Erythrocyte catalase inactivation (H_2O_2 production) by ascorbic acid and glucose in the presence of aminotriazole: role of transition metals and relevance to diabetes

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Erythrocytes exposed to ascorbic acid in the presence of aminotriazole undergo a dose- and time-dependent inactivation of endogenous catalase which is proportional to environmental hydrogen peroxide (H_2O_2) concentrations. The production of H_2O_2 seems to be dependent upon the availability of transition metal chelatable by *o*-phenanthroline (OPT), although the kinetics of catalase inactivation and H_2O_2 production by externally added copper ions in the presence of OPT is complex. Furthermore, although glucose is also able to undergo a transition-metal-catalysed oxidation yielding H_2O_2, the production of H_2O_2 by glucose seems to be a minor process by comparison with ascorbic acid oxidation. Indeed, on the basis of these data, transition-metal-catalysed ascorbic acid oxidation is likely to be a more important source of oxidative stress in the diabetic state than hyperglycaemia.

INTRODUCTION

Previous work from this laboratory and elsewhere has identified the processes of glucose 'autoxidation' and 'glycoxidation' as potentially important sources of oxidative stress in diabetes mellitus [1,2]. In brief, increased levels of glucose in diabetes are postulated to react with decompartmentalized transition metals (such as iron and copper) to form hydrogen peroxide (H_2O_2), ketoaldehydes and free radical oxidants *in vivo* [3]. In addition, the Amadori adduct on proteins (which forms to an increased extent as a result of hyperglycaemia) appears to encounter transition metals *in vivo* and oxidize in an analogous process to glucose autoxidation yielding H_2O_2, erythronic acid and carboxymethyl-lysine (CML) residues [4]. These oxidative processes have been suggested to contribute to antioxidant depletion and lipid peroxide accumulation in diabetes.

Both the glucose autoxidation and glycoxidation theories are dependent upon the notion that sufficient decompartmentalized iron and copper are available *in vivo* to allow oxidation of glucose and glycated protein to proceed. It seems probable that some transition metal is available *in vivo* for these oxidations to occur, as CML has been found within long-lived proteins in human diabetes [2]. However, if transition metal is available in a form permitting oxidation of susceptible biological reductants then it is likely that other reducing agents, such as ascorbic acid, thiols and lipids, would oxidize at a more rapid rate than glucose and its protein adducts. This, in its turn, leads to the suggestion that the level of transition metal, rather than the level of oxidizable substrate, is the critical factor in determining the extent of *in vivo* oxidative stress. To examine this possibility we have investigated the relative rates of H_2O_2 production in erythrocytes stressed with glucose and ascorbic acid using the H_2O_2-mediated inhibition of catalase in the presence of aminotriazole [5] as described previously [6]. We have also examined a particular role for copper ions in H_2O_2 production. The results suggest that ascorbic acid is likely to dominate intracellular H_2O_2 production even under conditions of extreme hyperglycaemia. Furthermore, chelatable transition metals (although not necessarily copper ions) seem to be required for intracellular H_2O_2 production.

EXPERIMENTAL

Ascorbic acid, glucose, H_2O_2, Xylenol Orange, sorbitol and glucose oxidase (from *Aspergillus niger*) were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). Ammonium ferrous sulphate, 3-amino-1,2,4-triazole (AMT), and *o*-phenanthroline (OPT) were obtained from the Aldrich Chemical Co. (Poole, Dorset, U.K.). Chelex (50–100 mesh) metal-chelating resin was obtained from Sigma and washed with 1 M HCl and distilled water before use. All solutions were prepared with Chelex-treated double-distilled water. Data presented are the means ± S.D. of duplicate samples from triplicate experiments. Statistical analyses (separate variance *t* tests) were performed using the Unistat Ltd. desktop statistical package (London, U.K.).

Catalase inactivation by H_2O_2 in the presence of AMT

AMT irreversibly inactivates catalase in the presence of a constant flux of H_2O_2 [7]. The extent of irreversible catalase inhibition in cells exposed to AMT is thus a function of the H_2O_2 flux within the cell. Catalase activity of erythrocytes after their exposure to agents generating H_2O_2 in the presence of AMT was discontinuously assayed using the FOX (ferrous oxidation in Xylenol Orange) method as described previously in detail [6].

