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Short Communication

THIOCTIC (LIPOIC) ACID: A THERAPEUTIC METAL-CHELATING ANTIOXIDANT?

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Abstract—Thioctic (α-lipoic) acid (TA) is a drug used for the treatment of diabetic polyneuropathy in Germany. It has been proposed that TA acts as an antioxidant and interferes with the pathogenesis of diabetic polyneuropathy. We suggest that one component of its antioxidant activity requiring study is the direct transition metal-chelating activity of the drug. We found that TA had a profound dose-dependent inhibitory effect upon Cu^{2+}-catalysed ascorbic acid oxidation (monitored by O_2 uptake and spectrophotometrically at 265 nm) and also increased the partition of Cu^{2+} into *n*-octanol from an aqueous solution suggesting that TA forms a lipophilic complex with Cu^{2+}. TA also inhibited Cu^{2+}-catalysed liposomal peroxidation. Furthermore, TA inhibited intracellular H_2O_2 production in erythrocytes challenged with ascorbate, a process thought to be mediated by loosely chelated Cu^{2+} within the erythrocyte. These data, taken together, suggest that prior intracellular reduction of TA to dihydrolipoic acid is not an obligatory mechanism for an antioxidant effect of the drug, which may also operate via Cu^{2+}-chelation. The R-enantiomer and racemic mixture of the drug (α-TA) generally seemed more effective than the S-enantiomer in these assays of metal chelation.

Key words: lipoic/thioctic acid; metal chelation; antioxidant; diabetes

TA§, the naturally-occurring coenzyme of pyruvate and α-ketoglutarate dehydrogenases, has been used in Germany for almost four decades for the treatment of diabetic polyneuropathy. The antioxidant properties of TA have been proposed to interfere with the pathogenesis of diabetic polyneuropathy but the precise mechanism of action of TA needs further evaluation. TA has been proposed to act as a chain-breaking antioxidant in its reduced form, DHLA, interacting with vitamin E to block lipid peroxidation; alternatively, TA may react with reactive oxidants directly [1–5]. It is currently unclear whether any *in vivo* antioxidant effect of TA is a direct effect of the compound or relies upon its prior reduction to DHLA.

An alternative, non radical-scavenging mechanism by which TA might feasibly act as an antioxidant is via its weak metal-chelating capacity [6, 7]. This proposal is of interest as it has previously been suggested that the complications of diabetes mellitus might be related to an increased oxidative stress initiated by decompartmentalized transition metal [8–10]. We show here that the enantiomeric mixture of TA, as well as the individual S- and R-forms of the drug are capable of inhibiting Cu^{2+}-catalysed oxidations *in vitro*. TA also assists the partition of Cu^{2+} into *n*-octanol and inhibits the flux of H_2O_2 induced within erythrocytes by exposure to ascorbic acid.

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§ Abbreviations: TA, thioctic (lipoic) acid; DHLA, dihydrolipoic acid; AMT, 3-amino-1,2,4-triazole; OPT, *o*-phenanthroline; AAPH, 2,2′-azobis (2-amidinopropane)hydrochloride; UV–Vis, ultraviolet–visible; HPLC, high-performance liquid chromatography; FOX, ferrous oxidation in xylenol orange; DETAPAC, diethylenetriamine penta-acetic acid.

Materials and Methods

Ascorbic acid, H_2O_2, xylenol orange, sorbitol, L-α-phosphatidylcholine, histidine and *n*-octanol were obtained from the Sigma Chemical Co. (Poole, U.K.). TA as the racemic mixture and R- and S-enantiomers was supplied by ASTA Medica (Germany). Ammonium ferrous sulphate, AMT, and OPT were obtained from the Aldrich Chemical Co. (Poole, U.K.). Chelex (50–100 mesh) metal chelating resin was obtained from Sigma and washed with 1 N HCl and distilled water before use. AAPH was obtained from Polysciences (Warrington, U.K.). All chemicals and reagents were of the highest purity available.

All solutions were prepared with chelex-treated double-distilled water. Data presented are the means ± SD of triplicate measurements from duplicate experiments. Statistical analyses (separate variance *t*-tests) were performed using the Unistat Ltd desktop statistical package (London, U.K.).

