

Glucose oxidation and low-density lipoprotein-induced macrophage ceroid accumulation: possible implications for diabetic atherosclerosis

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The exposure of proteins to high concentrations of glucose *in vitro* is widely considered a relevant model of the functional degeneration of tissue occurring in diabetes mellitus. In particular, the enhanced atherosclerosis in diabetes is often discussed in terms of glycation of low-density lipoprotein (LDL), the non-enzymic attachment of glucose to apolipoprotein amino groups. However, glucose can undergo transition-metal-catalysed oxidation under near-physiological conditions *in vitro*, producing oxidants that possess a reactivity similar to the hydroxyl radical. These oxidants can fragment protein, hydroxylate benzoic acid and induce lipid peroxidation in human LDL. In this study, glycation of LDL *in vitro* is accompanied by such oxidative processes. However, the oxidation of LDL varies with glucose concentration in a manner which does not parallel changes in

protein glycation. Glycation increases in proportion to glucose concentration, whereas in our studies maximal oxidation occurs at a glucose concentration of approx. 25 mM. The modification of LDL resulting from exposure to glucose alters macrophage ceroid accumulation, a process which occurs in the human atherosclerotic plaque. The accumulation of ceroid in macrophages is shown to be related to LDL oxidation rather than LDL glycation, *per se*, as it too occurs at a maximum of approx. 25 mM. Oxidative sequelae of protein glycation appear to be a major factor in LDL–macrophage interactions, at least with respect to ceroid accumulation. Our observations are discussed in the context of the observed increase in the severity of atherosclerosis in diabetes.

INTRODUCTION

The lipid-laden foam cells in human atherosclerotic lesions have been identified as monocyte-derived macrophages [1,2]. The main source of lipid in these cells might be oxidized low-density lipoprotein (LDL), as oxidized LDL has been detected in human lesions [3]. Within all atherosclerotic lesions, the lipid pigment, ceroid, is found within foam cells [4,5] and in the necrotic core of advanced lesions, as a result of macrophage necrosis [1,4,5]. Ceroid is an autofluorescent polymer of oxidized lipid and protein [5], which in atherosclerosis is probably partly composed of oxidized LDL [6,7]. Its presence supports the suggestion that LDL oxidation is involved in atherogenesis [4–8]. The protective effects of antioxidants against atherosclerosis in hyperlipidaemic rabbits [9] and possibly in human atherosclerosis [10–12] also support this suggestion. Studies *in vitro* which are consistent with this view include the inhibition of macrophage-mediated lipoprotein oxidation by antioxidants [13] and the accumulation of ceroid in macrophages exposed to oxidized LDL or to oxidizable artificial lipoproteins [14–17].

Atherosclerosis is more severe in diabetes mellitus [18]. Non-enzymic glycation of plasma lipoprotein has been shown to decrease its uptake by human fibroblasts, decrease its catabolism in cell culture and decrease its clearance from rabbit plasma *in vivo* [19,20]. Some studies have suggested that macrophages possess specific receptors for glycated proteins [21] and LDL glycation has therefore been proposed as the cause of the increase in atherosclerosis in diabetes [19–21]. Some human population studies suggest that enhanced atherosclerosis in diabetes may be due to hyperinsulinaemia [22] or altered lipid metabolism [23]; opinion is divided on hyperglycaemia as a cause [24,25a,b]. This suggests that the relationship between glucose control and atherosclerosis is not simple.

A recent proposal that might explain this complexity is that increased oxidative stress contributes to the enhanced development of atherosclerosis in diabetes mellitus [26,27]. The evidence includes the findings that plasma antioxidants such as ascorbic acid [28,29], vitamin E [30], uric acid [31] and glutathione [32] are all decreased in diabetes, but levels of plasma peroxides are elevated [33]. There also seems to be a link between diabetes and altered handling of transition metals [34–37], necessary catalysts of many oxidative processes. The enhanced oxidative stress in diabetes may be complicated, involving all of these factors and an increased availability of oxidizable substances, possibly including glucose [26,27]. This complexity may be obscuring an epidemiological link with hyperglycaemia, even if glucose were the eventual oxidizing substance. The idea is an attractive one because individual variations in susceptibility to diabetic complications, including atherosclerosis, might be derived from individual variations in antioxidant status, just as is postulated for atherosclerosis in general [16,17].

Therefore we have studied ceroid accumulation in macrophages cultured with LDL previously exposed to glucose. The relative contribution of LDL glycation and glucose-mediated oxidation to this ceroid accumulation in macrophages is investigated. The results suggest that studies on the effect of LDL glycation on its interactions with cells must take account of the oxidative reactions of glucose.

MATERIALS AND METHODS

Radiochemicals were obtained from Amersham (Aylesbury, Bucks., U.K.). All biochemicals were obtained from Sigma (Poole, Dorset, U.K.) or Aldrich (Gillingham, Kent, U.K.) and were of the highest purity available.

The incorporation of D-[U-¹⁴C]glucose into LDL and albumin

Abbreviations used: LDL, low-density lipoprotein; LPDFCS, lipoprotein-deficient fetal-calf serum; DETAPAC, diethylenetriamine penta-acetic acid.