Briefly, human blood was drawn from healthy volunteers and centrifuged to separate the plasma and buffy layer. Erythrocytes were washed three times in ice-cold PBS (15 mM potassium phosphate, 150 mM NaCl, pH 7.4). The erythrocytes were then incubated with ascorbic acid, glucose, copper and/or chelating agent in the presence of AMT (50 mM in PBS) at a final cell volume of 5% (packed cells v/v) in a shaking thermostatically controlled water-bath (120 strokes/min, 37 °C). (Details of the precise incubation conditions are given in the legends to Figures.)

At various time intervals, 15 μl aliquots of the cell suspension were removed and added, with vortexing, to 1 ml of lysis buffer (potassium phosphate buffer, 10 mM, pH 7.4) containing 200 μM

Abbreviations used: AMT, 3-amino-1,2,4-triazole; CML, carboxymethyl-lysine; OPT, *O*-phenanthroline.

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H_2O_2. After an incubation period of 3 min at room temperature, a 50 μl aliquot was withdrawn and added, in a 1 ml microfuge vial in duplicate, to 950 μl of FOX reagent composed of 100 μM Xylenol Orange, 250 μM ammonium ferrous sulphate and 100 mM sorbitol in 25 mM H_2SO_4 [6]. After an incubation period of 30 min at room temperature samples were directly read at 560 nm on a Pye–Unicam Series 8700 spectrophotometer and calibrated against H_2O_2 standards. Catalase inactivation was calculated by reference to residual H_2O_2 concentrations [6].

RESULTS

H_2O_2 production and catalase inactivation

Glucose in combination with glucose oxidase generates a flux of H_2O_2 which is linearly related to the extent of catalase inactivation within erythrocytes simultaneously exposed to AMT (Figure 1). Although the steady-state concentration of measurable H_2O_2 in the buffer surrounding the erythrocytes reflects a balance between H_2O_2 production and consumption by the cells, it is evident that the extent of catalase inactivation in the presence of AMT can be used as a measure of H_2O_2 production within the cell or in its vicinity. Glutathione peroxidase is, of course, also well known to metabolize H_2O_2. Under these conditions, however, co-incubation with diamide (500 μM), which depletes intracellular glutathione, increased catalase inactivation by less than 30% (results not shown).

Ascorbic acid and intracellular catalase inactivation

Catalase inactivation in the presence of AMT was used to demonstrate H_2O_2 production during exposure of erythrocytes to ascorbic acid (Figure 2). It is known that ascorbic acid reacts with iron and copper generating H_2O_2 [8]. The dose-dependent inactivation of catalase when erythrocytes are incubated with ascorbic acid in the presence of AMT suggests that ascorbic acid

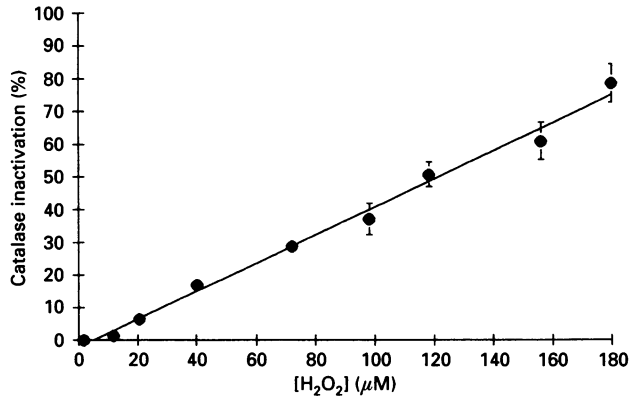

Figure 1 Catalase inactivation in erythrocytes exposed to different concentrations of H_2O_2 in the presence of AMT

