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Activation of Aldose Reductase in Rat Lens and Metal-Ion Chelation by Aldose Reductase Inhibitors and Lipoic Acid

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Sorbitol formation in rat lenses incubated with high levels of glucose was related to activation of aldose reductase (AR). The hyperglycaemia-activated aldose reductase was inhibited by α-lipoic (thioctic) acid, *O*-phenanthroline and aldose reductase inhibitors (ARIs) including Zeopolastat (ZPLS), Sorbinil (SBN) and AL-1576. This study also examined ARIs for the ability to chelate metal ions. We found that ARIs suppress copper-dependent ascorbate oxidation, lipid peroxidation and hydrogen peroxide production in erythrocytes. ARIs also increased partition of copper ions into n-octanol, which indicates formation of lipophilic complexes. Our data support the hypothesis that transition metals may be involved in activation of the polyol (aldose reductase) pathway. Also, ARIs function as metal-chelating antioxidants that may contribute to their therapeutic role for diabetic complications.

INTRODUCTION

In a chronic hyperglycaemic state, increased sorbitol production and NADPH consumption due to activation of aldose reductase may contribute to diabetic complications. Hyperglycaemia is known to cause an increase in aldose reductase activity in erythrocytes of diabetic subjects.[1] Aldose reductase inhibitors (ARIs) have been shown to inhibit the development of cataract as well as complications of the kidney and nerves in experimental diabetes.[2] Treatment with ARIs was also found to increase the GSH/GSSG ratio but not to normal values.[3,4]

Moreover, it has been suggested that some inhibitors of NADPH-dependent aldose reductase are potent inhibitors of copper and iron-catalysed ascorbate oxidation and thus possess the potential for antioxidant effects.[5] In ocular tissue, there are abundant biological reductants such as ascorbic acid, glucose and thiols, which are prone to be pro-oxidants in the presence of "decompartmentalized" transition metals, leading to oxidative tissue damage.[6] This study is a further investigation of the possible mechanisms of aldose reductase activation and ARIs action.

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MATERIALS AND METHODS

Preparation of Rat Lens Homogenate

After incision of the capsule, fresh rat lenses (n = 5–6, per group) were weighed and subsequently washed with cold potassium phosphate buffer (50 mM, pH 6.0). The lenses were then homogenised in potassium phosphate buffer (50 mM, pH 6.0) containing 2-mercaptoethanol (5 mM). The suspension was centrifuged at 1500 × g for 30 min and supernatant was then used for measurement of activity of aldose reductase.

Whole Lens Incubation

Fresh rat lenses (n = 5–6, per group) were weighed and subsequently washed with cold potassium phosphate (50 mM, pH 6.0). The samples were then incubated in 2ml of chelex-treated potassium phosphate buffer (50 mM, pH 7.4) containing varying concentrations of glucose in the presence or absence of sorbinil (SBN), α-lipoic acid (LA) or *O*-phenanthroline (OPT). Incubation was performed under sterile conditions at 22°C at room temperature for 20 hours. Sample medium was removed and the lenses were washed five times with cold distilled water. Cold potassium phosphate buffer (50 mM, pH 7.4) was added to make up 100mg tissue/ml and lens homogenates were prepared as described above. Insoluble tissue pellets were removed by centrifugation at 2500 × g for 15 min. Supernatant (0.2 ml) was used for sorbitol determination.

Analysis for Aldose Reductase (AR) Activity

(i) NADPH Oxidation Briefly, the appropriate amount of pure enzyme or lens homogenate was added to 1 ml of potassium phosphate buffer (50 mM, pH 6.0) containing 0.1 mM NADPH and 10 mM DL-glyceraldehyde. Rate of NADPH oxidation was followed by monitoring decrease in absorbance at 340nm at 25°C for 3 min.[1,6] One unit of enzyme activity is the amount of enzyme which catalyses the oxidation of 1μmol of NADPH/min.

