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SPIROHYDANTOIN INHIBITORS OF ALDOSE REDUCTASE INHIBIT IRON- AND COPPER-CATALYSED ASCORBATE OXIDATION *IN VITRO*

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Abstract—Transition metal-catalysed oxidations have been implicated in the complications of diabetes. We report here that some experimental inhibitors of the enzyme aldose reductase (implicated in diabetes mellitus via its ability to catalyse glucose reduction to sorbitol) are also potent inhibitors of transition metal-catalysed ascorbate oxidation. The inhibition appears to be dependent upon the presence of a spirohydantoin group. It is conceivable that the copper- and iron-binding capacity of these compounds may contribute to some of their observed biological effects and may provide a starting point for a new generation of experimental drugs for the treatment of diabetes mellitus.

It has been suggested that some complications of diabetes mellitus may derive from oxidative tissue damage catalysed by "decompartmentalized" transition metals [1–6]. Copper may contribute to a hypothetical pool of decompartmentalized transition metal in diabetes since total Cu^{2+} levels are higher in diabetic individuals than in normals, and are highest in diabetics with angiopathy and/or alterations in lipid metabolism [7, 8]. Certainly, it is by no means clear whether this copper increase is caused by an increase in caeruloplasmin or represents, in part at least, an increase in the pool of copper associated with albumin or low molecular weight chelates [7–9]. However, a small proportion of plasma copper is attached to amino acids and serum albumin [10–12] and, in this form, may well participate in oxidative reactions. A role for copper in the pathogenesis of diabetic complications is also suggested by the observation that levels of copper are higher in cataractous than clear lenses [13]. Oxidation products of glycated proteins have been found in the lens suggesting that transition metal is available in a form permitting oxidation *in vivo* [14].

Iron may also contribute to inappropriate oxidative events since diabetes mellitus is commonly associated with transfusion siderosis, dietary iron overload and idiopathic haemochromatosis [15], and is over-represented in occult haemochromatosis [16]. A specific link between iron overload and diabetic complications is further suggested by the observation that treatment with the iron-chelating agent desferrioxamine decreases hyperglycaemia and lowers hypercholesterolaemia and hyperlipidaemia (risk factors for atherosclerosis in diabetes) in diabetic individuals with high ferritin but without frank haemochromatosis [17]. Patients with iron overload often possess low levels of serum and white blood cell vitamin C [18, 19]. In diabetes, levels of plasma and white blood cell ascorbic acid are lower (despite similar levels of intake and excretion), and oxidation of this antioxidant to dehydroascorbate is higher than in normal individuals [20, 21]. Oxidative stress, perhaps initiated by decompartmentalized iron and copper, may thus contribute to the pathogenesis of the diabetic complications.

Here we report that some inhibitors of the NADPH-dependent enzyme aldose reductase (the activity of which is associated with polyol accumulation, cellular osmotic damage and NADPH depletion [22]) are potent inhibitors of copper and iron-catalysed ascorbate oxidation and thus possess the potential for antioxidant effects. These observations may explain some of the biological effects of these inhibitors and suggest a starting point for the design of a new generation of experimental and therapeutic compounds which may diminish diabetic complications.

MATERIALS AND METHODS

All materials and biochemicals, unless specified otherwise, were obtained from the Sigma Chemical Co. (Poole, U.K.) or Fluka (Glossop, U.K.) and were of the highest purity available. The aldose reductase inhibitor sorbinil was generously provided by Pfizer (Sandwich, U.K.). AL-1576 was kindly provided by Alcon Inc. (Fort North, TX). ICI 105552 was donated by ICI Pharmaceuticals (Macclesfield, U.K.). ONO 2235 was the gift of the ONO Pharmaceutical Co. (London, U.K.). 3,3-Tetramethylene glutamic acid was obtained from Sigma. All stock solutions of aldose reductase inhibitors were prepared by rapid dissolution in dilute alkali (10 mM NaOH) followed by rapid neutralization.