Well-washed human erythrocytes (5%, v/v) were incubated with glucose (2.5 mM) and various concentrations of glucose oxidase (0–100 m-units/ml) in the presence of aminotriazole (AMT) (50 mM) in PBS at 37 °C with shaking for 30 min. Concentrations of H_2O_2 in the buffer produced by the glucose/glucose oxidase system were measured using the FOX reagent after removal of erythrocytes. Catalase inactivation in the erythrocytes was measured by adding 15 μl aliquots of the cell suspension to 1 ml of lysis buffer (potassium phosphate buffer, 10 mM, pH 7.4) containing 200 μM H_2O_2. The reaction was allowed to proceed for 3 min at room temperature and then stopped by rapidly transferring 50 μl samples to 950 μl of FOX reagent in a 1 ml microfuge vial with vortexing. The admixture was incubated for 30 min before reading the absorbance at 560 nm. Data shown are the means ± S.D. of duplicate samples from triplicate parallel experiments.

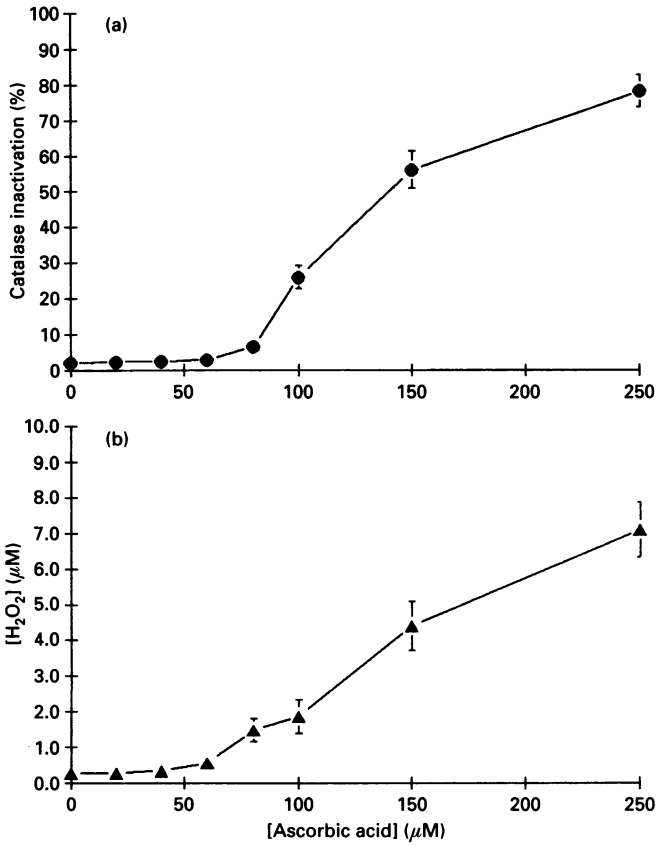

Figure 2 Dose–response relationship between added ascorbic acid concentration, inactivation of intracellular catalase and H_2O_2 generation

(**a**) Human erythrocytes (5%, v/v) were incubated with ascorbic acid at various concentrations in PBS with AMT (50 mM) for 45 min at 37 °C. The samples were withdrawn for analysis of catalase inactivation (%) as described in Figure 1. (**b**) In a parallel incubation without erythrocytes, H_2O_2 production by ascorbic acid was measured using the FOX assay. Data shown are the means ± S.D. of duplicate samples from triplicate parallel experiments.

oxidation occurs rapidly within, or in the proximity of, erythrocytes (Figure 2a). There is an apparent threshold for catalase inactivation, which may reflect glutathione peroxidase activity at low concentrations of H_2O_2. Alternatively, the threshold may simply reflect the kinetics of H_2O_2 production from ascorbic acid, which shows a similar apparent threshold in a parallel incubation of ascorbic acid in PBS in the absence of erythrocytes (Figure 2b).

Precise calibration of catalase inactivation by ascorbic acid by reference to steady-state levels of H_2O_2 associated with glucose oxidase/glucose data is clearly not possible. However, the extent of catalase inactivation in erythrocytes exposed to a physiological level of ascorbic acid (100 μM ascorbic acid producing approx. 30% catalase inactivation in the presence of AMT) would correspond to approx. 75 μM H_2O_2 generated by glucose oxidase/glucose. Although this is almost certainly an overestimate it is quite clear that ascorbic acid would seem to produce substantial intracellular fluxes of H_2O_2 under normal circumstances. By contrast, another reducing agent, 2-mercaptoethanol, used as a control, produced less than 8% of the H_2O_2 produced by ascorbic acid at an identical concentration in free solution and, correspondingly, virtually no inhibition of erythrocyte catalase inactivation in the presence of AMT (Figures 3a and 3b).