(ii) $NADP^+$ Formation Aliquot of lens homogenate (50 μl) was mixed with potassium phosphate buffer (50 mM; pH 6.0) containing 0.4 M lithium sulphate, 5 mM 2-mercaptoethanol. The sample was incubated for 3 min at 37°C. Subsequently, NADPH (0.1mM) was added and the reaction mixture was incubated for an additional one min. The reaction was started by the addition of DL-glyceraldehyde (10mM). The reaction was terminated after 5 min by adding 0.2 ml of HCl (0.5 M). After an additional 10 min, 2ml of a solution containing NaOH (6 M) and imidazole (10mM) was added. Signal was monitored (Excitation 360nm and Emission 460nm) as described elsewhere.[6]

(iii) Sorbitol measurement by gas chromatography (GC)

Sample Preparation: Lens homogenate (20 mg) was mixed with 1 ml of methanol containing 250 mmol mannitol as internal standard and the sample was kept at –20°C for 20 min. The sample was centrifuged at 1000 × g for 10 min and supernatant was transferred to glass tube. Hexane (1 ml) was added and the mixture was vortex mixed. The sample was centrifuged and hexane (upper) layer was discarded. The methanol layer (containing polar substances) was dried under a stream of nitrogen at room temperature.

Derivatisation: The dry methanolic extract was mixed with 100 ml of N,O-bis(trimethylsilyl)-trifluoroacetamide (Pierce, Rockford, Ill). After 60 min incubation at 60 °C, sample (1 μl) was injected onto a CP-Sil 5 CP (25 m × 0.25 ID, film thickness 0.25 mm, Chrompack, The Netherlands). Splitless injection was performed at 240°C. Column temperature was programmed from 140°C (2 min hold) with 2°C/min to 240°C (10 min hold). Detector (flame ionization) was held at 250°C.

Drug-Induced Catalase Inactivation (H_2O_2 Production) in Erythrocytes

The rate of catalase inactivation in erythrocytes after their exposure to ascorbic acid in the presence of AMT was assayed discontinuously using the Ferrous Oxidation in Xylenol orange (FOX) method, version 1 (for H_2O_2), as described previously.[7–9]

Blood was drawn from healthy volunteers. The sample was centrifuged to separate the plasma and buffy layer. Erythrocytes were washed three times in ice-cold potassium phosphate-buffered saline (PBS:15 mM potassium phosphate, 150 mM NaCl, pH 7.4). The erythrocytes were then incubated with ascorbic acid (250 μM) in the presence of AMT (50 mM in PBS) at a final cell volume of 5% (packed cells v/v) in a shaking thermostatic water bath (120 strokes/min, 37°C). Where the effect of ARIs (ZPLS, SBN and AL-1576) was studied, erythrocytes were pre-incubated with the agent at various concentrations for 5 min prior to the addition of ascorbic acid. At appropriate time intervals, 15 μl of the cell suspension was removed and mixed with 1 ml of lysis buffer (potassium phosphate buffer, 10 mM, pH7.4) containing 200 μM of H_2O_2. After an incubation period of 3 min at room temperature, a 50 μl aliquot was withdrawn, mixed with 950 μl of FOX1-reagent and the samples were incubated for 30 min at room temperature. Absorbance was read at 560 nm. Catalase inactivation was calculated by reference to residual H_2O_2 concentrations.[9,10]

Lipid Hydroperoxide Assay

Hydroperoxides were assay using the ferrous oxidation in xylenol orange (FOX, version 2 for lipid hydroperoxides) as described previously.[10] Briefly, samples (50 μl) were mixed with FOX2-reagent (950 μl). After 30 min incubation at room temperature, the samples were centrifuged at 12000 × g for 5 min and absorbance was read at 560nm.

RESULTS

Hyperglycaemia Activates AR Activity

Aldose reductase activity was evaluated by exposing rat lenses to varying concentrations of glucose. Figure 1a shows a dose-related increase of aldose reductase activity as measured by the rate of $NADP^+$ formation and NADPH oxidation. This coincided with an increase in sorbitol content in the lenses (Figure 1b; Table 1). Sorbitol content of lenses incubated with 50 mM and 100 mM glucose for 20 hours was 39.7 ± 3.2 and 44.6 nmol/100 mg tissue respectively. This was 4–6 fold higher than the lenses exposed to a physiological level of glucose.

Inhibition of AR Activity by Metal-Chelating Agents

Whole rat lenses were incubated with OPT, SBN and α-LA, as metal chelating agents, in the presence of glucose (100 mM) for 20 hours. Sorbitol accumulation in lenses exposed to glucose (100 mM) was inhibited by OPT, α-LA and SBN in an increasing order (Table 1). Effects of the metal chelating agents on $NADP^+$ formation as well as sorbitol generation are shown in Figure 2.