Ascorbate oxidation. All materials were dissolved in chelex-treated (50–100 dry mesh; Sigma) double

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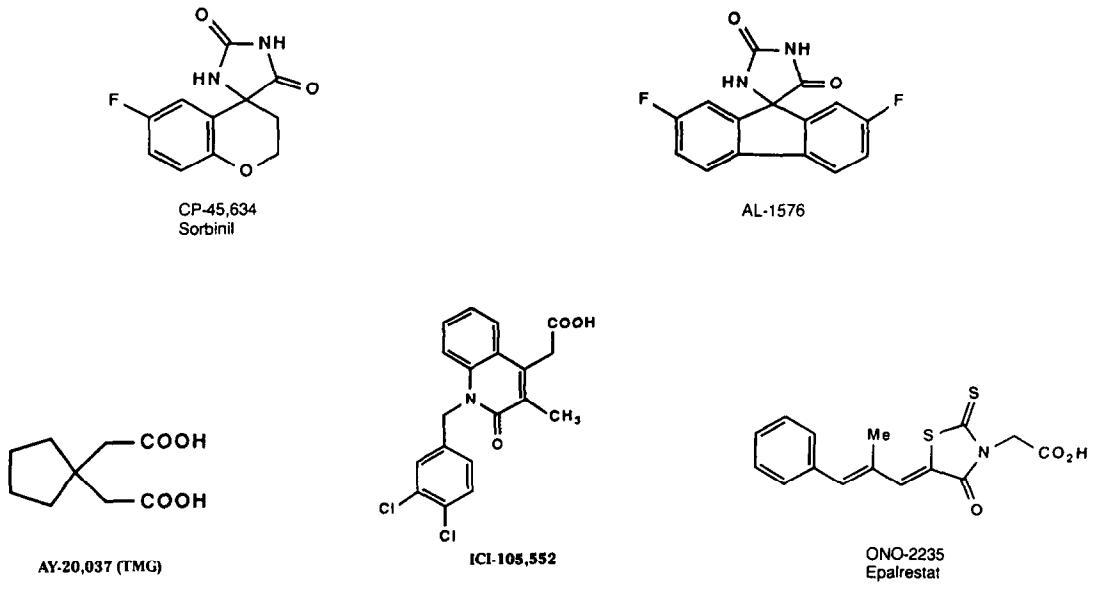

Fig. 1. The structure of aldose reductase inhibitors.

Table 1. The effects of some aldose reductase inhibitors on Cu^{2+}-induced ascorbate oxidation

	Concentration (μM)	Ascorbate oxidation rate (nmol/min)	Percentage activity
Control (Cu^{2+})		7.84 ± 0.08	100
Sorbinil	100	3.60 ± 0.08	45.9
AL-1576	100	3.04 ± 0.04	38.8
ONO 2235	100	7.68 ± 0.16	98
ICI 105552	100	8.08 ± 0.16	103
TMG	100	7.52 ± 0.08	95.9
Hydantoin	100	6.24 ± 0.16	79.6
EDDA	100	0.24 ± 0.04	3
NTA	100	0.24 ± 0.04	3
BCA	10	0.24 ± 0.04	3

Copper sulphate was dissolved in chelex-treated distilled water. Other reagents were prepared and treated as described in Materials and Methods. Final concentrations of ascorbate, potassium phosphate buffer (pH 7.4) and Cu^{2+} were 100 μM, 20 mM and 200 nM, respectively. Ascorbate oxidation rate ($-O.D._{265}$ nm/min) was monitored at 37° for 3 min.

Results are expressed as means +/− SD of four measurements.

distilled water. Solutions were stored over chelex and made fresh each day. Vitamin C oxidation was monitored over an initial 3 min period at 265 nm in a Pye Unicam 8720 UV/VIS spectrophotometer thermostatted at 37°. Ascorbate oxidation was catalysed by Cu^{2+} (copper sulphate), Cu^{2+}–1,10 orthophenanthroline (OP*) complex, Fe^{3+} (ferric chloride) or Fe^{3+}–ethylenediaminetetra-acetic acid (EDTA) and H_2O_2 (see legends to figures). The reaction was initiated by addition of ascorbate (final concentration, 100 μM). Low baseline rates of ascorbate oxidation were ensured by prior chelex treatment of stock solutions and water used to dissolve components. This yielded very reproducible rates of oxidation (coefficient of variation < 2%).