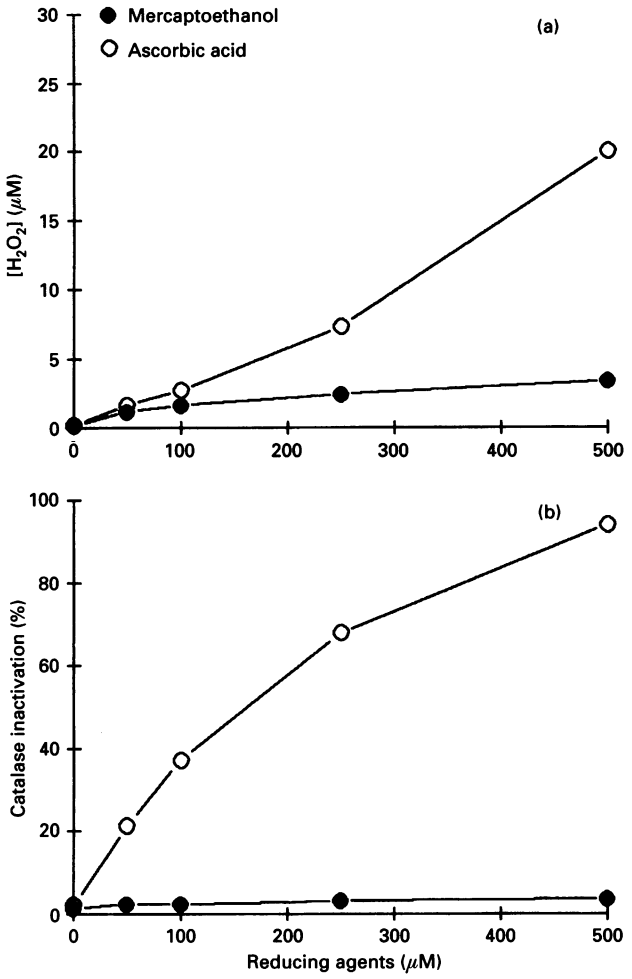

Figure 3 Production of H_2O_2 and catalase inactivation by mercaptoethanol

Experiments performed were the same as those in Figure 2 except that 2-mercaptoethanol was substituted for ascorbic acid.

