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Is glucose the sole source of tissue browning in diabetes mellitus?

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Reactions between glucose and protein are held to be responsible for the protein 'browning' reactions which occur in diabetes mellitus. In vitro, however, the formation of such novel protein fluorophores is dependent upon the metal-catalysed oxidation of the monosaccharide (glucose 'autoxidation'). Since other small oxidisable molecules are capable of 'browning' proteins via similar metal-catalysed oxidative mechanisms we suggest that protein modification in diabetes may not be restricted to reactions with monosaccharides but may also include other small autoxidisable molecules.

Protein browning; Non-enzymic glycation; Diabetes; Oxidative stress

1. INTRODUCTION

A number of metabolic abnormalities characterise diabetes, including altered lipid metabolism [1,2], ascorbic acid metabolism [3,4] and hyperglycaemia [4,5]. Elevated glucose levels are the most obvious feature of the diabetic individual. As a result, the non-enzymic glycation theory of glucose toxicity, described in the first instance by food chemists, has often been used as a potential explanation for the underlying tissue damage associated with diabetic complications [6,7]. Non-enzymic glycation, often studied in vitro by incubating protein with glucose [8] occurs via glucose condensation, Schiff's base formation and rearrangement to form the Amadori product. Amadori products may subsequently degrade and lead to chromo/fluorophoric adducts and result in the protein becoming 'browned' and fluorescent [9].

We have demonstrated, however, that reactions between glucose and proteins in vitro are considerably more complex than such simple addition of glucose to protein amino groups. Glucose can oxidise, catalysed by trace amounts of transition metal, under physiological conditions, generating hydrogen peroxide, reactive oxidants, and ketoaldehydes which contribute to protein fluorescence alterations and structural damage [10]. Indeed, in vitro at least, browning reactions appear to be largely inhibited if such oxidative reactions are suppressed. Since other small molecules such as polyunsaturated fatty acids and ascorbic acid are also implicated in diabetic tissue damage and are also subject to transition metal catalysed oxidation, we have compared their protein oxidation and modification properties with those of glucose.

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2. MATERIALS AND METHODS

2.1. *Protein fragmentation*

Free radicals are known to fragment protein to trichloroacetic acid (TCA)-soluble peptide [11]. α-Crystallin, prepared from homogenised bovine lens using gel filtration [12], was incubated with 25 mM glucose or ascorbate as described in legends to the figures. The production of TCA-soluble peptide was determined using the fluorescamine assay [13]. 150 μl samples of a 5% TCA-supernatant were added to 850 μl of 0.5 M potassium phosphate buffer (pH 6.8). This was then mixed with 1 ml fluorescamine solution (0.1 mg/ml in acetone). Fluorescence was determined after an incubation of 20 min at room temperature at an excitation wavelength of 392 nm and emission wavelength of 476 nm using a Perkin-Elmer LS 5 fluorometer. Protein standards of bovine serum albumin (BSA) between 0 and 100 μg/ml were used to estimate protein concentration. Neither glucose nor ascorbic acid, at the concentration of 25 mM, interfered in this assay.

2.2. *Generation of novel protein fluorescence*

Protein (α-crystallin or BSA), previously exposed to ascorbic acid or glucose as described in legends to the figures, was precipitated and washed with 5% trichloroacetic acid prior to resuspending in 100 mM potassium phosphate (pH 7.2). The suspension was fluorimetrically assessed by measuring emission between 350 nm and 500 nm when exciting at 350 nm [14]. In the case of arachidonic acid exposure, the protein was delipidated by the Folch-Lees method [15] prior to fluorimetric assessment.

Incubations were performed using filter sterilised (0.25 μm filter) reaction mixtures, except in the case of fatty acid incubations, in which 100 μg/ml penicillin and streptomycin were included.

3. RESULTS AND DISCUSSION

α-Hydroxyaldehydes, such as glucose, can enolize and undergo a transition metal-catalysed oxidation under physiological conditions [14,16]. This produces protein-reactive α-ketoaldehydes capable of browning proteins. The exposure of α-crystallin to glucose (Fig.