* Abbreviations: OP, orthophenanthroline; EDDA, ethylenediaminediacetic acid; NTA, nitrilotriacetic acid; TMG, tetramethyleneglutarate; BCA, 2,2′-bicinchoninic acid; EDTA, ethylenediaminetetra-acetic acid; ARIs, aldose reductase inhibitors.

RESULTS

The structures of the aldose reductase inhibitors tested are shown in Fig. 1. The spirohydantoin compounds sorbinil and AL-1576 inhibited copper-catalysed ascorbate oxidation whereas the carboxylate inhibitors ICI 105552, ONO 2235 and

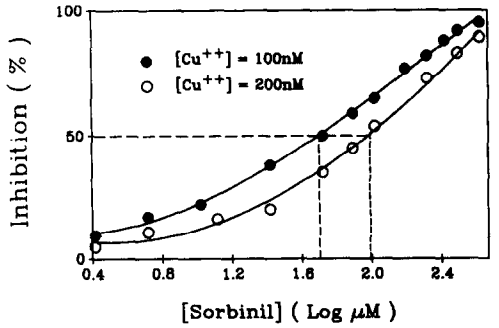

Fig. 2. The inhibitory effect of sorbinil on Cu^{2+}-induced ascorbate oxidation. Cu^{2+} was dissolved in chelex-treated distilled water. Sorbinil and ascorbate stock solutions and potassium phosphate buffer (pH 7.4) were made using chelex-treated double distilled water. Potassium phosphate buffer (20 mM), 100 μM ascorbate, 100 nM or 200 nM Cu^{2+} and 2.5–600 μM sorbinil were present in a 1 mL reaction volume. Ascorbate was added last. Ascorbate oxidation rate ($-O.D._{265}$ nm/min) was monitored at 37° for 3 min. The figure shows the percentage inhibition of ascorbate oxidation against concentration (log μM) of sorbinil.

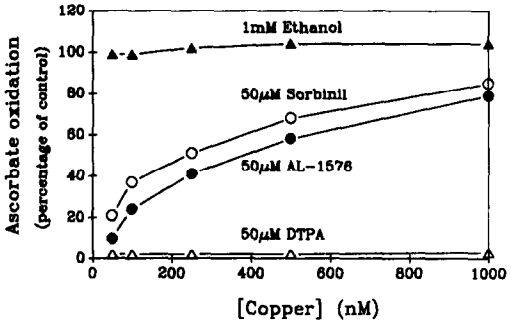

Fig. 3. The effects of sorbinil, AL-1576, DTPA and ethanol on ascorbate oxidation induced by different concentrations of Cu^{2+}. Ascorbate (100 μM) oxidation was initiated by the addition of different concentrations of Cu^{2+} (in the range of 50 to 1000 nM) in 20 mM potassium phosphate buffer (ph 7.4) at 37° and was monitored for 3 min. The effects of sorbinil (50 μM), AL-1576 (50 μM), DTPA (50 μM) and ethanol (1 mM) were examined.

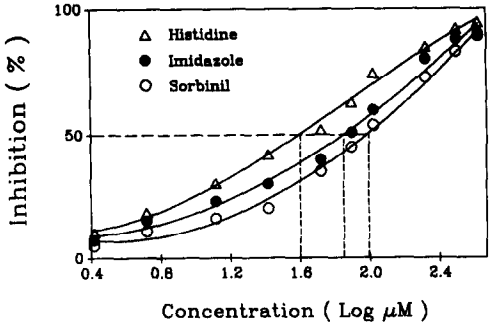

Fig. 4. Comparison of the effects of sorbinil, histidine and imidazole on Cu^{2+}-induced ascorbate oxidation. The inhibitory effects of different concentrations (1–600 μM) of sorbinil, histidine and imidazole were observed under the conditions described in the legend to Fig. 2. Ascorbate oxidation was initiated by the addition of 150 nM Cu^{2+}.