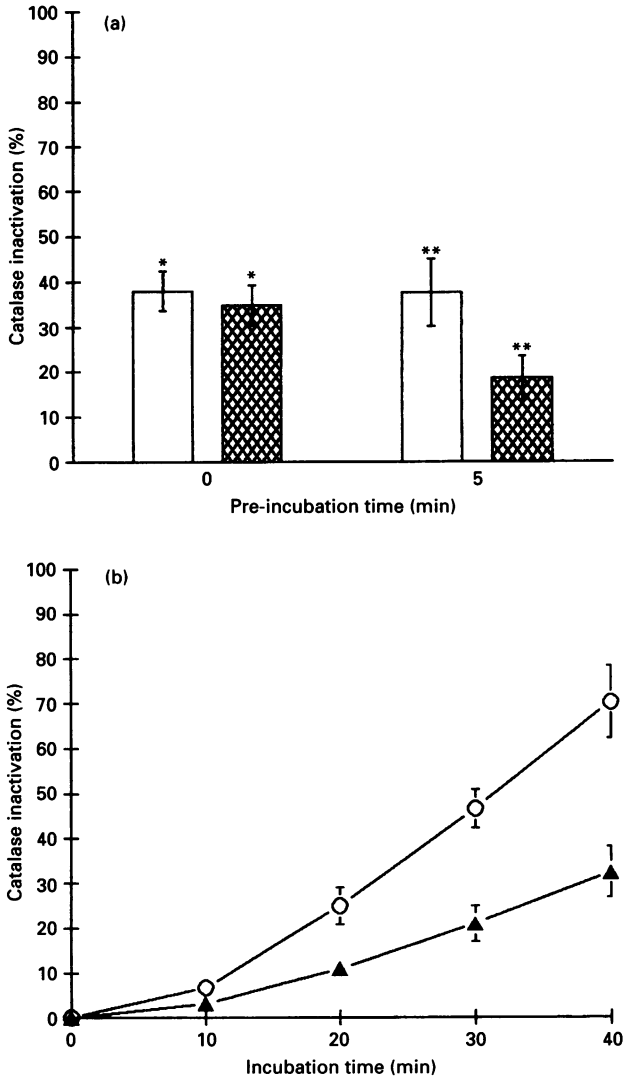

Figure 4 Inhibition of ascorbic acid-mediated catalase inactivation requires preincubation with the metal-chelating agent OPT

(**a**) Erythrocytes were exposed to OPT (hatched bars; 100 μM) for 0 to 30 min at 37 °C in PBS before addition of ascorbic acid (250 μM) followed by a further incubation for 15 min at 37 °C. [AMT (50 mM) was added at the start of the incubation.] Control cells were treated with ascorbic acid alone (open bars). Catalase was then measured as described in the legend to Figure 1. Data shown are the means ± S.D. of duplicate samples from triplicate parallel experiments at 0 and 5 min pre-incubation. Statistical analyses shown are results of separate variance *t*-test: * versus *, not different; ** versus **, different at $P < 0.05$. (**b**): Erythrocytes were exposed to OPT (▲, 100 μM) at 37 °C in PBS for 5 min before addition of ascorbic acid (250 μM) and a further incubation for 0 to 40 min at 37 °C in PBS. AMT (50 mM) was added with OPT. Controls were treated with ascorbic acid alone (○). Catalase activity was measured as described in the legend to Figure 1.

Transition metal involvement

The possible role of transition metals in the inactivation of erythrocyte catalase by ascorbic acid was examined using OPT, a membrane-permeant chelating agent [9]. OPT inhibited ascorbic acid-mediated catalase inactivation but pre-incubation of the cells with the drug was required, suggesting that transition-metal-catalysed ascorbic acid oxidation to H_2O_2 occurs intracellularly (Figure 4a). A 5 min preincubation of the erythrocytes with OPT before addition of ascorbic acid produced a 45% inhibition of catalase inactivation (Figure 4a). In fact, maximal inhibition is observed after 5 min pre-incubation with OPT before addition of ascorbic acid. Longer periods of pre-incubation had no further effect (results not shown). Figure 4(b) shows the results of an experiment in which erythrocytes were incubated with ascorbic acid and AMT in the presence and absence of OPT following the inhibition of catalase with time. The latter experiment is obviously an integration of penetration of OPT into the cell combined with the inhibitory effect of OPT on intracellular H_2O_2 production but it supports a role for chelatable metals, probably intracellular, in catalase inactivation by ascorbic acid.

We attempted to examine a particular role for copper ions in catalase inactivation by ascorbic acid by examining the effects of externally added copper ions in the presence and absence of OPT (Figure 5). We observed (Figure 5a) that a combination of externally added copper ions and OPT caused a greater inhibition of catalase inactivation than added copper ions in the absence of OPT. OPT caused, by contrast, a substantial inhibition of copper-catalysed ascorbic acid oxidation, at least as monitored by loss of ascorbic acid *in vitro* (Figure 5b). Two possibilities may account for the effect of copper ions plus OPT on erythrocyte catalase

