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Ascorbic acid oxidation: a potential cause of the elevated severity of atherosclerosis in diabetes mellitus?

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The exposure of mouse peritoneal macrophages to cholesterol linoleate-containing artificial lipoproteins can lead to intracellular ceroid accumulation. This can be used as a model to study the role of oxidation in macrophage uptake of lipoproteins containing unsaturated fatty acids, considered by many as a primary event in atherosclerotic plaque formation. Our studies show that ascorbic acid can both inhibit and promote the formation of ceroid in such a model system. The transition metal copper (Cu(II)) further elevates ceroid accumulation and EDTA, a metal chelator, inhibits it. When trace levels of transition metals are present, low concentrations of ascorbic acid can elevate ceroid formation. This pro- and antioxidant characteristic of ascorbic acid was confirmed by monitoring the generation of oxidants by various concentrations of ascorbic acid, assessed by benzoic acid hydroxylation or the fragmentation of BSA. We discuss these observations in the context of an apparent increase in ascorbic acid oxidation and elevated severity of atherosclerosis in diabetes mellitus.

Ascorbic acid; Diabetes; Atherosclerosis; Oxidative stress; Transition metal-catalysis

1. INTRODUCTION

Complications of diabetes mellitus may be, in part, due to an increase in oxidative stress [1,2]. An enhanced level of oxidative stress in an organism may result from an increase in free radical production, a reduction in antioxidant defences, or both. Indeed, antioxidant defences seem to be compromised in diabetes [3–5] and an increase in plasma markers of lipid peroxidation occurs in diabetic microangiopathy [6].

Among the antioxidants preventing free radical-induced oxidation of lipids is ascorbic acid (AA), a water-soluble antioxidant [7] which may be able to recycle vitamin E [8], a major lipid-associated antioxidant. In diabetes there appears to be an alteration in AA metabolism, characterised by decreased levels of plasma AA and increased levels of the oxidation product, dehydroascorbic acid [9]. AA supplementation has therefore been proposed as a therapeutic approach to oxidative stress in diabetes.

However, AA can oxidise in the presence of transition metals such as iron and copper, generating free radicals and protein-reactive aldehydes [10,11]. The handling of transition metals appears to be altered in diabetes [12,13]. The possibility that protein-reactive aldehydes, produced during transition metal-catalysed AA oxidation, contribute to diabetic complications has been raised [11]. AA can induce changes in protein structure and fluorescence similar to those caused by exposure of protein to glucose [11]. This was discussed in the context of alterations in protein fluorescence known to occur in vivo [11]. However, the possible contribution of AA oxidation to oxidative stress in diabetes must also be considered, especially in the light of the likely role of oxidative stress in the life-threatening complication of atherosclerosis. The severity of atherosclerosis is elevated in diabetes and accounts for over 50% of deaths [14].

Evidence in favour of oxidative stress as a contributory factor to atherosclerosis has become extensive [15,16]. In atherosclerotic lesions, the lipid-laden foam cells have been identified as macrophages [17,18]. The main source of lipid is thought to be LDL, probably oxidatively modified LDL, which has been detected in human lesions using monoclonal antibodies [19]. Oxidised LDL is avidly taken up by macrophages and results in the appearance within macrophages of ceroid pigment, an autofluorescent polymer of oxidised lipid and protein, probably at least partly composed of oxidised LDL [20–23]. The cause of oxidation of LDL during atherogenesis is uncertain. One possible factor is the availability of transition metals as catalysts for such oxidative reactions [24].

We have used a model system to study ceroid accumulation in mouse peritoneal macrophages cultured with artificial lipoproteins consisting of a cholesterol ester and albumin. Since LDL has a variable fatty acid and antioxidant composition, this circumvents any such variations [25]. These artificial lipoproteins may have a naturally-occurring counterpart, since cholesterol ester-rich albumin particles have been found in human ath-

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erosclerotic plaques [26], but they also serve as a model of cholesterol esters in LDL.