Effects of Metal Chelation by Aldose Reductase Inhibitors (ARIs): Inhibition of Copper-Catalysed Ascorbate Oxidation

ARIs were also examined for their metal chelation abilities by assessment of the Cu^{2+}-dependent oxidation of ascorbic acid. Zeopolastat (ZPLS), SBN and AL-1576 inhibited ascorbate oxidation by 34%, 78.2% and 92.1% respectively (Figure 3a). This occurred simultaneously with a descrease in oxygen uptake. Figure 3b shows the effect of sorbinil on oxygen uptake during ascorbate oxidation in the presence of $CuSO_4$ (0.5μM).

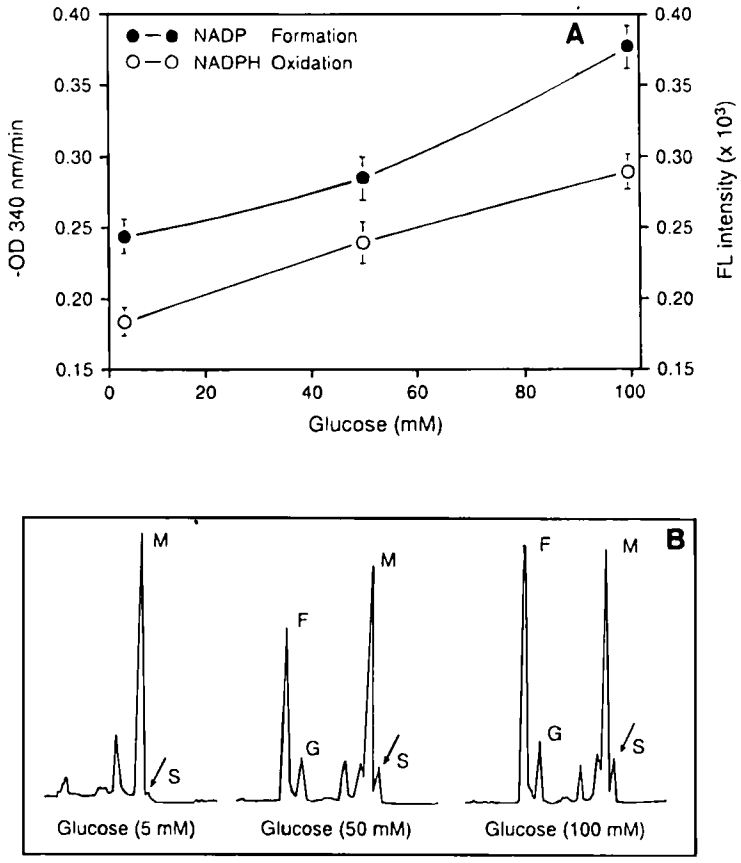

FIGURE 1 Effect of glucose on aldose reductase activity in rat lens. Whole rat lenses (n = 5–6 per group) were incubated with varying concentrations of glucose in potassium phosphate buffer at 22°C under sterile conditions. After 20 hours, the lenses were washed and homogenised as described in Materials and Methods. Aldose reductase activity was assessed by monitoring: A) Rate of NADPH oxidation (–OD 340/min) and $NADP^+$ formation (Ex. 360 nm, Em. 460 nm); B) Sorbitol generation (measured by GC); Abbreviations: Fructose (F); Glucose (G); Mannitol (M) and Sorbitol (S).

TABLE I Sorbitol Formation and Aldose Reductase Activity in Rat lenses Incubated with Glucose in the Presence or Absence of Sorbinil (SBN), α-Lipoic acid (α-LA) or *O*-Phenanthroline (OPT).

Lens + (100mg/ml)	AR Activity in Vitro			Sorbitol Formation		
	Unit(-mM/min)	+(%)*	–(%)*	nmol/100mg	+(%)*	–(%)*
G (5mM)	3.65 ± 0.18	100		7.09 ± 0.4	100	
G (50mM)	4.27 ± 0.09	117		39.7 ± 3.3	559	
G (100mM)	5.09 ± 0.06	140		44.6 ± 5.2	628	
G (100mM) + SBN	1.72 ± 0.07		66.3	0.81 ± 0.1		97.8
G (100mM) + α-LA	2.61 ± 0.12		48.7	10.8 ± 2.1		75.5
G (100mM) + OPT	2.87 ± 0.18		43.6	16.8 ± 3.4		61.3