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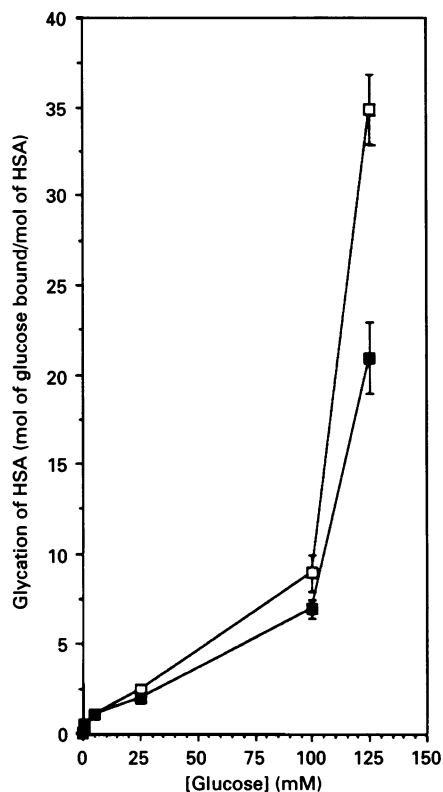


Figure 1 Glycation of HSA: effect of glucose concentration

The exposure of 20 mg/ml HSA to various concentrations of radiolabelled glucose over a period of 7 days led to the attachment of glucose to this protein. EDTA (1 mM) significantly reduced this level of attachment. Unless stated otherwise all reactions in this and subsequent studies were performed in the presence of 10 mM potassium phosphate (pH 7.4) at 37 °C. Glucose attachment increased linearly with glucose concentration either in the absence (□) or presence (■) of EDTA. Values in this and subsequent figures are means \pm S.D. from a minimum of three experiments during which assays were performed in triplicate.

was performed as previously described [38,39]. Before use, the absence of other protein-reactive aldehydes was confirmed [28,29] and any transition-metal contaminants were removed by treating with chelating resin, as previously described [27]. Briefly, protein was incubated with various glucose concentrations (from 5 to 500 mM) containing 10 μ Ci/ml D-[U- 14 C]glucose in the presence of 10 mM potassium phosphate buffer (pH 7.4). After 7 days, samples were withdrawn and precipitated by the addition of trichloroacetic acid to a final concentration of 5%. The pellet was washed twice in 5% trichloroacetic acid and finally dissolved in formic acid before scintillation counting.

The generation of oxidants by glucose was assessed by monitoring the hydroxylation of benzoic acid to fluorescent products (308 nm excitation/410 nm emission) [38–40]. Incubations at 37 °C consisted of 1 mM benzoic acid, 10 mM potassium phosphate (pH 7.2) and various concentrations of glucose (from 5 to 500 mM).

Fragmentation of human serum albumin (HSA; 20 mg/ml) during exposure to glucose was used as a measure of free radical damage, as previously described [39,41]. Albumin (fraction V; Boehringer–Mannheim, Mannheim, Germany) was labelled by reductive methylation of 0.1% of total lysine groups (50000 d.p.m./mg of protein) with [14 C]formaldehyde and 10 mM NaCNBH₃. Fragmentation of radiolabelled HSA during

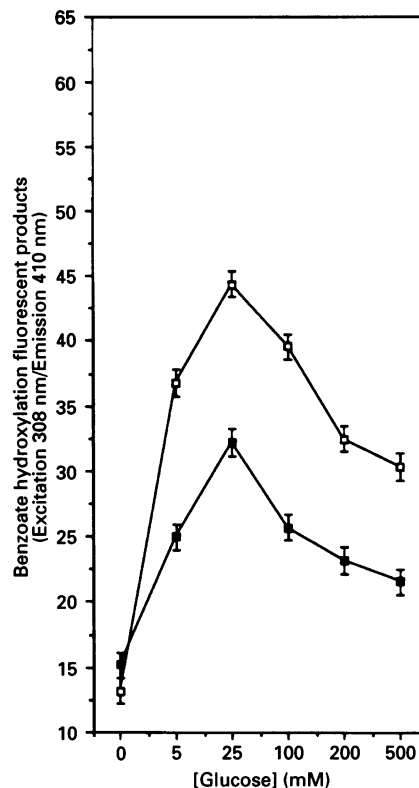


Figure 2 Glucose-derived oxidant formation: effect of glucose concentration

The exposure of 1 mM benzoic acid to various concentrations of glucose for 7 days resulted in the formation of fluorescent hydroxylation derivatives, inhibited by 1 mM EDTA. Hydroxylation products are expressed as salicylic acid equivalents. For reasons of clarity, glucose concentrations are shown on a non-linear x-axis. Levels of benzoate hydroxylation were not directly proportional to glucose concentration either in the absence (□) or presence (■) of EDTA.

exposure to glucose was monitored by the determination of radiolabelled fragments soluble in 5% trichloroacetic acid.

Human LDL was prepared from non-diabetic subjects as previously described [38]. Blood was centrifuged in the presence of 1 mg/ml EDTA to obtain plasma. Lipoprotein fractions were then obtained from pooled plasma by ultracentrifugation and flotation through KBr gradients. Centrifugations were performed at 100000 g for 18 h at 16 °C in the presence of EDTA. The fraction that floated at a density of 1.019–1.063 g/ml was taken as LDL.

Throughout, LDL was incubated at a concentration of 3.5 mg/ml total LDL at 37 °C for 7 days. Sterility was achieved by filter sterilization (0.22 μ m pore diam. filter). LDL oxidation was assessed as previously described by reaction with thio-barbituric acid [38] and confirmed by reaction with Xylenol Orange [38,40].