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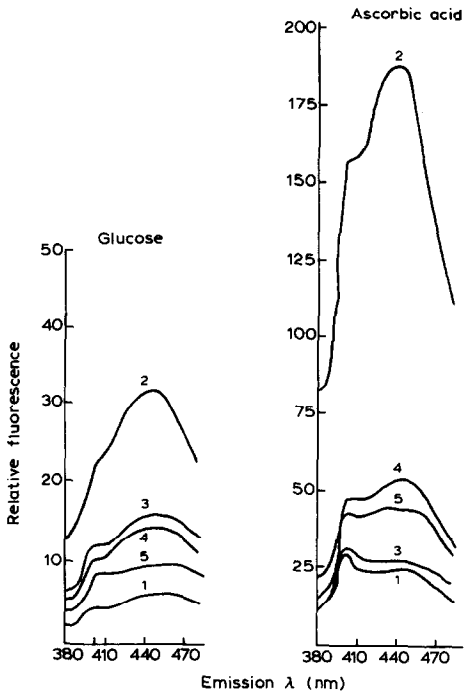

Fig. 1. The browning of α-crystallin by glucose and ascorbic acid. 5 mg/ml α-crystallin was incubated at 37°C with 25 mM glucose or ascorbic acid in the presence of 100 mM potassium phosphate (pH 7.2), for 1 and 14 days, respectively. After incubation, protein was washed free of unbound reagents by trichloroacetic acid precipitation and assessed fluorimetrically (Excitation 350 nm). Profile 1, protein and buffer; 2, protein and glucose or ascorbic acid; 3, protein, glucose or ascorbic acid, 50 mM glutathione (reduced); 4, proteins, glucose or ascorbic acid and 50 mM glutathione (oxidised); 5, proteins, glucose or ascorbic acid and 1 mM DETAPAC.

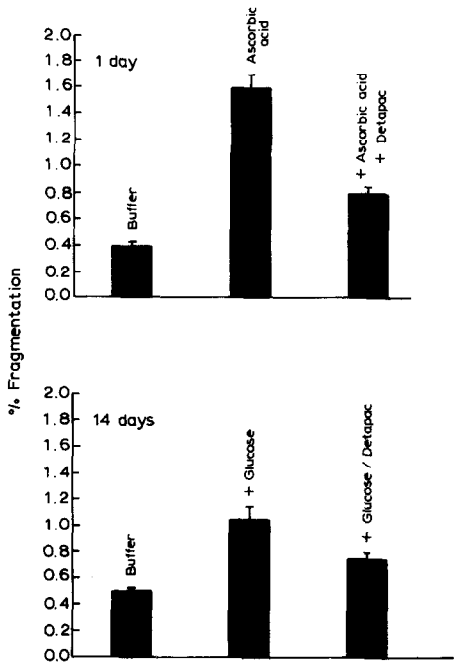

Fig. 2. The exposure of α-crystallin to ascorbic acid and glucose leads to the production of trichloroacetic acid-soluble peptide. 5 mg/ml α-crystallin was incubated as described in the legend to Fig. 1. Exposure to ascorbic acid or glucose over 1 and 14 days, respectively, leads to protein fragmentation to trichloroacetic acid-soluble fluorescamine-reactive peptide.

1a) leads to fluorophore formation which is inhibited by the addition of the metal chelator diethylene-triaminepenta-acetic acid, (DETAPAC), indicating the role of metal-catalysis during in vitro 'browning' of this particular protein by glucose. Ascorbic acid, a permanent enediol, is also able to undergo metal-catalysed oxidation [18–20] and modify proteins [21–23] in a similar manner to glucose. DETAPAC also inhibited fluorophore formation in the case of ascorbic acid (Fig. 1b). Both oxidised and reduced glutathione inhibited fluorophore formation. However, in the case of ascorbic acid, reduced glutathione (which maintains ascorbic acid in the reduced state [24]) led to profound inhibition of fluorophore formation. In contrast, glucose-mediated fluorophore formation was inhibited to a lesser degree, and equally, by both oxidised and reduced glutathione, suggesting that the amino groups rather than the thiol groups are important in the inhibition seen with glucose. Metal-catalysed oxidative reactions are thus required for both glucose- and ascorbate-mediated browning reactions. However, comparison of Fig. 1a and 1b demonstrates that fluorophore generation by ascorbic acid (performed over 1 day) far exceeded that of glucose (performed over 14 days).