*+(%) increased rate; –(%) decreased rate

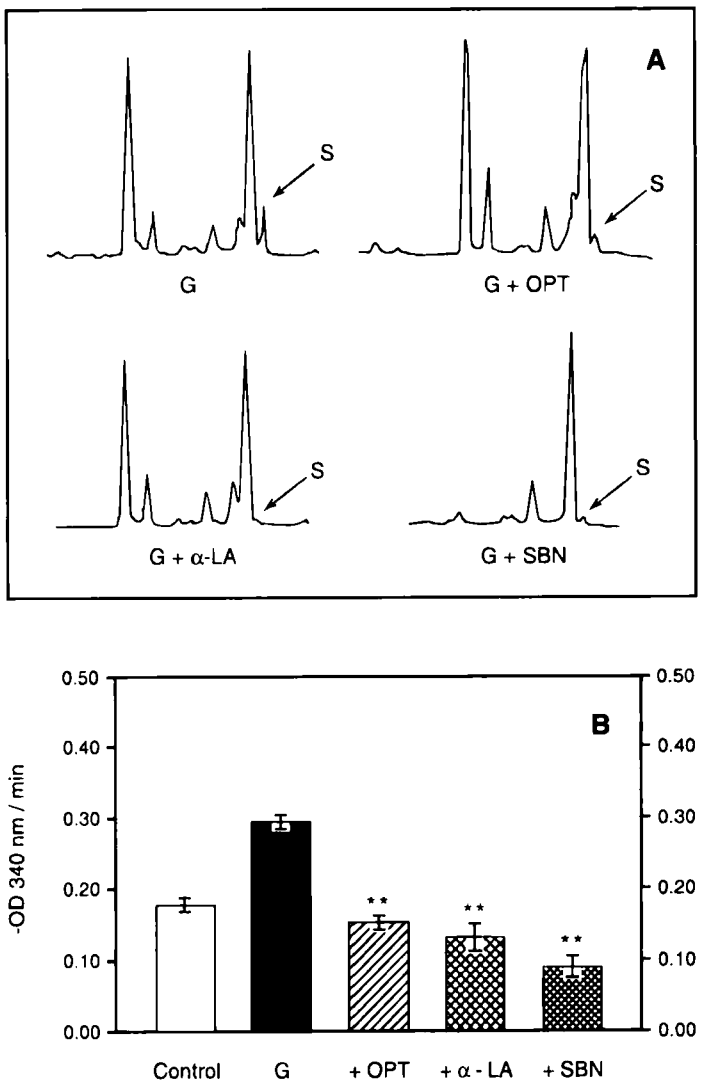

FIGURE 2 Effect of metal-chelating agents on aldose reductase activity in rat lens. Whole rat lenses (n = 5–6 per group) were incubated with SBN, OPT or α-LA (all at 200 μM) in the presence of glucose (100 mM) at 22°C for 20 hours. The lenses were treated as described under Materials and Methods. Aldose reductase activity was assessed by monitoring: A) Sorbitol formation (measured by GC); B) Rate of NADPH oxidation (–OD 340/min). Data shown are the means ± SD of triplicate samples. * or ** represent $P < 0.05$ or $P < 0.01$ respectively (compared to control).

ARIs Inhibit Copper-Catalysed Lipid Peroxidation and Facilitate Copper Ion Partition in N-Octanol

The ability of the ARIs to inhibit transition metal-catalysed oxidation may not only be restricted to their effects upon copper-catalysed ascorbate oxidation. Therefore, we examined their effect on copper-dependent lipid peroxidation. When phosphatidylcholine liposomes exposed to 0.5μM $CuSO_4$ were incubated with the ARIs at final concentration of 200μM, SBN and Al-1576 exhibited a significant inhibition of lipid hydroperoxide production compared to ZPLS (Figure 4).