Evidence of a handling defect of iron and copper in diabetes, as well as an increased oxidation of AA [9], implies that AA oxidation may contribute to oxidative stress in this disease [1,2,11]. The effects of AA on the events following culture of macrophages with cholesteryl linoleate-containing artificial lipoproteins were therefore investigated.

2. MATERIALS AND METHODS

Radiochemicals were obtained from Amersham (Aylesbury, UK). All biochemicals were obtained from Sigma (Poole, UK) or Aldrich (Gillingham, UK) unless stated otherwise and were of the highest purity available.

The generation of hydroxyl radicals by AA was assessed by monitoring the hydroxylation of benzoic acid to fluorescent products (308 nm excitation/410 nm emission) with salicylic acid as a standard [27–29]. 1 mM benzoic acid, 100 mM potassium phosphate (pH 7.2) and increasing concentrations of AA (from 0 to 3 mM) were incubated for 24 h at 37°C in the presence of 1 μM copper sulphate under air within sealed sterile vessels.

Fragmentation of bovine serum albumin (BSA; 20 mg/ml) during exposure to AA was used as a measure of free radical damage as previously described [28,30]. Albumin (fraction V; Boehringer-Mannheim, Mannheim, Germany) was labelled by reductive methylation of 0.1% of total lysine groups (50,000 dpm/mg protein) with [^{14}C]formaldehyde and $NaCNBH_3$. Fragmentation of radiolabelled BSA during exposure to AA was monitored by the determination of 5% trichloroacetic acid (TCA)-soluble radiolabelled fragments. All incubations contained ascorbic acid, 100 mM potassium phosphate (pH 7.2) and 10 μM copper sulphate and were performed at 37°C.

Peritoneal macrophages were isolated from male BALB/c mice aged 6–8 weeks as previously described [22]. Cells were resuspended in RPMI 1640 medium containing 10% lipoprotein-deficient fetal calf serum (LPDFCS) and plated at 2×10^6 cells/culture dish. All media contained 100 IU/ml penicillin and 100 μg/ml streptomycin sulphate. Cell cultures were performed in 3 ml (5 cm diameter) culture dishes (Anumbra dishes, Payne, Slough, Berks, UK) throughout. The macrophages were allowed to adhere by incubating for 3 h. Non-adherent cells were removed by washing with fresh medium. Fresh medium was then added, and cells incubated overnight at 37°C in an atmosphere of 5% CO_2 prior to introduction of lipoprotein. The cultures were then incubated with lipoprotein for 24 h.

Cholesteryl-linoleate/bovine serum albumin (CL/BSA) was prepared as previously described [22]. The molar ratio of CL to BSA was 60:1. BSA was used at 10 mg/ml in phosphate-buffered saline (PBS). CL was dissolved in acetone (BDH-Analar), added to 10 mg/ml BSA whilst vortexing and then sonicated for 1 min. Acetone was evaporated by gassing with nitrogen.

Cells were harvested by removing the medium and adding 3 ml 0.33% ethylenediamine tetraacetic acid (EDTA) in RPMI with 10% LPDFCS. After 15 min, his medium was removed and retained. Remaining adherent cells were scrape-harvested in 3 ml of Hanks' balanced salt solution. A cell pellet was prepared, from the EDTA-containing medium and scrape-harvested cells, by centrifugation. Cells were resuspended in 0.25 ml fixative (1.5% formaldehyde, 0.1% BSA in PBS) by vortexing. The fixed cells were stored at 4°C and analysed within 2 weeks, during which period no change in fluorescence is observed. Fluorescence in cells was analysed using a FACStar Plus flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with a Spectra Physics 2025 argon ion laser. The laser was tuned to emit 300 mW of light in the UV wavelengths 351.1–363.8 nm. Intact macrophages were identified by forward light scatter. Computerised selection of live populations of macrophages was determined using propidium iodide to stain dead cells. Fluorescence was monitored using a 490 nm long-pass filter in front of the detector. Results were expressed relative to a control in which cells were exposed to medium alone, since this instrument, in common with all fluorimeters, measures light intensities in arbitrary units.