Cu^{2+}-catalysed ascorbate oxidation in vitro. Ascorbate oxidation was monitored over an initial 3 min period at 265 nm in a Pye Unicam 8720 UV–Vis spectrophotometer at 37° in acid-washed quartz cuvettes. Oxidation of ascorbic acid (100 μM) was initiated by the addition of Cu^{2+} (200 nM) in 20 mM potassium phosphate buffer (pH 7.4) in the presence or absence of TA. All solutions and hardware were shielded as far as possible from contamination by environmental heavy metals. Buffer, ascorbic acid and drug solutions were made up in chelex-treated water. Under these conditions the observed initial rates of ascorbate oxidation had coefficients of variation of less than 2%.

Measurement of oxygen uptake. Oxygen uptake measurements were performed in a Clark-type oxygen electrode in an incubated chamber with a magnetic stirrer at a constant temperature of 37°. Reaction mixtures consisted of ascorbic

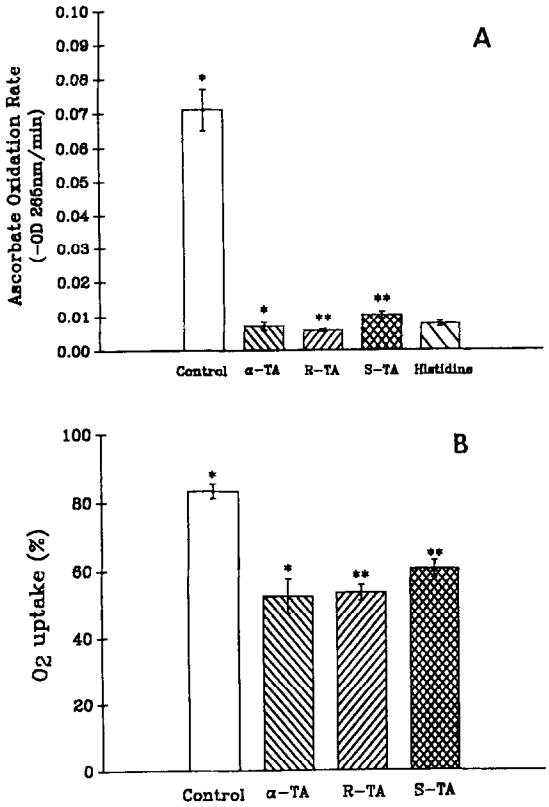

Fig. 1. Thioctic acid inhibits Cu^{2+}-catalysed ascorbate oxidation. (A) Oxidation of ascorbic acid monitored spectrophotometrically at 265 nm. Oxidation of ascorbic acid (100 μM) was initiated by the addition of copper ions (Cu^{2+}: 200 nM) in the presence and absence of α-TA, R-TA, S-TA or histidine (all at 100 μM). Oxidation of ascorbic acid was monitored for 3 min at 265 nm at 37°. Data given are the mean ± SD of triplicate measurements obtained from duplicate experiments (* versus * $P < 0.001$; ** versus ** $P < 0.001$). (B) Oxidation of ascorbic acid monitored by oxygen uptake. Oxidation of ascorbic acid (1 mM) in potassium phosphate buffer (20 mM, pH 7.4) was initiated in a 10 mL oxygen uptake chamber by the injection of a small volume of a concentrated Cu^{2+} solution to give a final concentration of 5 μM. Oxygen uptake was calculated as a percentage of the total oxygen in solution. All drugs were present at 250 μM. The results represent the mean ± SD of triplicate measurements obtained from duplicate experiments (* versus * $P < 0.05$; ** versus ** $P < 0.05$).