Resident peritoneal macrophages were isolated from male Balb/c mice aged 6–8 weeks as previously described [42–44]. Cells were resuspended in RPMI 1640 medium containing 10% (v/v) lipoprotein-deficient fetal-calf serum (LPDFCS; prepared by ultracentrifugation and flotation through KBr as previously described [45]), and plated at a density of 2×10^6 cells per dish. All media contained 100 i.u./ml penicillin and 100 μ g/ml streptomycin sulphate. Cell cultures were performed in 3 ml (5 cm diam.) culture dishes (Anumbra dishes, Payne, Slough, Berks.,

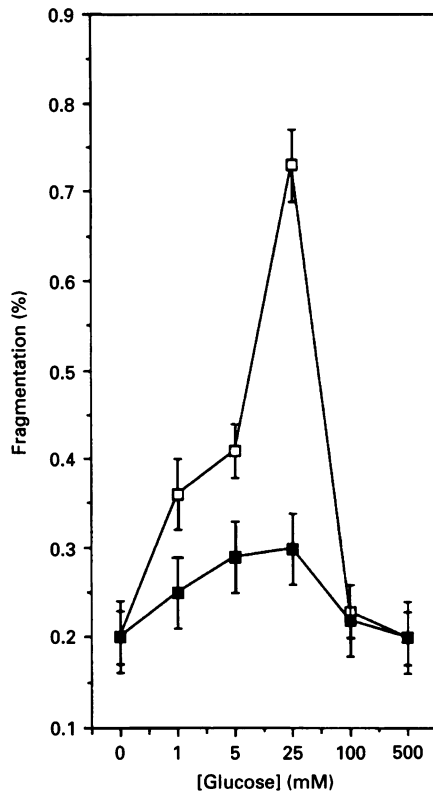


Figure 3 Effect of glucose concentration on fragmentation of HSA

The exposure of 20 mg/ml radiomethylated HSA to various concentrations of glucose over 7 days led to the production of trichloroacetic acid-soluble radiolabelled peptide fragments. EDTA (1 mM) reduced the attachment at high concentrations of glucose. For reasons of clarity, glucose concentrations are shown on a non-linear x-axis. Fragmentation of HSA by glucose in the absence (□) or presence (■) of EDTA was not directly proportional to glucose concentration.

U.K.). The macrophages were allowed to adhere by incubating for 3 h at 37 °C in an atmosphere of 5% CO₂. Non-adherent cells and erythrocytes were then removed by washing with fresh medium. Fresh medium was then added, and cells incubated overnight at 37 °C in an atmosphere of 5% CO₂ before experiments were performed.

The LDL samples, previously exposed under defined conditions (described above and in the figure legends), were dialysed free of glucose and any other reagents as previously described [38]. Before ceroid analysis, cells (2×10^6 cells per dish) were cultured with 200 µg/ml pre-treated LDL for 24 h.

Cells were harvested by removing the medium and adding 0.33% EDTA in RPMI 1640 medium containing 10% (v/v) LPDFCS. This 'EDTA medium' was then removed and retained. Remaining adherent cells were scrape-harvested in Hanks' balanced salt solution and mixed with the 'EDTA medium'. A cell pellet was then prepared by centrifugation, as previously described [44]. Cells were resuspended in 0.25 ml of fixative (1.5% formaldehyde/0.1% BSA in PBS) by vortexing. Samples prepared in this way were stored at 4 °C and were analysed within 7 days for ceroid accumulation by flow cytometry, during which period no change in fluorescence was observed. Fluorescence of ceroid in cells was analysed by a FACstar flow cytometer [44,46] equipped with a Spectra Physics 2025 argon ion laser. The laser was tuned to emit 300 mW of light in the u.v.

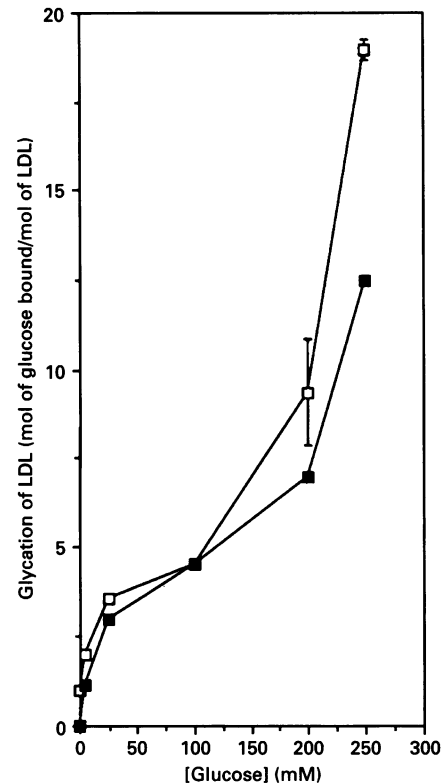


Figure 4 Effect of glucose concentration on glycation of human LDL

The exposure of 3.5 mg/ml human LDL to various concentrations of radiolabelled glucose for a period of 7 days led to the attachment of glucose to this protein. EDTA (1 mM) reduced the attachment at high concentrations of glucose. Glucose attachment increased linearly with glucose concentration either in the absence (□) or presence (■) of EDTA.

wavelengths 351.1–363.8 nm. Intact macrophages were identified by forward light scatter. Selection of live populations of macrophages was determined, as previously described [44,46], using propidium iodide to stain dead cells. Fluorescence was monitored using a 490 nm long-pass filter in front of the detector. Flow cytometers, in common with all fluorimeters, measure light intensities in arbitrary unit. Low-level autofluorescence, present in control cells cultured in medium alone, varied by up to 10% between experiments. Therefore, in each experiment results for ceroid were calculated relative to the control (as a ratio). Such low-level autofluorescence in control cells cultured in medium alone is due to other cellular components, including nicotinamide dinucleotides and flavins [47,48], which bear similarities in fluorescence characteristics to that of ceroid. Thus, the values shown are corrected for the contribution of autofluorescence found in control cells.

