKC) and formation of nuclear factor κβ (NF-κβ) and activator protein 1 (AP1) and inflammatory diseases which can be prevented by inhibition of aldose reductase enzyme (Ramana, 2011). Hence, research on aldose reductase presents a confusing picture.

Another major pathway equally implicated in the pathogenesis of secondary complications of diabetes is protein glycation (Singh, Barden, Mori, Beilin, 2001). Glycation of proteins triggers formation of dicarbonyls and a series of advanced glycation end products (AGEs) through Amadori rearrangement and Maillard reactions (Thornalley, Langborg, & Minhas, 1999). Such products of glycation participate in establishment of secondary complications (Ahmed, 2005). Inhibitors of protein glycation have demonstrated to be effective in amelioration of these secondary complications (Bolton et al., 2004; Figarola et al., 2003).

It is essential to understand the relative contribution of the aldose reductase and protein glycation inhibitors to prevent the devastating

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effects of diabetes (Boel, Selmer, Flodgaard, & Jensen, 1995). Enhanced hyperglycemia leads to a large influx of glucose in insulin independent tissues leading to activation of these two major pathways. It has been suggested that 30% of the glucose entering inside the cells is metabolized through the polyol pathway (Suryanarayana, Kumar, & Saraswat, 2004). Likewise extensive intracellular protein glycation is known to be associated with hyperglycemia. The present study deals with an analysis of inhibition of aldose reductase vis-à-vis inhibition of protein glycation. We hypothesized that if these are the two major pathways for metabolism of glucose, inhibition of aldose reductase should increase the flux of glucose towards protein glycation enhancing the formations of AGEs, or else the aldose reductase inhibitors may also be inhibiting protein glycation.

The objective of the present study is to evaluate the effect of aldose reductase inhibitors and protein glycation inhibitors on cataract formation, fructosamine formation, dicarbonyl formation and fluorescent AGEs formation. Toxicity studies of the components were carried out using MTT and comet assays. The study deals with usage of an inhibitor that is purely aldose reductase inhibitor (citronellol), one that can exclusively act as a protein glycation inhibitor (limonene) and an inhibitor that can inhibit both the pathways (epalrestat). We have identified limonene as a potent glycation inhibitor (Joglekar, Panaskar, Chougale, Kulkarni, & Arvindekar, 2013) isolated from *Aegle marmelos* Correa and also determined its mechanism of action. Recently, we have reported citronellol from C*ymbopogon citratus* as an exclusive inhibitor of aldose reductase (Jagdale, Kamble, Nalawade, & Arvindekar, 2015). Both citronellol and limonene are considered for Generally Recognized As Safe (GRAS) status for use as a flavoring ingredient.

2. Materials and methods

2.1. Materials

Xylose, lithium sulfate, TCA, DNPH, nitro blue tetrazolium (NBT), ethyl acetate, streptozotocin (STZ), sodium tungstate, picric acid, low melting point agarose, and normal melting point agarose are all AR grade chemicals and were procured from local supplier. Nicotoneamide adenine dinucleotide phosphate hydrogen (NADPH), aminoguanidine, 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT), HEPES, BSA, citronellol, and limonene were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Aldose reductase enzyme isolation and inhibition

Aldose reductase enzyme was isolated from goat eye lens and the enzyme assay was performed as previously described (Hayman & Kinoshita, 1965) with some modifications, Fresh goat eyeballs were obtained from slaughter house immediately after sacrifice and transported to the laboratory at 0–4 °C. One gram lenses were washed with saline and homogenized in 3 volumes of saline containing 10 mM beta-mercaptoethanol. The homogenate was subjected for centrifugation and the supernatant was used as crude enzyme and protein content was measured by Lowry's method (Lowry, Rosenbrough, Farr, & Randall, 1951). Partial purification of enzyme was carried out by ammonium sulfate precipitation in 3 stages at 40%, 50%, and 75% saturation. The precipitated enzyme was dialyzed overnight. Dialyzed enzyme was used for determination of AR activity and the enzyme activity was expressed in μM of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidized/min/gm lenses. The reaction mixture contained 0.2 ml of freshly prepared enzyme, 0.067 M phosphate buffer (pH 6.2), 0.125 mM NADPH, 400 mM $LiSO_4$ and 40 mM xylose (substrate) and different concentrations. The decay of absorbance (due to the NADPH oxidation) was measured at 340 nm for 5 min using an Eppendorf UV-spectrophotometer.