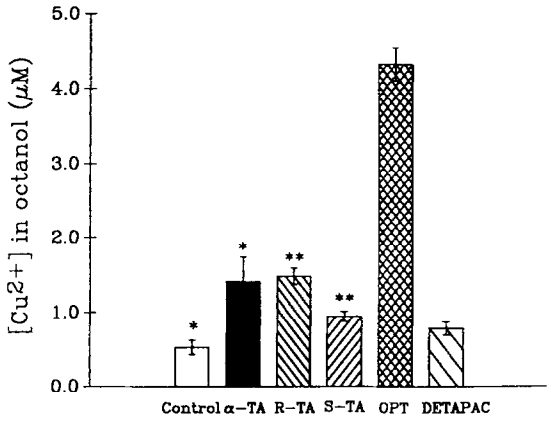

Fig. 2. Thioctic acid, like *or*-phenanthroline, partitions copper ions into octanol. Two millilitres of a solution of Cu^{2+} (20 mM) and test compound (2.5 mM) in PBS were mixed vigorously with 2 mL of *n*-octanol (pre-equilibrated with PBS). The *n*-octanol layer was then removed and evaporated to dryness. The residue was redissolved in concentrated nitric acid and analysed for Cu^{2+} concentration by calibrated atomic absorption spectrometry. The results represent the mean ± SD of triplicate measurements obtained from duplicate experiments (* versus * $P < 0.05$; ** versus ** $P < 0.05$).

acid (1 mM) in the presence and absence of various concentrations of TA in potassium phosphate buffer (20 mM, pH 7.4). Oxygen uptake was initiated by the injection of small volumes of concentrated Cu^{2+} solutions (final concentration = 5 μM) and monitored for 3 min. Total oxygen uptake was calculated as a percentage of the initial oxygen concentration.

Measurement of n-*octanol partition of copper.* The ability of a compound to assist the partition of Cu^{2+} into *n*-octanol can be used as an index of the ability of a compound to form a lipid-soluble complex with the metal. Solutions of Cu^{2+} (20 mM) were prepared in 2 mL volumes of phosphate-buffered saline, pH 7.4 in the presence or absence of 2.5 mM TA. Two millilitres of *n*-octanol (pre-equilibrated with PBS) were then added to the mixtures of drug/Cu^{2+} and mixed vigorously by vortexing. The mixture was allowed to separate at room temperature with gentle shaking for 20 min and then centrifuged at 750 × *g* for 5 min. One millilitre of the octanol (upper) layer was then transferred to another glass tube and evaporated to dryness under a nitrogen stream at 100°. The residue was redissolved by the addition of 1 mL of 1% HNO_3. Copper concentration in the nitric acid was then determined by atomic absorption spectrophotometry (Pye Unicam SP9 Series, Philips, U.K.) at 324.8 nm.

Measurement of liposomal peroxidation. Artificial liposomes were prepared by dissolving 400 mg L-α-phosphatidylcholine in 4 mL HPLC-grade ethyl acetate. Sixteen millilitres of potassium phosphate buffer (10 mM, pH 7.4) were then added and the resulting emulsion was ultrasonicated for 2 min on ice. Ethyl acetate was removed by vigorous bubbling under a nitrogen stream to yield a stock solution of small liposomes with a final concentration of 25 mg/mL L-α-phosphatidylcholine. Liposomes (final concentration, 2.5 mg/mL) were then incubated with Cu^{2+} (500 nM) in the presence and absence of TA at 37° for 24 hr in phosphate buffer (10 mM, pH 7.4). Lipid hydroperoxide was determined with the FOX assay, version 2 (for lipid hydroperoxides) as described previously [11, 12]. Briefly, 50 μL of liposome incubation mixture was added to 950 μL FOX2 reagent composed of 100 μM ammonium ferrous sulphate, 250 μM xylenol orange, 4 mM butylated hydroxytoluene, 25 mM H_2SO_4 in 90% (v/v) HPLC-grade methanol. The mixture was incubated at room temperature for 30 min and then read at 560 nm.

Concentration of lipid hydroperoxide was calculated using an extinction coefficient of $4.3 \times 10^4 M^{-1}cm^{-1}$ [11]. The FOX2 reagent was also obtained as the commercial preparation (PeroXOquant Quantitative Peroxide Assay: lipid compatible formulation) from Pierce (Rockford, IL, U.S.A.).