Statistical analysis, including Student's *t* tests and analysis of variance, were performed using 'Microsoft Excel 5.0' (Apple Macintosh).

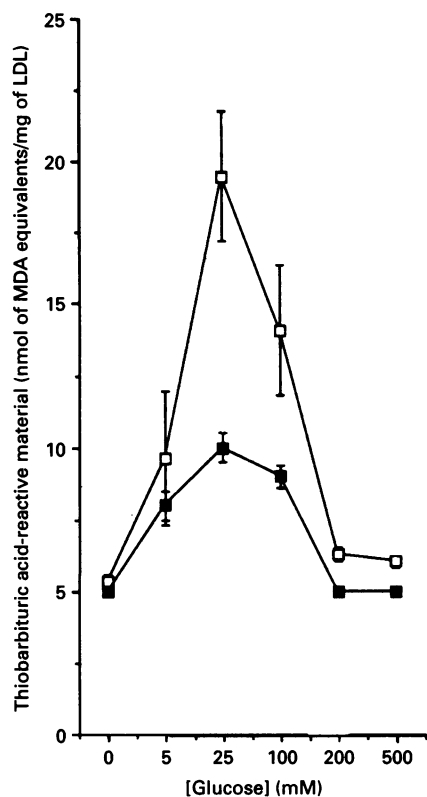
RESULTS

The attachment of glucose to HSA (Figure 1) increased in a linear manner with glucose concentration under these conditions. Although the metal chelator EDTA decreased the extent of glucose attachment slightly, especially at glucose concentrations above 25 mM, the linear relationship was unaffected. However, oxidant formation and protein fragmentation appeared to have

Table 1 Effect of different metal chelators on the attachment of glucose to LDL

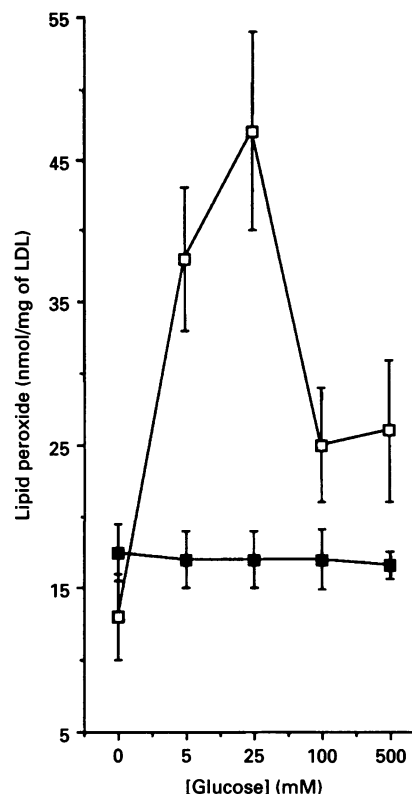
LDL (3.5 mg/ml) was incubated at 37 °C in the presence of radiolabelled glucose for a period of 1 week. The effect upon glucose attachment of 1 mM EDTA, DETAPAC, desferrioxamine mesylate (DES) and sodium diethyldithiocarbamic acid (DDC) is shown. Values of glucose attachment (mol of glucose bound/mol of LDL) and the extent of inhibition (%) are provided and are expressed as the mean \pm S.D. from a minimum of three experiments.

Experimental conditions	Attachment of glucose to LDL			
	25 mM Glucose		500 mM Glucose	
	(mol/mol)	(% inhibition)	(mol/mol)	(% inhibition)
No chelator	3.52 \pm 0.14	0	19 \pm 2.58	0
DETAPAC	0.73 \pm 0.06	79.17 \pm 6.95	11 \pm 3.47	41.47 \pm 12.9
EDTA	2.75 \pm 0.09	2.4 \pm 0.7	14 \pm 1.05	26.3 \pm 1.97
DES	3.3 \pm 0.05	5.7 \pm 0.08	17.6 \pm 3.3	7.15 \pm 1.33
DDC	1.96 \pm 0.69	44 \pm 15	14.8 \pm 0.55	22.15 \pm 0.82

**Figure 5** Effect of glucose concentration upon the formation of thiobarbituric acid-reactive material

To monitor the extent of LDL oxidation after 7 days incubation, dialysed LDL, which had previously been exposed at 3.5 mg/ml to various concentrations of glucose with or without 1 mM EDTA, was assessed by the thiobarbituric acid assay. For reasons of clarity, glucose concentrations are shown on a non-linear *x*-axis. Oxidation was not directly proportional to glucose concentration either with (■) or without (□) EDTA. Abbreviation: MDA, malondialdehyde.

glucose concentration maxima (approx. 25 mM) and were not linearly related to glucose concentration. Figure 2 shows the effect of various glucose concentrations on the process of oxidant

**Figure 6** Effect of glucose concentration upon the formation of lipid peroxides

To monitor the extent of LDL oxidation after 7 days incubation, dialysed LDL, which had previously been exposed at 3.5 mg/ml to various concentrations of glucose with or without 1 mM EDTA, was assessed by the Xylenol Orange assay of lipid peroxides. For reasons of clarity, glucose concentrations are shown on a non-linear *x*-axis. Oxidation was not directly proportional to glucose concentration either with (■) or without (□) EDTA.