As shown in Figure 5, all the ARIs (final concentration 2.5mM) enhanced partitioning of cop-

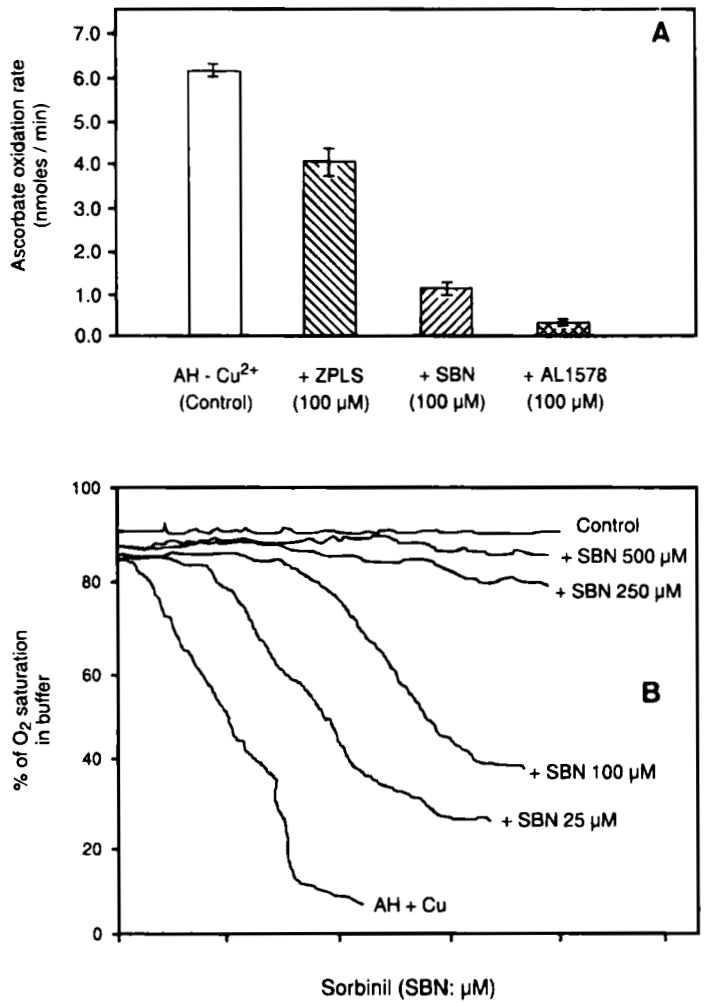

FIGURE 3 Effect of aldose reductase inhibitors on Cu^{2+} -catalysed ascorbate oxidation in vitro. Potassium phosphate buffer containing Cu^{2+} (200 nM) was mixed with aldose reductase inhibitors including ZPLS, SBN or AL-1576 (all at 200 µM). The reaction was initiated by adding ascorbic acid (100 µM) and changes in absorbance at 260 nm for monitored for 3 min. A) Cu^{2+}-dependent ascorbate oxidation; B) Dose-response inhibition of oxygen uptake in the presence of SBN. Data shown are means ±SD of triplicate samples.

per ion into n-octanol. Of these, OPT was more effective in partitioning copper ions into n-octanol than the hydrophilic metal chelator EDTA.

ARIs Inhibit H_2O_2 Production in Human Erythrocytes Incubated with Ascorbate

Catalase inactivation in the presence of aminotriazole (AMT) has been used as a model for estimation of intracellular H_2O_2 production (7–9). In this study, human erythrocytes exposed to ascorbate (100 µM) in the presence of AMT had catalase activity inhibited by 63% (Figure 6a). This implies that H_2O_2 production driven by ascorbate oxidation is likely to be catalysed by copper and iron ions within the cells.[9] ARIs including SBN, ZPLS and AL-1576 decreased catalase inactivation by 33%, 62% and 75% respectively. Inhibition of intracellular catalase was also observed using other metal chelators such as imidazole (IMDZ), histidine (HSTD) and OPT (Figure 6b).

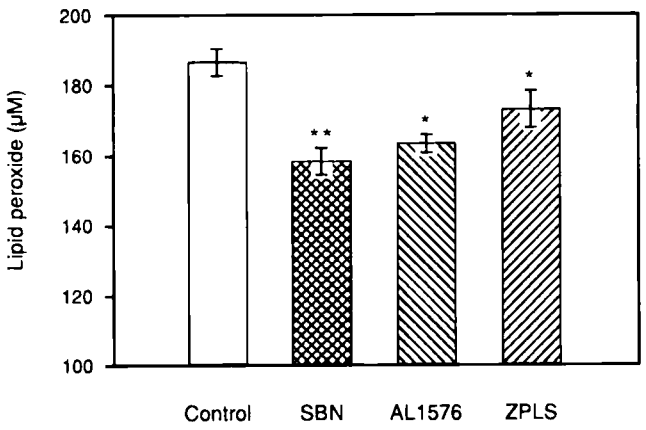

FIGURE 4 Effect of aldose reductase inhibitors on Cu^{2+}-mediated lipid peroxidation. Phosphatidylcholine liposomes (25 mg/ml in phosphate buffer) were incubated with Cu^{2+} (0.5 μM) with ZPLS, SBN or AL-1576 (all at 200 μM) for 6 hours at 37°C. Lipid hydroperoxide was measured using FOX2-assay. Data represent the mean ± SD of triplicate samples. * or ** represent $P < 0.05$ or $P < 0.01$ respectively (compared to control).