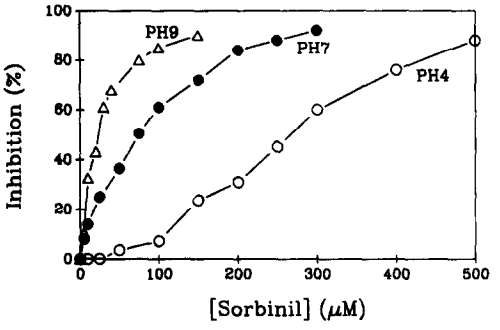

Fig. 5. The effect of pH on the inhibitory effect of sorbinil on Cu^{2+}-induced ascorbate oxidation. Potassium phosphate buffer (20 mM) of varying pH values (4.0, 7.0 or 9.0) was used to see the effect of pH on the inhibitory effect of sorbinil on ascorbate oxidation. In a 1 mL reaction volume, 150 nM Cu^{2+}, 1–500 μM sorbinil and 100 μM ascorbate were used. The treatment of solutions and the condition for the measurement of ascorbate oxidation rate were the same as described in the legend to Fig. 2. The figure shows the percentage inhibition of ascorbate oxidation against concentration (μM) of sorbinil.

tetramethyleneglutarate (TMG) had no inhibitory effect over the time scales of ascorbate oxidation studied (Table 1). Consistent with the observation that hydantoin inhibitors block copper-catalysed ascorbate oxidation we found that hydantoin itself had a moderate inhibitory effect. Table 1 also shows the inhibitory behaviour of known metal-chelating reagents for comparison. The inhibitory effect of sorbinil and AL-1576 on Cu^{2+}-catalysed ascorbate oxidation was much lower than the inhibition achieved by ethylenediaminedia-acetic acid (EDDA), nitrilotriacetic acid (NTA) and 2,2'-bicinchoninic acid (BCA). Figure 2 gives log dose–inhibition curves for sorbinil at two concentrations of added catalytic copper (100 and 200 nM) showing that at lower concentrations of copper the IC_{50} for inhibition by sorbinil was decreased. Conversely, over a 20-fold copper concentration range (Fig. 3) it can be seen that the inhibitory effect of the aldose reductase inhibitors (ARIs) decreased as copper concentration was increased for a fixed concentration of ARIs. By contrast, DTPA was a potent inhibitor over the entire copper concentration range. In addition, ethanol had no effect, thereby excluding a role for hydroxyl radical scavenging in the inhibitory effect of the ARIs. Taken together, these data would seem to suggest the existence of a saturable process in the inhibition of ascorbate oxidation by the ARIs.

Figure 4 shows log dose–inhibition curves for sorbinil and for the selective copper-complexing agents histidine and imidazole in the presence of 200 nM copper ion. The IC_{50}s for the three compounds (histidine 38 μM, imidazole 75 μM and sorbinil 95 μM) are within the same order of magnitude. (In

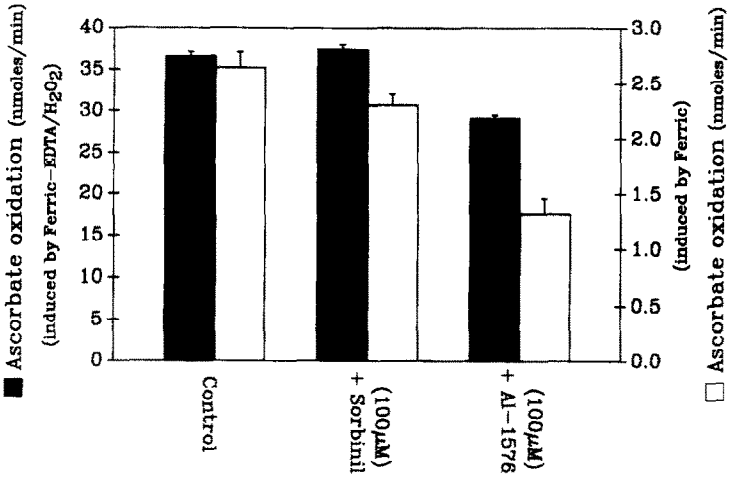

Fig. 6. The effects of sorbinil and AL-1576 on Fe^{3+}- or Fe^{3+}–EDTA–H_2O_2-induced ascorbate oxidation. Ascorbate oxidation was induced by 20 μM $FeCl_3$ or by 20 μM $FeCl_3$, 100 μM EDTA and 2 mM H_2O_2 in 20 mM sodium carbonate buffer (pH 7.4) at 37°. The effects of sorbinil (100 μM) and AL-1576 (100 μM) on Fe^{3+} or Fe^{3+}–EDTA–H_2O_2-induced ascorbate oxidation were monitored for 3 min. Ascorbate oxidation rate is given as nanomoles of ascorbate oxidized per minute.