formation, monitored by the hydroxylation of benzoic acid, and the inhibitory effect of EDTA. Addition of 1 μ M Cu(II) (CuSO₄) greatly increased benzoic acid hydroxylation but not the concentration of glucose at which oxidant formation was maximal (results not shown). The exposure of 20 mg/ml (300 mM) HSA to increasing glucose concentrations exhibited a similar trend in protein fragmentation (Figure 3), a measure of protein oxidation [29,41], which again had a maximal glucose concentration of approx. 25 mM and was inhibited by EDTA. However, whereas the formation of oxidants above 100 mM glucose was inhibited by EDTA (Figure 2) the fragmentation of HSA was unaffected by EDTA at 100 mM glucose (Figure 3). This apparent contradiction is unsurprising as above 100 mM glucose fragmentation was minimal, presumably due to the antioxidant properties of albumin and high concentrations of glucose.

Studies with human LDL showed a linear increase in glucose attachment with increasing concentrations of glucose (Figure 4). Figure 4 also shows the inhibitory effect of EDTA upon glucose attachment, which is most obvious at high glucose concentrations. Previous studies with BSA showed that EDTA is a relatively poor inhibitor of glucose attachment [49] when compared with chelators of a higher metal affinity, such as diethylenetriamine penta-acetic acid (DETAPAC). Table 1 shows the effect of 1 mM EDTA, DETAPAC, desferrioxamine mesylate and sodium diethyldithiocarbamic acid upon glucose attachment to LDL

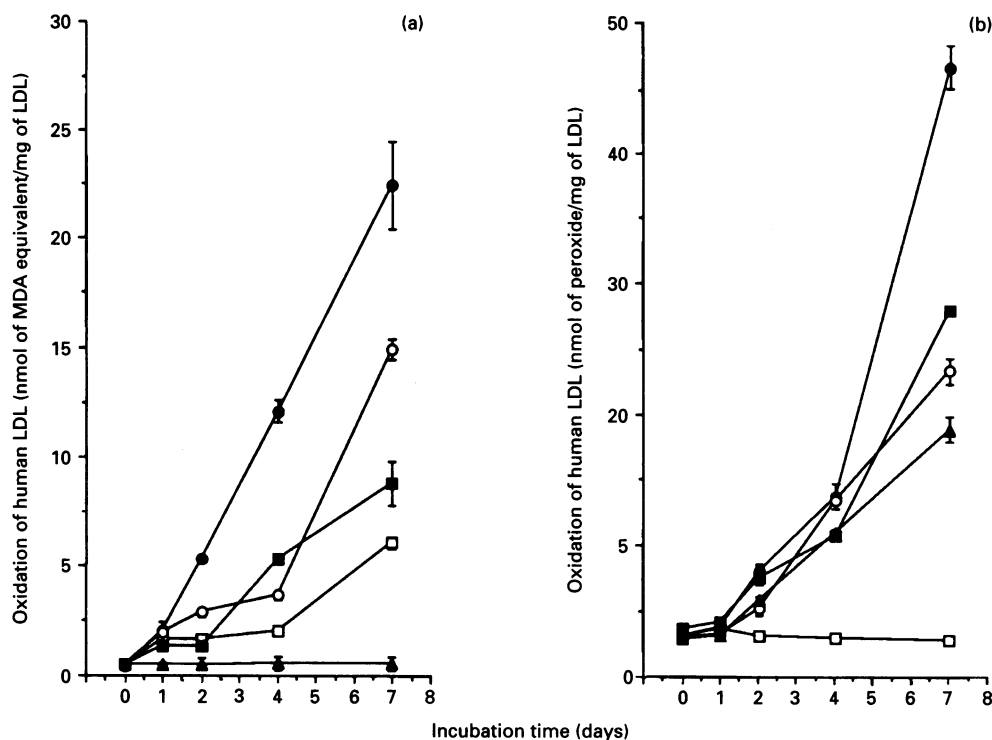


Figure 7 Time-dependent effect of glucose concentration on the oxidation of human LDL

The oxidation of LDL (3 mg/ml) incubated with various concentrations of glucose (0, \square ; 5, \blacksquare ; 25, \bullet ; 100, \circ ; and 500 mM, \blacktriangle) over a 7-day period was monitored by (a) the thiobarbituric acid assay and (b) the Xylenol Orange assay.

over 7 days in the presence of 25 and 500 mM glucose. DETAPAC proved the most effective inhibitor of LDL glycation.

On the other hand, studies showed a 25 mM maximum glucose concentration for the oxidation of the lipid moiety of LDL, assessed as thiobarbituric acid-reactive material (Figure 5) and by the Xylenol Orange assay of lipid peroxides (Figure 6). Figures 7(a) and 7(b) show the time-dependent increase in the accumulation of thiobarbituric acid-reactive-material and Xylenol Orange-detectable lipid peroxide respectively. The effect of the metal chelator (EDTA) upon lipid peroxidation was similar to that with HSA fragmentation. EDTA both reduced glucose attachment at higher glucose concentrations and inhibited lipid oxidation at all concentrations of glucose. EDTA inhibited the formation of thiobarbituric acid-reactive material (Figure 5) and greatly inhibited lipid peroxide formation (Figure 6). The contrast between the thiobarbituric acid and Xylenol Orange assays may have resulted from a difference in sensitivity. However, EDTA did not alter the concentration of glucose required for maximal oxidation nor the linear manner with which glucose attachment increased with glucose concentration. The accumulation of thiobarbituric acid-reactive-material and Xylenol Orange-detectable lipid peroxide (Figures 7a and 7b) confirmed a continuing increase in LDL oxidation with the maximal level of lipid peroxidation occurring with 25 mM glucose.