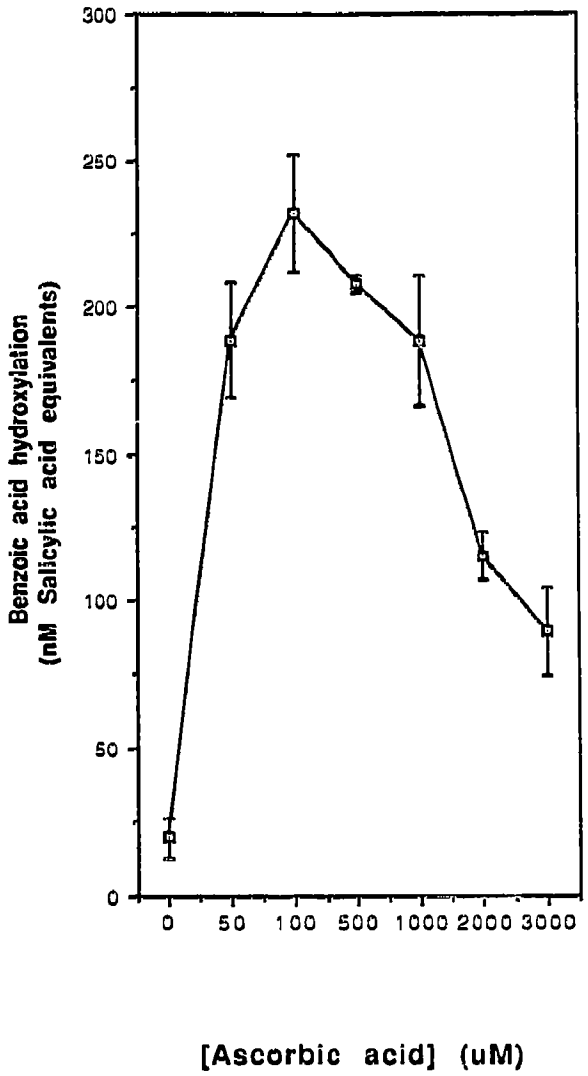

Fig. 1. Effect of ascorbic acid on benzoic acid hydroxylation. Values in all figures are the mean ± SD from 3 experiments, and in Figs. 1–3, plotted against a non-linear x-axis of AA concentration.

3. RESULTS AND DISCUSSION

The hydroxylation of benzoic acid to fluorescent derivatives is used to monitor the presence of hydroxyl radicals [27,28]. Incubation of benzoic acid with AA in the presence of Cu(II) showed maximum hydroxylation at an AA concentration of 100 μM (Fig. 1). This suggests that AA has either pro- or antioxidant characteristics depending on concentration. The same was true for BSA fragmentation, a measure of protein oxidation under these circumstances [28,30], which was maximal