DISCUSSION

Glucose autoxidation is believed to be the main cause of protein damage in experimental glycation models of diabetes mellitus.[11] In this study, rat lenses incubated with glucose (50–100mM) accumulated sorbitol and showed increased aldose reductase activity. The activation of aldose reductase from glucose-exposed lenses (well washed to remove remaining glucose after incubation) is hard to reconcile with a pure effect of increased substrate of the enzyme. During the period of incubation (twenty hours), glucose autoxidation somehow affected the activity of aldose reductase.

The ability of metal chelating agents to inhibit the aldose reductase activation suggests that transition metals are implicated. First, metals may be required for the enzyme activation. Second, metals may initially catalyse glucose

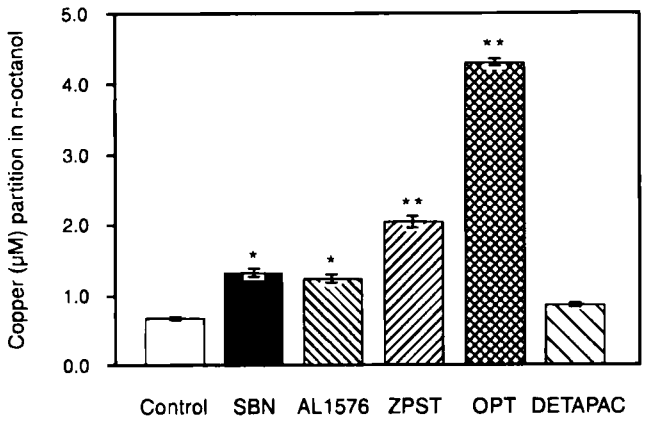

FIGURE 5 Effects of Aldose reductase inhibitors on Cu^{2+} partitioning into octanol. Stock solutions of drugs (all at 2.5 mM) were prepared in PBS in the presence of 1 mM Cu^{2+}. Aliquots (1 ml) of the drugs were mixed with octanol (2 ml) and were shaken for 10 min. After centrifugation at 1000 × g, Cu^{2+} content in the organic layer was analysed by atomic absorption spectrophotometry. Data represent the mean ± SD of triplicate samples. * or ** represent $P < 0.05$ or $P < 0.01$ respectively (compared to control).