When LDL, previously exposed to various concentrations of glucose, was added to cultures of mouse peritoneal macrophages there was a change in intracellular ceroid accumulation, measured by cytometric analysis of cellular fluorescence (Figure 8). Again, ceroid accumulation was greatest on culture with LDL previously incubated with 25 mM glucose ($P > 0.02$, student's t test). The inclusion of EDTA during LDL incubation with various con-

centrations of glucose diminished the subsequent accumulation of ceroid in macrophages ($P > 0.01$, analysis of variance).

This was further confirmed in a study during which LDL was exposed to glucose in the presence of NaCNBH_3 or $10 \mu\text{M}$ Cu(II) for 24 h. NaCNBH_3 is a reducing agent able to lead to extensive lysine modification (30 mol/mol of LDL) in the absence of oxidative events [49]. Figure 8 shows extensive glucose attachment in the presence of NaCNBH_3 but negligible change in thiobarbituric acid reactivity and no macrophage ceroid accumulation. Also shown is a control of LDL exposed to glucose alone for 24 h. Such a short time of exposure leads to little glucose attachment and no oxidation nor subsequent ceroid accumulation in cells. Copper-incubated LDL exhibited an elevated level of oxidation and ceroid accumulation, further supporting the role of LDL oxidation in ceroid accumulation (Table 2).

DISCUSSION

The contribution of protein glycation to diabetic tissue damage is frequently examined by the exposure of macromolecules to glucose. Glucose concentrations selected for *in vitro* studies have varied between 25 mM [38] (considered relevant to hyperglycaemic levels of glucose) and 200 mM [50,51]. Modifications that result have then been monitored over various periods of exposure, from a few days to several weeks. Under these circumstances some alterations in protein function and structure may be due, not to glycation itself, but to oxidative processes.

Observations on such oxidative processes have led to the suggestion of several mechanisms by which glucose may oxidize [52]. Oxidation is transition-metal-dependent, as parameters of

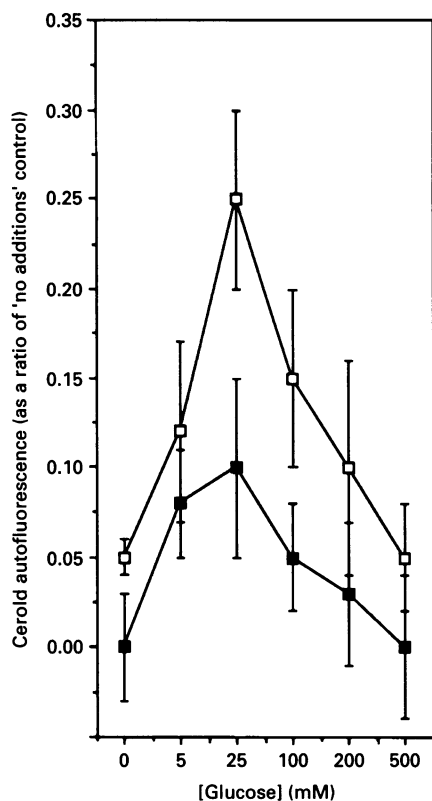


Figure 8 Effect of LDL pre-exposed to glucose on macrophage ceroid accumulation

Dialysed LDL (200 $\mu\text{g}/\text{ml}$), previously exposed at 3.5 mg/ml to various concentrations of glucose (0, 5, 25, 100, 200 and 500 mM) for 7 days with or without 1 mM EDTA, was cultured with 2×10^6 mouse peritoneal macrophages for 24 h, as described. The accumulation of ceroid, monitored as intracellular fluorescence by flow cytometry, was measured. There was no direct relationship between the intracellular fluorescence and glucose concentration either in the absence (\square) or presence (\blacksquare) of EDTA.

Table 2 Attachment of glucose to LDL does not cause ceroid accumulation in macrophages

LDL (3.5 mg/ml) was incubated for 24 h with buffer alone, 25 mM radiolabelled glucose, 10 μM Cu(II), 10 mM NaCNBH₃ or 25 mM radiolabelled glucose plus 10 mM NaCNBH₃. Levels of thiobarbituric acid-reactive material in dialysed LDL and the extent of glucose attachment were determined and the accumulation of ceroid on subsequent culture of macrophages with 200 $\mu\text{g}/\text{ml}$ pre-treated LDL was determined as described. Abbreviations: MDA, malondialdehyde; N/A, not applicable; ND, not detectable.

Experimental conditions	Glucose attachment (mol of glucose bound/mol of LDL)	Lipid peroxidation (mol of MDA equivalents/mg of LDL)	Macrophage ceroid accumulation (ratio of 'no additions' control)
Buffer alone	N/A	2.0 \pm 0.1	ND
25 mM Glucose	2.0 \pm 0.1	2.0 \pm 0.2	0.05 \pm 0.002
10 μM Cu(II)	N/A	144 \pm 8.0	2.4 \pm 0.05
10 mM NaCNBH ₃	N/A	3.5 \pm 1.0	ND
10 M NaCNBH ₃ + 25 mM Glucose	30 \pm 5.0	3.6 \pm 1.0	ND

oxidation measured *in vitro* are inhibited by metal chelators such as EDTA. Indeed, the inhibition of glucose attachment to protein by metal chelators led to the hypothesis of autooxidative glycosylation, during which aldehydic oxidation products of glucose were proposed to contribute to overall protein glycation [39,49]. Figures 1 and 4 show that EDTA does indeed reduce the level of glucose attachment at higher concentrations of glucose, but the attachment still increases in proportion to glucose concentration. That EDTA inhibited glycation at high concentrations of glucose alone is unsurprising. EDTA is a relatively poor inhibitor of glycation when compared with chelators of a higher affinity, such as DETAPAC, as demonstrated in previous glycation studies [38,49] on albumin and also shown in Table 1.