Concomitant with this generation of protein

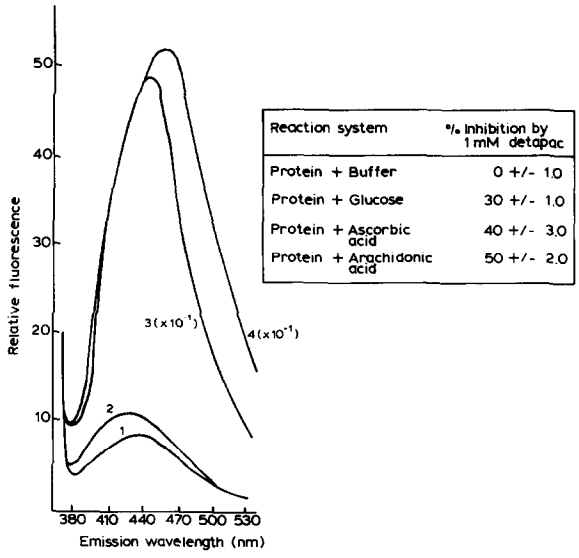

Fig. 3. A number of small autoxidisable molecules lead to protein browning. 5 mg/ml bovine serum albumin was exposed to 25 mM ascorbic acid, arachidonic acid (2 days) or glucose (7 days) in the presence of 100 mM potassium phosphate (pH 7.2). Reactions were performed in the presence or absence of 1 mM DETAPAC. The protein was then assessed fluorimetrically (Excitation 350 nm). Profile 1, protein and buffer; 2, protein and glucose; 3, protein and ascorbic acid; 4, protein and arachidonic acid. INSET: Percentage inhibition of fluorophore formation by 1 mM DETAPAC of the maximal emission wavelength.

fluorophores during the reactions of ascorbic acid and glucose with protein, the protein undergoes free radical-mediated fragmentation to TCA-soluble peptides [14,16,17] (Fig. 2). Fragmentation by both glucose and ascorbic acid was inhibited by the inclusion of 1 mM DETAPAC. The control reaction mixture, consisting of protein and buffer, contained ca 0.4% TCA-soluble peptide which did not change with time.

Thus, in Figs 1 and 2, we have demonstrated that browning reactions mediated by glucose are less extensive than those mediated by ascorbic acid, and that modification reactions by ascorbate as well as glucose are associated with free radical damage to the protein.

Finally, we have also demonstrated that exposure of bovine serum albumin to polyunsaturated fatty acids, such as arachidonic acid, is also able to generate protein fluorophores (Fig. 3) and that this process is also inhibited by DETAPAC (Fig. 3 inset). Fig. 3 also shows the similarity in fluorescence characteristics for the three protein modifying agents studied. Other studies have shown that unsaturated lipids also produce protein fragmenting oxidants, as a result of their metal-catalysed oxidation [11,25].

Our investigations suggest that the reaction of glucose with protein may not be the sole cause of protein modification in diabetes and imply that oxidative reactions are commonly involved. Indeed, both ascorbic acid and arachidonic acid are able to oxidise and brown proteins. Perhaps their involvement in metal-catalysed oxidative reactions contributes to the alteration in polyunsaturated fatty acids and ascorbic acid in diabetics [2-5,26]. However, oxidative processes are not commonly considered as a contributory factor in protein alterations observed either in vivo, or during in vitro experimental glycation studies. In this communication, we reiterate the role of metal-catalysed oxidation of small molecules such as glucose during in vitro exposure of protein to such molecules and question the assumption that glucose is the sole contributor to tissue browning and associated alterations in diabetes.

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