Catalase inactivation by H_2O_2 *in the presence of aminotriazole.* Aminotriazole irreversibly inactivates cata-

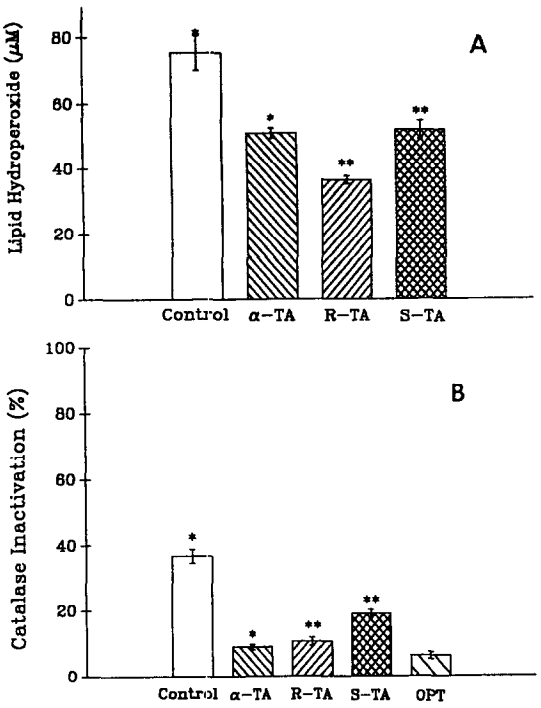

Fig. 3. Thioctic acid inhibits liposomal peroxidation and intracellular H_2O_2 production by ascorbic acid. (A) Inhibition of liposomal peroxidation by thioctic acid. Liposomes were prepared as described in the Materials and Methods section and incubated at a final concentration of 2.5 mg/mL in phosphate-buffered saline with 500 nM Cu^{2+} for 20 hr at 37°. Drugs were included at a final concentration of 250 μM. The results represent the mean ± SD of triplicate measurements obtained from duplicate experiments (* versus * $P < 0.02$; ** versus ** $P < 0.05$). (B) Inhibition of erythrocyte catalase produced by ascorbic acid in the presence of aminotriazole. Well-washed human erythrocytes (5%, v/v) were pre-incubated for 5 min with TA and OPT prior to addition of ascorbic acid (250 μM) and AMT (50 mM) in PBS and then incubated at 37° with shaking for 30 min. 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 FOX reagent in a 1 mL microfuge vial with vortexing. The admixture was incubated for 30 min before reading absorbance at 560 nm. The results represent the mean ± SD of triplicate measurements obtained from duplicate experiments (* versus * $P < 0.005$; ** versus ** $P < 0.005$).

lase in the presence of a constant flux of H_2O_2 [13]. 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 ascorbic acid in the presence of AMT was discontinuously assayed using the FOX method, version 1 (for H_2O_2), as described previously [14, 15].

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 potassium 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 thermostatted water bath (120 strokes/min, 37°). When the effect of TA was studied, erythrocytes were pre-incubated with TA at various concentrations for 5 min prior to the addition of ascorbic acid.

At appropriate time intervals, 15 μL 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 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 [12]. After an incubation period of 30 min at room temperature samples were 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 [14, 15]. The FOX1 reagent was also obtained as the commercial preparation (PeroXOquant Quantitative Peroxide Assay: aqueous compatible formulation) from Pierce (Rockford, IL, U.S.A.).

Results and Discussion

Thioctic acid inhibits ascorbic acid oxidation. Oxidation of ascorbic acid by Cu^{2+} is rate-limited by the availability of uncomplexed metal. Agents which complex copper ions will thus inhibit the rate of Cu^{2+}-catalysed ascorbic oxidation. Figure 1(A) shows the effects of histidine, a known copper ion complexing agent, and various forms of TA (100 μM) on ascorbic acid oxidation. α-TA inhibited ascorbic acid oxidation by 90%; R-TA inhibited ascorbic acid oxidation by 92% and S-TA inhibited ascorbic acid oxidation by 85%. All forms of TA seems capable of complexing Cu^{2+} although the α- and R-forms of the drug seemed slightly more effective in this assay of metal complexing ability than the S-form.