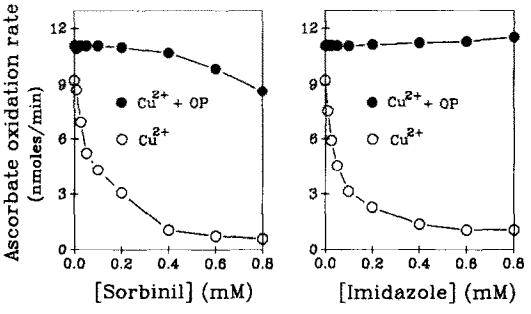

Fig. 7. The effects of sorbinil and imidazole on Cu^{2+}- or Cu^{2+}-orthophenanthroline-induced ascorbate oxidation. Ascorbate oxidation was introduced by Cu^{2+}- or Cu^{2+}-OP complex in potassium phosphate buffer (pH 7.4). Cu^{2+} and OP were added to the cuvette and mixed in buffer prior to the addition of 5–800 μM sorbinil or imidazole. The final concentrations of Cu^{2+}, OP, ascorbate and potassium phosphate in the cuvette were 500 nM, 30 μM, 100 μM and 20 mM, respectively.

the presence of 100 nM Cu^{2+} the IC_{50} for sorbinil decreases to 50 μM, Fig. 2.) The inhibitory effect of sorbinil was strongly pH dependent (Fig. 5) which might be explained by enolization of the spirohydantoin group at higher pH values and thus a greater availability of nitrogen lone pair electrons. These play an important role in the chelating ability of other nitrogen-containing copper-complexing agents.

Sorbinil and AL-1576 also had some influence upon ascorbate oxidation catalysed by Fe^{3+} alone or Fe^{3+} in the presence of EDTA and hydrogen peroxide (H_2O_2) (Fig. 6). In the presence of Fe^{3+} (20 μM) alone the rate of ascorbate oxidation was low relative to that seen with Cu^{2+} (Fig. 6), as reported previously [23], but was accelerated over 10-fold by the simultaneous addition of EDTA (100 μM) and H_2O_2 (2 mM). Sorbinil had a negligible inhibitory effect upon Fe^{3+}–EDTA–H_2O_2-catalysed ascorbate oxidation (Fig. 6) but inhibited ascorbate oxidation catalysed by free Fe^{3+} by 10%. AL-1576 was a more effective inhibitor than sorbinil in both cases (Fig. 6) and inhibited Fe^{3+}–EDTA–H_2O_2-catalysed oxidation of ascorbate by 21% and that catalysed by free Fe^{3+} by 50%. Similarly, when ascorbate oxidation was catalysed by Cu^{2+} in the presence of orthophenanthroline (OP) (Fig. 7), there was little evidence of inhibition by sorbinil until very high (> 500 μM) concentrations were achieved (Fig. 7). Imidazole, at all concentrations, was found to be inert with respect to inhibition of ascorbate oxidation catalysed by copper–orthophenanthroline (Fig. 7). Neither TMG, ICI 105552 nor ONO 2235 had any effect upon Fe^{3+}- or Fe^{3+}–EDTA–H_2O_2-catalysed ascorbate oxidation (data not shown), as well as having no effect on Cu^{2+}-catalysed ascorbate oxidation. Neither magnesium nor zinc decreased the inhibition of Cu^{2+}-catalysed ascorbate oxidation by sorbinil and AL-1576 (data not shown).