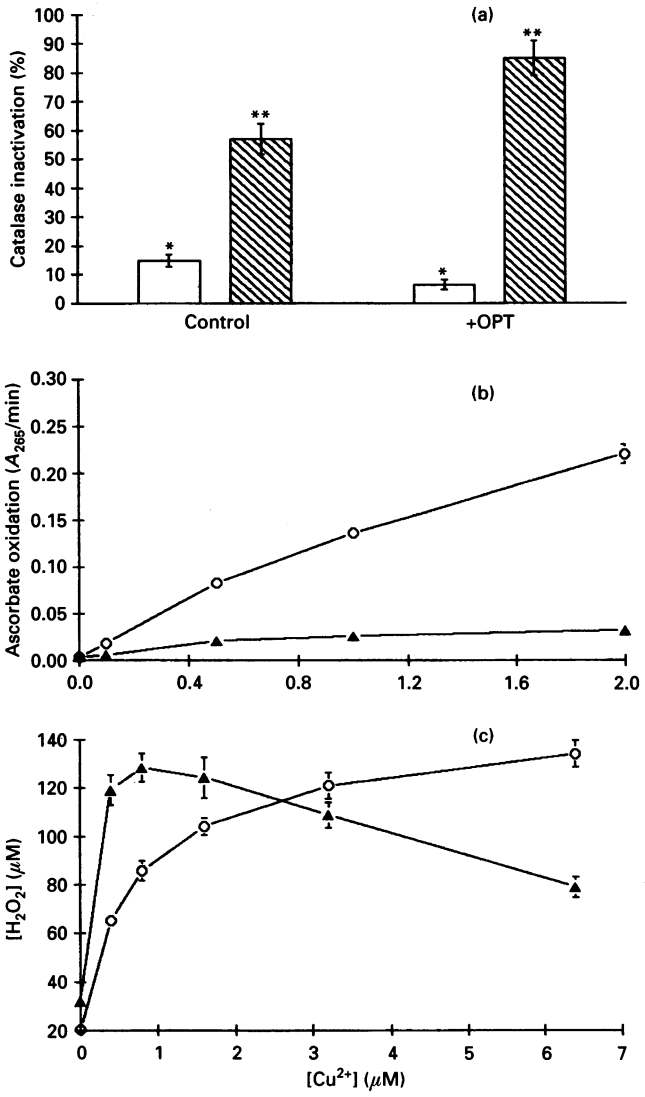

Figure 5 OPT enhances copper-catalysed catalase inactivation and enhances H_2O_2 accumulation during copper-catalysed ascorbic acid oxidation but inhibits copper-catalysed ascorbic acid oxidation

(**a**) Erythrocytes were exposed to ascorbic acid (250 μM) and AMT (50 mM) in the presence of OPT (100 μM) and/or copper ions (hatched bars; 1 μM Cu^{2+}) at 37 °C in PBS for 30 min before the measurement of catalase activity as described in the legend to Figure 1. Statistical analyses shown are the results of separate variance *t*-tests: * versus *, different at $P < 0.005$; ** versus **, different at $P < 0.001$. (**b**) Oxidation of ascorbic acid (100 μM) was monitored directly at 265 nm for 3 min at 37 °C in PBS in the presence of various concentrations of copper ions (Cu^{2+}) and in the presence (▲) or absence (○) of OPT (100 μM). Data shown are the means ± S.D. of triplicate measurements. (**c**) Ascorbic acid (100 μM) was incubated at 37 °C in PBS for 15 min in the presence of various concentrations of copper ions (Cu^{2+}) and in the presence (▲) or absence (○) of OPT (100 μM). The reaction was allowed to proceed for 3 min at room temperature and then stopped by rapidly transferring 50 μl samples to 950 μl of FOX reagent in a 1 ml microfuge vial with vortexing. The admixture was incubated for 30 min before reading the absorbance at 560 nm. Data shown are the means ± S.D. of triplicate samples.

inactivation. First, OPT may transport copper ions across the erythrocyte membrane so that, even though copper is inhibited with respect to ascorbic acid oxidation in the presence of OPT (Figure 5b), there is a greater net catalytically active concentration of copper ions in the copper/OPT-treated cells. Secondly, the enhanced catalase inactivation in the presence of