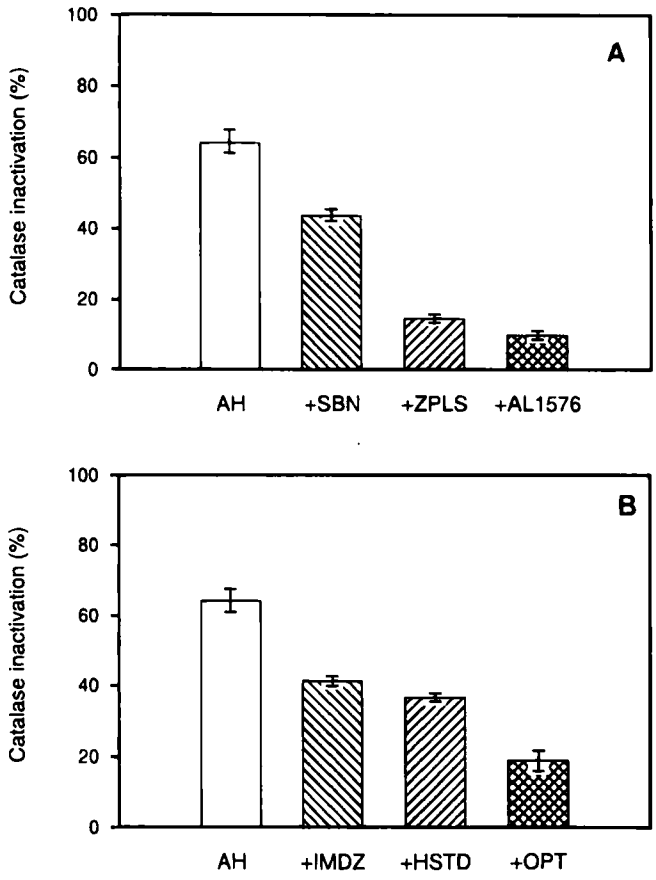

FIGURE 6 Effect of aldose reductase inhibitors and metal chelators on ascorbate mediated H_2O_2 production (assessed by catalase inactivation in the presence of AMT) in erythrocytes. Human erythrocytes (5% v/v) were preincubated with ARIs or metal chelating agents (all at 200 μM) for one minute at 37°C in phosphate buffer (containing AMT 50 mM). Ascorbic acid (100mM) was added and the samples were incubated for another 15 min. Catalase inactivation assay was carried out as described in Materials and Methods. A) Effect ARIs; B) Effect of metal-chelating agents. Data represent the mean ± SD of triplicate determinations.

autoxidation, and then induce the activation of the enzyme. Aldose reductase contains sulfhydryl groups,[12] which are capable of undergoing metal catalysed oxidation. Indeed, some of the observed biological effects of ARIs are not easily explained by simple inhibition of aldose reductase activity. For example, ARIs enhance the lens's oxidative resistance, perhaps by decreasing competition for NADPH by aldose reductase.[13] Sorbinil can also block lipid peroxidation in the rat lens.[14] This may be attributed to their metal chelation ability. Under the circumstance of hyperglycaemia, the therapeutic action of ARIs may involve blocking of NADPH oxidation and prevention of transition metal-ion-catalysed monosaccharide oxidation.[15]

The inhibitory function of ARIs in this observation seems to be dependent upon their hydantoin-like structure (Figure 7) within the molecule. Hydantoin itself partially inhibits copper-catalysed ascorbate oxidation and hydantoins bear some chemical resemblance to the 1-alkyl-2-methyl-3-hydroxypyrid-4-ones which are potent iron chelating agents.[16]

In conclusion, this study suggests that increased oxidant production by metal-catalysed glucose oxidation may be an important mechanism in activation of aldose reductase. Therefore,

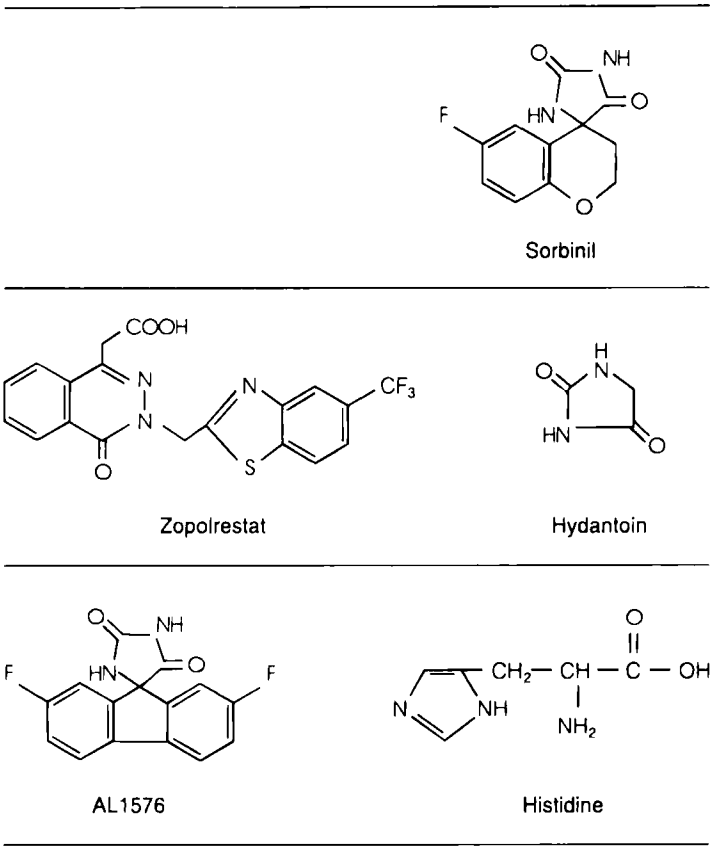

FIGURE 7 Structures of the compounds used in this study.

selective metal-complexing agents might be a useful experimental approach to the treatment of the complications of diabetes mellitus.

Acknowledgments

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