DISCUSSION

The data described above suggest, but cannot prove, that sorbinil and AL-1576 have the ability to chelate redox active transition metal ions and thus inhibit transition metal-catalysed ascorbate oxidation. This ability to inhibit ascorbate oxidation, by whatever mechanism, seems dependent upon the presence of a spirohydantoin group within the molecule. This is suggested by: (1) comparison of those structures which inhibit oxidation with those which do not; (2) structural analogy of the hydantoin group with imidazole and histidine; (3) implication of a role for nitrogen lone pair electrons on the basis of greater inhibition of ascorbate oxidation at higher

pH; as well as by (4) the ability of hydantoin itself to inhibit copper-catalysed ascorbate oxidation partially. No specific mechanism can yet be advanced to account for the postulated binding of iron and copper to the compounds, except to note that the hydantoins bear some chemical resemblance to the 1-alkyl-2-methyl-3-hydroxypyrid-4-ones which are potent iron chelating agents [24]. However, the affinity constants, at least for Cu^{2+}, would have to be orders of magnitude lower than the respective affinity constants of the polynitrogenated and polycarboxylated compounds EDTA, NTA and BCA for Cu^{2+} (Table 1). The mechanism of Cu^{2+} chelation by sorbinil is, furthermore, somewhat different to that of imidazole since sorbinil, but not imidazole, is able to inhibit Cu^{2+}-ortho-phenanthroline-catalysed ascorbate oxidation which presumably relates to the ability of sorbinil, but not imidazole, to gain access to coordination sites on the metal which are otherwise required for ascorbate oxidation.

Whatever the precise mechanism of chelation, it is conceivable that this secondary activity of the compounds may contribute to some of the observed biological effects which are not easily explained by simple inhibition of aldose reductase activity. For example, the aldose reductase inhibitor Tolrestat inhibits the plasma ascorbic depletion associated with experimental diabetes, but by unknown mechanisms [25]. Similarly, the inhibitory effect of sorbinil on the increased kidney glomerular filtration rate observed in experimental diabetes appears not to be related to inhibition of glomerular polyol production but rather suppression of the formation of vasodilatory prostaglandins [26]. Sorbinil can also block lipid peroxidation in the diabetic rat lens [27]. In addition, AL-1576 is able to retard naphthalene-induced cataract [28]. Aldose reductase is difficult to implicate in this form of cataract, but it may involve oxidation of lens components. Finally, although the aldose reductase inhibitor Statil improves erythrocyte deformability in patients, this effect is not related to the lowering of erythrocyte polyol levels but appears to be a separate membrane effect [29]. The observations made here are consistent with previous observations on the ability of some aldose reductase inhibitors to block free radical NADPH oxidation stimulated by transition metal-catalysed monosaccharide enediol oxidation *in vitro* [30].

No judgement can, however, be made concerning the extent to which the putative metal complexation by spirohydantoin drugs may relate to the efficacy of the compounds in the treatment of diabetic complications, since the therapeutic efficacy of ARIs in human diabetes mellitus is not well established. Most clinical trials of these drugs have been performed to assess efficacy against diabetic neuropathy. Although some trials have indicated that aldose reductase inhibitors produce an objective benefit, at least in terms of an increase in nerve conduction velocity [31–35], other studies have shown no such effect [36–41]. Furthermore, although aldose reductase was originally implicated as a causative factor of cataract in diabetes, the only drugs which appear to have a protective effect against human cataract are aspirin, paracetamol and other "aspirin-like analgesics" [42]. The latter drugs are poor inhibitors of aldose reductase but do show various forms of antioxidant activity, including metal chelation [43, 44]. Of note, here, is the observation that as the concentration of copper used to catalyse ascorbate oxidation was decreased so the IC_{50} for inhibition by sorbinil was decreased. This may be of relevance to the question of the putative level of decompartmentalized transition metal *in vivo* and whether transition metal-binding capacity contributes to the biological effects of the drug. Plasma steady-state levels of sorbinil during its administration in clinical trial are in the range of 10 to 60 μM [45] which is of the order that would be necessary for the chelation of low levels of free transition metal ion. In conclusion, we would like to suggest that the design of selective metal-complexing agents might be a useful experimental approach to the treatment of the complications of diabetes mellitus.

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