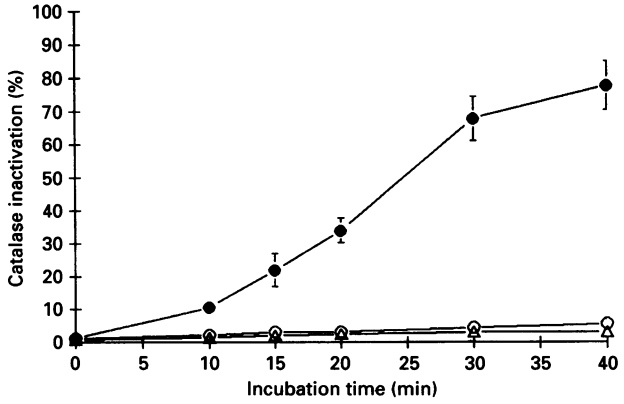

Figure 6 Time course of inactivation of intracellular catalase by ascorbic acid and glucose in the presence of AMT

Well-washed human erythrocytes (5%) were incubated with glucose (○; 100 mM) or ascorbic acid (●; 100 μM) in PBS (15 mM, pH 7.4) with AMT (50 mM) at 37 °C with shaking. Controls were treated with AMT alone (△). Catalase inactivation was analysed at various time intervals.

externally added copper ions and OPT may relate to the fact that despite a lower rate of ascorbic acid oxidation, there is a greater net accumulation of detectable H_2O_2 resulting from ascorbic acid oxidation in the presence of copper ions and OPT than with copper ions alone (Figure 5c). The kinetics of this latter reaction are complex (Figure 5c) with a distinct 'cross-over' at different ratios of copper ions and OPT. We could not determine whether this chemistry was directly relevant to inhibition of catalase in the erythrocyte as extracellular H_2O_2 levels in the presence of erythrocytes were consistently found to be close to zero, presumably because H_2O_2 is rapidly metabolized by the cells during catalase inactivation (results not shown). We cannot thus conclude whether the effect of copper ions in the presence of OPT is an effect upon levels of H_2O_2 in the general environment of the cells or an effect upon facilitated copper ion permeation into the cells.

The overall results are, however, consistent with a hypothesis that intracellular chelatable metals are required for catalase inactivation by ascorbic acid. The results are also consistent with a suggestion that ascorbic acid must traverse the erythrocyte membrane as ascorbic acid before its oxidation to yield H_2O_2. This latter conclusion contrasts with results from other cell types where ascorbic acid uptake is postulated to require its prior extracellular oxidation to dehydroascorbate [10].

Relative rates of H_2O_2 production: glucose versus ascorbic acid

Finally, a comparative study of AMT-induced inactivation of catalase by ascorbic acid and glucose showed that inactivation of catalase is much greater when erythrocytes are exposed to ascorbic acid than when erythrocytes are exposed to glucose (Figure 6). Although glucose at an extreme, unphysiological hyperglycaemic level (100 mM) seems capable of producing a small flux of H_2O_2, as judged by the small extent of erythrocyte catalase inhibition (Figure 6), the extent of catalase inactivation over short time periods is far less than that induced by a physiological level of ascorbic acid (100 μM). Although glucose has the potential for H_2O_2 generation, actual production of H_2O_2 from glucose within cells, even at high concentrations of glucose, appears trivial compared with the flux of H_2O_2 which would result from transition-metal-catalysed ascorbic acid oxidation.

DISCUSSION

The data presented here suggest that it is questionable whether the processes of glucose autoxidation and glycoxidation are of significance to oxidative stress, if this results from increased H_2O_2 production, *in vivo*. Clearly, even small changes in plasma and tissue ascorbic acid concentrations will have a greater impact upon intracellular H_2O_2 fluxes than increases in glucose levels, even up to very high concentrations. Furthermore, small variations in the concentration of chelatable transition metal ions may be expected to greatly modify the rate of ascorbic acid-mediated intracellular H_2O_2 production as well as the oxidation of other reducing agents. These findings, of a relatively low rate of H_2O_2 production from glucose by comparison with ascorbic acid, do not exclude the possibility that hyperglycaemia may be damaging through 'site-specific' protein damage [1,2] or related mechanisms, but specifically exclude a hypothesis that glucose contributes to substantial fluxes of H_2O_2 *in vivo*. Erythrocytes are here used, of course, as a model for H_2O_2 production in cells in general but it is unlikely that cell types specifically affected in diabetes, such as endothelial cells, would experience much greater fluxes of H_2O_2 from glucose.