In contrast, there is a maximum concentration for oxidant production by glucose (Figures 2, 3, 5 and 7), suggesting that glucose possesses both pro-oxidant and antioxidant properties. The same is true for ascorbic acid and other reducing sugars [53,54]. Thus, at high concentrations, oxidant formation is probably subject to scavenging by unoxidized substrate. On the other hand, the formation of protein-reactive aldehydes is unlikely to be affected by the presence of unoxidized glucose. Thus, glucose attachment increases in linear fashion with glucose concentration, whereas oxidant formation is subject to anti-oxidant activity at high glucose concentrations. In the light of these differences, we were prompted to conduct the present studies, which were designed to investigate the relative importance of glucose attachment and oxidation to the formation of LDL-derived ceroid in macrophages.

Ceroid accumulation was greatest when LDL had been exposed to 25 mM glucose (Figure 8), the same was true for glucose-mediated LDL oxidation (Figures 5, 6 and 7) and protein fragmentation (Figure 3). This suggests that glucose-mediated oxidation, rather than glucose attachment *per se*, is responsible for macrophage ceroid accumulation. This is further supported by studies during which maximal levels of glucose attachment were achieved under the reducing conditions induced by the inclusion of NaCNBH₃ [49]. By reducing Schiff bases, reversibly formed between protein and glucose in the open chain form, high levels of glucose attachment are achieved over a short period [49]. However, LDL treated in this manner resulted in virtually no ceroid accumulation, whereas copper-oxidized LDL led to very high levels of ceroid (Table 1).

There is an active debate about the roles of glycation and free radical production in the complications of diabetes. It is important to recognize the role of oxidative reactions during glycation reactions *in vitro*, appreciating the effect of transition metals and glucose concentrations. Where oxidation is to be minimized it is important to include metal-chelating agents, as the oxidative reactions are accelerated by trace transition-metal contaminants present in all physiological buffers. Certainly, studies of LDL modification by glucose and its subsequent effect, if any, upon cellular interactions, must ensure that oxidative reactions are both monitored and controlled. The fact that oxidative sequelae of glycation vary with glucose concentration may have important implications for the interpretation of studies on LDL glycation, cellular interactions and atherosclerosis.

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REFERENCES

- 1 Aqel, N. M., Ball, R. Y., Waldmann, H. and Mitchinson, M. J. (1985) *J. Pathol.* **146**, 197-204