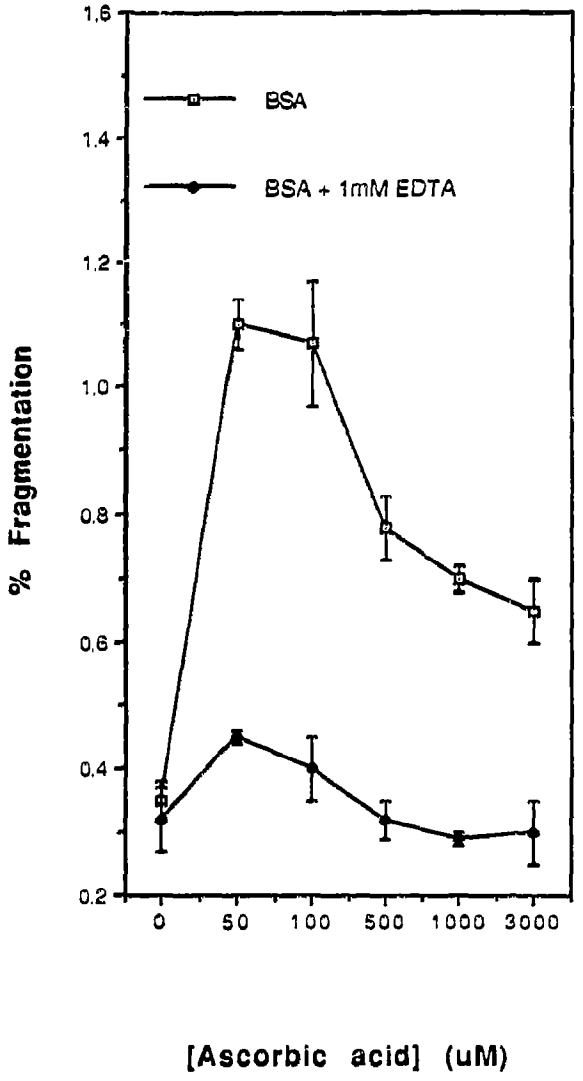

Fig. 2. Effect of ascorbic acid on protein fragmentation.

at 50–100 μM AA (Fig. 2). EDTA inhibited the fragmentation by 90% but did not affect the concentration producing maximal fragmentation.

The accumulation of ceroid in macrophages exposed to oxidisable lipoprotein is a model system for investigating oxidative events relevant to atherosclerosis [25]. Using this model we have shown that vitamin E inhibits ceroid accumulation (Hunt et al., submitted for publication). Murine peritoneal macrophages exposed to CL/BSA led to ceroid accumulation, assessed by flow cytometry (Fig. 3). Fig. 3 also shows the effect of including various concentrations of AA with or without EDTA. Again, AA can either elevate or inhibit ceroid accumulation depending on AA concentration. However, inhibition was evident only at the non-physiologically high concentrations of 3 mM. In the presence of EDTA, AA inhibited at lower concentrations (Fig. 3). Thus, AA behaves as an antioxidant only in the near absence of catalytically active transition metals. When transition metals are available, the oxidation of unsaturated fatty acids is favoured and physiological concentrations of AA are pro-oxidant. This suggests that the pro-oxidant activity of AA requires transition metals. This is supported by the experiment shown in Fig. 4. Cu(II) increased ceroid accumulation modestly in the absence of AA but the presence of 3 mM AA led to greatly increased ceroid accumulation.

These results lead us to suggest that, in the presence of transition metals, AA behaves as a pro-oxidant and therefore might even contribute to the increased severity of atherosclerosis in diabetes mellitus. Short-term supplementation of AA in diabetic individuals with and without complications improved their plasma AA levels only transiently [9]. Indeed, such studies also showed that AA supplements led to an increase in products of AA oxidation [9]. This suggests that AA supplementation does little to alleviate oxidative stress in the short term and might even contribute to it. Also, oral AA can