The ability of TA to inhibit copper ion-catalysed ascorbic acid oxidation was confirmed using the oxygen electrode (Fig. 1(B)). Comparing the effect of a single concentration of the various forms of TA indicated that the α- and R-forms of the drug seemed to inhibit Cu^{2+}-catalysed ascorbic acid oxidation with the same efficacy, and to a slightly greater extent than the S-form.

Thioctic acid facilitates the partition of copper ions into octanol. Direct evidence for the copper-complexing activity of TA was obtained by examining the ability of TA to facilitate the partition of Cu^{2+} into *n*-octanol. This manoeuvre can be employed where there is a need to examine the lipophilicity of a drug [16]. We reasoned that drugs which formed lipophilic complexes with Cu^{2+} would increase the partitioning of the metal into *n*-octanol.

Figure 2 shows the results of an experiment in which TA and various known metal-complexing agents were incubated with Cu^{2+} prior to the vigorous mixing of the solutions with *n*-octanol and subsequent atomic absorption analysis of the *n*-octanol layer. The metal-chelator OPT increased the concentration of Cu^{2+} in the octanol layer by six-fold. By contrast, DETAPAC, which chelates Cu^{2+} to form a negatively charged complex, barely increased Cu^{2+} partition into *n*-octanol over the control. α-TA and R-TA both doubled the level of Cu^{2+} detected in *n*-octanol relative to the control, and were slightly more effective than S-TA, in agreement with the data obtained for inhibition of ascorbic acid oxidation. Since inhibition of ascorbic acid oxidation and partition of metal into *n*-octanol mediated by TA are purely chemical assays of metal complexation it would suggest that the stereochemistry of TA must play some small role in the complexation process.

Thioctic acid inhibits lipid peroxidation. We also examined the ability of TA to inhibit Cu^{2+}-catalysed

liposomal peroxidation. We have shown before that measurement of hydroperoxide accumulation is a more sensitive measure of liposomal peroxidation than the measurement of peroxidation-derived aldehydes using thiobarbituric-acid [12]. Figure 3(A) shows data for the inhibition of Cu^{2+}-catalysed liposomal peroxidation by TA. The R-form of the drug inhibited hydroperoxide accumulation by 52% and was more effective than α-TA and S-TA which generated only 32% and 31% inhibition of hydroperoxide accumulation, respectively. In order to confirm that the inhibition afforded by TA was related to metal-chelation, rather than a chain-breaking antioxidant effect, we also tested the ability of TA to inhibit lipid peroxidation initiated by the thermolabile pro-oxidant 2,2′-azobis (2-amidinopropane)hydrochloride. No form of TA inhibited lipid peroxidation produced by this reagent (data not shown).

TA inhibits H_2O_2 production within the erythrocyte. We have shown previously that the exposure of erythrocytes to ascorbic acid *in vitro* is associated with an intracellular flux of H_2O_2 probably generated by the intracellular reaction of ascorbic acid with redox-active Cu^{2+} [14]. Experimentally, this flux of H_2O_2 is detected by the inhibition of erythrocyte catalase in the presence of AMT, which acts as a suicide substrate for catalase in the presence of an H_2O_2 flux. Figure 3(B) shows the inhibitory effect of TA on erythrocyte catalase inactivation produced by ascorbic acid (250 μM) in the presence of AMT (50 mM). α-TA was found to be as active as the R-form of the drug in the inhibition of catalase inactivation under these conditions and was more effective than S-TA (Fig. 3(B)). The inhibitory effect of OPT, a known membrane-permeant chelating agent, is also shown for comparative purposes. In this experiment, we cannot exclude the possibility that TA is undergoing intracellular reduction to DHLA, which may be preferential for the physiological R-form. DHLA might protect catalase via metal complexation or other effects. However, the results are consistent with the hypothesis that TA can complex Cu^{2+} and thus protects catalase via inhibition of ascorbic acid.

The data presented here suggest that TA in its oxidized form may be an antioxidant via its ability to complex Cu^{2+}. In addition, there is the suggestion that the R-form and enantiomeric mixtures of the compound are more effective than the S-form in the battery of tests of metal complexation employed here. The ability of TA to act as a metal-chelator would be in accord with the view that the complications of diabetes mellitus involve transition metal overload and certainly requires further examination.

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