The suggestion that ascorbic acid may cause a transition-metal-catalysed flux of H_2O_2 *in vivo* is of importance since there are marked derangements of ascorbic acid metabolism in diabetes. Ascorbic acid plasma levels are reported to be decreased in diabetes, whereas dehydroascorbic acid levels are increased [11,12]. One possibility is that there may be an increased rate of oxidation of this important vitamin via elevations in decompartmentalized metal. Indeed, although ascorbic acid is undoubtedly an important antioxidant, its ability to act as a pro-oxidant under various conditions is well known (e.g. [8,13]). Thus, although emphasis has been placed upon the pro-oxidant effects of hyperglycaemia in diabetes, it seems that ascorbic acid is a more probable precursor of H_2O_2 through oxidation catalysed by decompartmentalized transition metal *in vivo*. By this analysis, the presence of oxidation products of the Amadori product, such as CML, would suggest extensive metal-catalysed oxidation of ascorbic acid. Indeed, it has been suggested previously that part of the cytoprotective activity of spirohydantoin-derivative aldose reductase inhibitors in diabetes may relate to their ability to chelate copper ions weakly and thus inhibit ascorbic acid oxidation [14]. Furthermore, there is some evidence that ascorbic acid does contribute to oxidative stress in diabetes. For example, supplementation of ascorbic acid to the streptozotocin-induced diabetic rat appears to result in greater levels of plasma lipid peroxidation [15].

In conclusion, although hyperglycaemia may be an important contributory factor to long-term tissue damage in diabetes via adverse covalent modification of macromolecules, it seems unlikely that it contributes appreciably to oxidative stress, at least not via H_2O_2 production. The possibility that ascorbic acid oxidation catalysed by transition metals is responsible for accumulative protein damage in diabetes and ageing requires closer attention [16].

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REFERENCES

1 Wolff, S. P. (1993) Br. Med. Bull. **49**, 642–652
2 Baynes J. W. (1991) Diabetes **40**, 415–412
3 Wolff, S. P., Jiang, Z. Y. and Hunt, J. V. (1991) Free Rad. Biol. Med. **10**, 339–352
4 Ahmed, M. U., Thorpe, S. R. and Baynes, J. W. (1986) J. Biol. Chem. **261**, 4889–4894
5 Margoliash, E., Novogrodsky, A. and Schejter, A. (1960) Biochem. J. **74**, 339–348
6 Ou, P. and Wolff, S. P. (1993) Biochem. Pharmacol. **46**, 1139–1144
7 Margoliash, E. and Novogrodsky, A. (1958) Biochem. J. **68**, 468–475
8 Wolff, S. P., Wang, G.-M. and Spector, A. (1987) Exp. Eye Res. **45**, 777–789
9 Krishnamurti, C., Sayna, L. A. and Petering, D. H. (1980) Cancer Res. **40**, 4092–4099
10 Washko, P. W., Wang, Y. and Levine, M. (1993) J. Biol. Chem. **268**, 15531–15535
11 Yue, D. K., Mclennan, S., Fisher, E., Heffernan, S., Ross, G. R. and Turtle, J. R. (1989) Diabetes **38**, 257–261
12 Sinclair, A. J., Girling, A. J., Gray, L., Le Guen, C., Lunec, J. and Barnett, A. H. (1991) Diabetologia **34**, 171–175
13 Hunt, J. V., Bottoms, M. A. and Mitchinson, M. J. (1992) FEBS Lett. **331**, 161–164
14 Jiang, Z.-Y., Zhou, Q.-L., Eaton, J. W., Koppenol, W. H., Hunt, J. V. and Wolff, S. P. (1991) Biochem. Pharmacol. **42**, 1273–1278
15 Young, I. S., Torney, J. J. and Trimble, E. R. (1992) Free Rad. Biol. Med. **13**, 41–46
16 Dunn, J. A., Ahmed, M. U., Murtiashaw, M. H., Richardson, J. M., Walla, M. D., Thorpe, S. R. and Baynes, J. W. (1990) Biochemistry **29**, 10964–10970

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