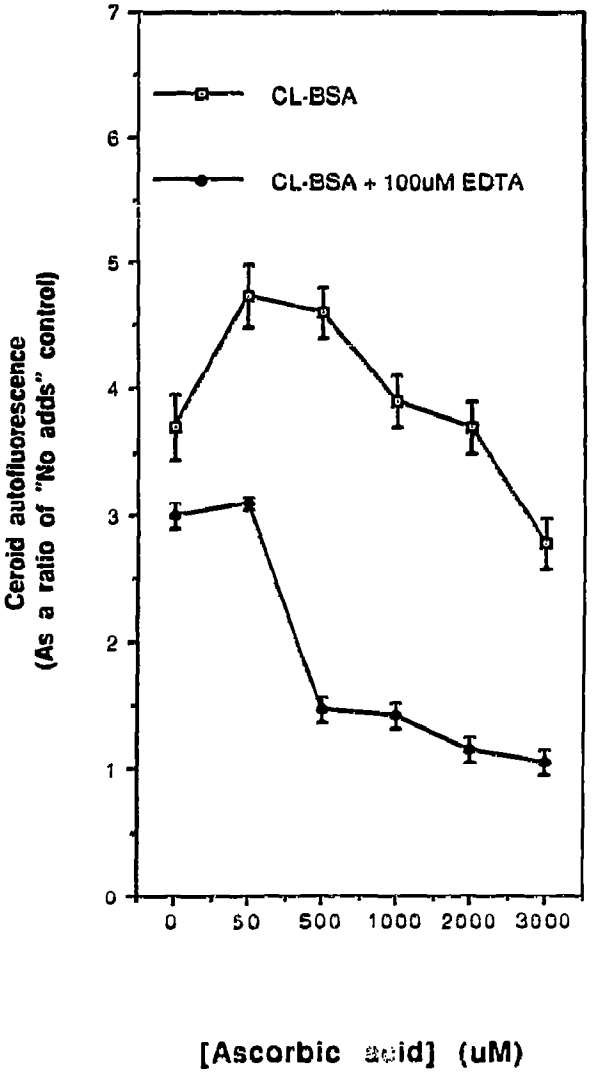

Fig. 3. Effect of AA on ceroid accumulation in macrophages.

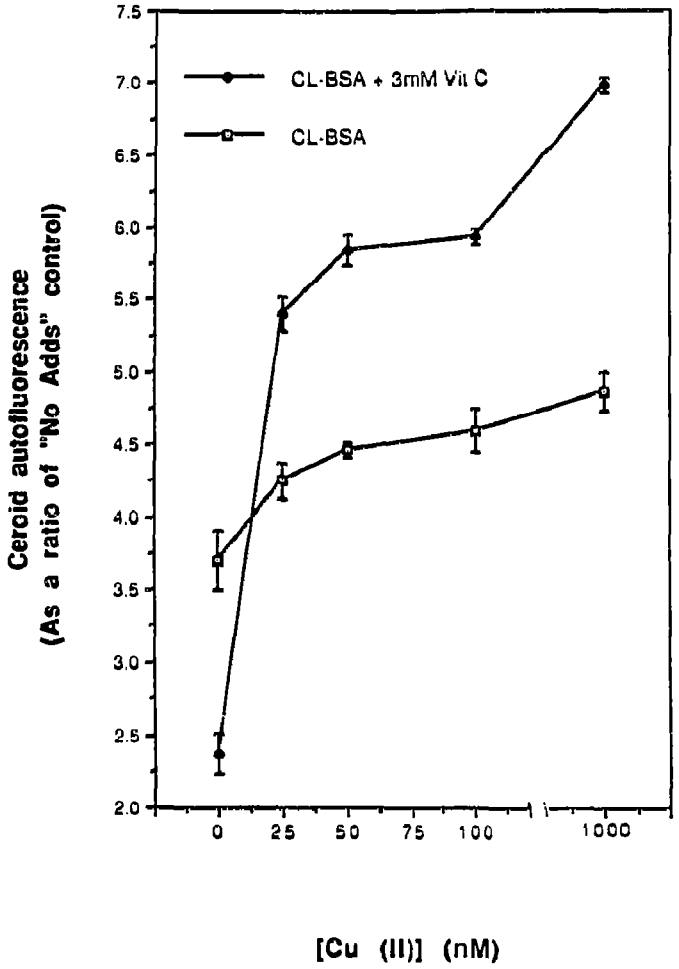

Fig. 4. Effect of cupric ions on ceroid accumulation in macrophages, in the presence and absence of AA.

cause increased iron absorption and be harmful in states of iron overload [31]. Since altered handling of iron has been shown in diabetes, along with beneficial effects of metal chelators [32], there remains a distinct possibility that ascorbic acid supplementation may do more harm than good.

Albeit using only an in vitro model of certain oxidative events, these studies suggest that ascorbic acid enhances oxidative reactions which may be pertinent to the development of atherosclerosis. Therefore, prior to advocating ascorbic acid supplementation to alleviate oxidative stress in diabetic individuals, there is a real need to establish whether the antioxidant activity of ascorbate indeed predominates in vivo. Long-term ascorbic acid supplementation could theoretically exacerbate oxidative stress, and actually enhance the risk of the life-threatening diabetic complication, atherosclerosis.

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