- 2 Jonasson, L., Holm, J., Skalli, I., Bondjers, G. and Hanssen, G. K. (1986) *Arteriosclerosis* **6**, 131–138
- 3 Ylä-Herttua, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L. and Steinberg, D. (1989) *J. Clin. Invest.* **84**, 1086–1095
- 4 Mitchinson, M. J. (1982) *Atherosclerosis* **5**, 11–15
- 5 Mitchinson, M. J., Hothersall, D. D., Brooks, P. N. and De Burbure, C. Y. (1985) *J. Pathol.* **145**, 177–183
- 6 Mitchinson, M. J., Ball, R. Y., Carpenter, K. L. H. and Parums, D. V. (1988) in *Hyperlipidaemia and Atherosclerosis* (Suckling, K. E. and Groot, P. H. E., eds.), pp. 117–134, Academic Press, London
- 7 Parums, D. V., Brown, D. L. and Mitchinson, M. J. (1990) *Arch. Pathol. Lab. Med.* **114**, 383–387
- 8 Ball, R. Y., Carpenter, K. L. H. and Mitchinson, M. J. (1987) *Arch. Pathol. Lab. Med.* **111**, 1134–1140
- 9 Kita, T., Nagano, Y., Yokode, M., Ishii, K., Kume, N., Ooshima, A., Yoshida, H. and Kawai, C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5928–5931
- 10 Gey, K. F., Puska, P., Jordan, P. and Moser, U. K. (1991) *Am. J. Clin. Nutr.* **53**, 326S–343S
- 11 Stampfer, M. J., Herreken, C. H., Manson, J. E., Colditz, G. A., Rosner, B. and Willett, W. C. (1993) *New Engl. J. Med.* **328**, 1444–1449
- 12 Rimm, E. B., Stampfer, M. J., Ascherio, A., Giovannucci, E., Colditz, G. A. and Willett, W. C. (1993) *New Engl. J. Med.* **328**, 1450–1456
- 13 Esterbauer, H., Gebicki, J., Puhl, H. and Jurgens, G. (1992) *Free Rad. Biol. Med.* **13**, 341–390
- 14 Ball, R. Y., Bindman, J. P., Carpenter, K. L. H. and Mitchinson, M. J. (1986) *Atherosclerosis* **60**, 173–181
- 15 Ball, R. Y., Carpenter, K. L. H., Enright, J. H., Hartley, S. L. and Mitchinson, M. J. (1987) *Br. J. Exp. Pathol.* **68**, 427–438
- 16 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) *New Engl. J. Med.* **320**, 915–924
- 17 Mitchinson, M. J., Ball, R. Y., Carpenter, K. L. H., Enright, J. H. and Brabbs, C. E. (1990) *Biochem. Soc. Trans.* **18**, 1066–1069
- 18 Deckert, T., Poulsen, J. E. and Larsen, M. (1978) *Diabetologia* **14**, 363–370
- 19 Sasaki, J. and Cottam, G. L. (1982) *Biochim. Biophys. Acta* **713**, 199–207
- 20 Steinbrecher, U. P. and Witztum, J. L. (1984) *Diabetes* **33**, 130–134
- 21 Vlassara, H., Brownlee, M. and Cerami, A. (1988) *Diabetes* **37**, 456–461
- 22 Beach, K. W. and Strandness, D. E. (1980) *Diabetes* **29**, 882–888
- 23 Laakso, M. and Pyörälä, K. (1988) *Atherosclerosis* **74**, 55–63
- 24 Mykkanen, L., Laakso, M., Penttilä, I. and Pyörälä, K. (1991) *Atherosclerosis* **88**, 153–161
- 25a Pirart, J. (1973) *Diabetes Care* **1**, 168–188
- 25b Pirart, J. (1973) *Diabetes Care* **1**, 252–263
- 26 Baynes, J. W. (1991) *Diabetes* **40**, 405–412
- 27 Wolff, S. P., Jiang, Z. Y. and Hunt, J. V. (1991) *Free Rad. Biol. Med.* **10**, 339–352
- 28 Som, S., Basu, D., Mukherjee, S., Deb, S., Choudary, P. R., Mukherjee, S. N. and Chatterjee, S. N. (1981) *Metabolism* **30**, 572–577
- 29 Jennings, P., Chirico, S., Jones, A. F., Lunec, J. and Barnett, A. H. (1987) *Diabetes Res.* **6**, 151–154
- 30 Karpen, C. W., Cataland, S. D., O'Dorisio, T. M. and Panganamala, R. (1984) *Diabetes* **33**, 239–243
- 31 Herman, J., Medalie, J. and Goldbourt, U. (1976) *Diabetologia* **12**, 47–52
- 32 Illing, E. K., Gray, C. H. and Lawrence, R. D. (1951) *Biochem. J.* **48**, 637–640
- 33 Sato, Y., Hotta, N., Sakamoto, N., Matsuoka, S., Ohishi, N. and Yagi, K. (1979) *Biochem. Med.* **21**, 104–107
- 34 Phelps, G., Hall, P., Chapman, I., Braund, W. and McKinnon, M. (1989) *Lancet* **i**, 233–234
- 35 Cutler, P. (1989) *Diabetes* **38**, 1207–1210
- 36 Mateo, M. C. M., Bustamante, J. B. and Cantalapiedra, M. A. G. (1978) *Biomed.* **29**, 56–58
- 37 Noto, R., Alicata, R. and Sfogliano, L. (1983) *Acta Diabetol. Latina* **20**, 81–85
- 38 Hunt, J. V., Smith, C. T. and Wolff, S. P. (1990) *Diabetes* **39**, 1420–1424
- 39 Hunt, J. V., Dean, R. T. and Wolff, S. P. (1988) *Biochem. J.* **256**, 205–212
- 40 Jiang, Z. Y., Hunt, J. V. and Wolff, S. P. (1992) *Anal. Biochem.* **202**, 384–389
- 41 Hunt, J. V., Simpson, J. A. and Dean, R. T. (1988) *Biochem. J.* **250**, 87–93
- 42 Carpenter, K. L. H., Ballantine, J. A., Fussell, B., Enright, J. H. and Mitchinson, M. J. (1990) *Atherosclerosis* **85**, 217–229
- 43 Ball, R. Y., Carpenter, K. L. H., Enright, J. H., Hartley, S. and Mitchinson, M. J. (1987) *Br. J. Exp. Pathol.* **68**, 427–438
- 44 Hunt, J. V., Carpenter, K. L. H., Bottoms, M. A., Carter, N. P., Marchant, C. E. and Mitchinson, M. J. (1993) *Atherosclerosis* **98**, 229–239
- 45 Ball, R. Y., Bindman, J. P., Carpenter, K. L. H. and Mitchinson, M. J. (1986) *Atherosclerosis* **60**, 173–181
- 46 Ormerod, M. G. (1990) in *An Introduction to Flow Cytometry: A Practical Approach* (Ormerod, M. G., ed.), I.R.L. Press, Oxford
- 47 Aubin, J. E. (1979) *J. Histochem. Cytochem.* **27**, 36–43
- 48 Benson, R. C., Meyer, R. A., Zaub, M. Z., McKhann, G. M. (1979) *J. Histochem. Cytochem.* **27**, 44–48
- 49 Wolff, S. P. and Dean, R. T. (1987) *Biochem. J.* **245**, 243–250
- 50 Sasaki, J., Okamura, T. and Cottam, G. L. (1983) *Eur. J. Biochem.* **131**, 535–538
- 51 Witztum, J. L., Mahoney, E. M., Brana, M., Fisher, M., Elam, R. L. and Steinberg, D. (1982) *Diabetes* **31**, 283–291
- 52 Hunt, J. V., Bottoms, M. A. and Mitchinson, M. J. (1993) *Biochem. J.* **291**, 529–535
- 53 Hunt, J. V., Bottoms, M. A. and Mitchinson, M. J. (1993) *FEBS Lett.* **311**, 529–535
- 54 Aruoma, O. I. and Halliwell, B. (1987) *Biochem. J.* **241**, 273–278