2.3. In vitro glycation inhibition studies of BSA

Bovine serum albumin (BSA) 20 mg/ml, in phosphate buffer (0.1 M, pH 7.4) contained sodium azide (0.02%), 50 μg/ml, 100 μg/ml of epalrestat, 25 μg/ml, 50 μg/ml of citronellol, 100 μg/ml aminoguanidine and 50 μg/ml, 100 μg/ml of limonene. Each reaction mixture was pre-incubated with test molecules for 10 min. before the addition of 250 mM glucose. Reaction mixture was incubated at 37 °C for 21 days. At the end of the incubation, unbound glucose was removed by extensive dialysis against the same buffer. Protein concentration was determined by Lowry's method using BSA as standard.

2.3.1. Fructosamine analysis

The fructosamine formed during non-enzymatic glycation of protein was determined by using nitro blue tetrazolium (NBT) assay as described previously (Baker, Zyzak, & Thorpe, 1994). The aliquots of glycation materials (0.2 ml) and 0.8 ml 300 μM NBT reagent in sodium carbonate buffer (100 mM, pH 10.3) were added in the reaction mixture and incubated at room temperature for 15 min, and absorbance was measured at 530 nm on UV–visible spectrophotometer. The fructosamine content was calculated based on the molar extinction coefficient of 12,640 M^{-1} cm^{-1} for monoformazan (Ansari & Ali, 2011).

2.3.2. Determination of protein bound carbonyls (PCO)

Protein bound carbonyl content as an indicator of oxidation of protein was measured as described previously (Nakamura & Goto, 1996). Briefly, dialyzed protein solution was mixed with equal volume of 20% trichloroacetic acid (TCA). The precipitate formed was retained and treated with 2 N HC1 containing 10 mM DNPH at room temperature for 1 h. After that, the reaction mixture was centrifuged at 11,000*g* for 10 min. The precipitates were washed with an ethanol-ethyl acetate (1:1) mixture three times and the final precipitates were dissolved in 8 M urea (in 2 mM potassium phosphate, pH 2.3). The absorbance was measured at 360 nm and the carbonyl content was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}. Samples processed similarly but without DNPH treatment were used as controls. Protein concentration was determined by Lowry's method.

2.3.3. Measurement of total AGEs

The fluorescence intensity of total AGEs was measured using a JASCO Model F-750 (Japan) spectrofluorometer with excitation and emission maxima at 370/440 nm, respectively (Panaskar, Joglekar, Taklikar, Haldavnekar, & Arvindekar, 2013). The results were expressed as AU mg^{-1} protein. Percentage inhibition of total AGE formation was calculated using the following formula;

$$\% \text{ Inhibition} = (1 - F \text{ sample} / F \text{ control}) \times 100$$

where F = fluorescence intensity at $370_{ex}/440_{em}$.

2.3.4. Measurement of specific AGEs (pentosidine)

The pentosidine specific fluorescence was measured at excitation and emission wavelengths 335/385 nm. (Sell & Monnier, 1989). Specific AGE formation was calculated by using a formula similar to total AGE measurement.

2.4. Long-term animal experiments

All animal experiments were carried out according to the guidelines of animal ethical committee of the Shivaji University and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) formed by the Government of India (Registration no. 1825/PO/EReBi/S/15/CPCSEA). Male Wistar rats weighing about 190–200 g were used for