Autoxidative Glycosylation and Possible Involvement of Peroxides and Free Radicals in LDL Modification by Glucose

JAMES V. HUNT, CHRISTOPHER C.T. SMITH, AND SIMON P. WOLFF

It has been postulated that the etiology of the complications of diabetes involves oxidative stress, perhaps as a result of hyperglycemia. Consistent with this hypothesis, it has been shown that glucose, under physiological conditions, produces oxidants that possess reactivity similar to the hydroxyl free radical. These oxidants hydroxylate benzoic acid, fragment protein, and induce peroxidation in phosphatidylcholine liposomes and low-density lipoprotein (LDL) when LDL is incubated with hyperglycemic levels of glucose in vitro. These reactions are accelerated by transition metals and inhibited by a metal-chelating agent. The atherosclerotic potential of LDL in diabetes mellitus is often discussed in terms of protein glycosylation, which may affect cellular interactions. Our studies demonstrate, however, that peroxidative reactions also accompany LDL glycosylation in vitro. Peroxidative modification of LDL has also been implicated in LDL atherogenicity. Our studies indicate that glycosylation and peroxidation occur concomitantly in LDL modified by glucose in vitro and may both contribute to the behavioral changes of this lipoprotein. *Diabetes* 39:1420–24, 1990

It has been proposed that oxidative stress contributes to development of the diabetic complications (1). Antioxidants such as ascorbic acid, vitamin E, uric acid, and glutathione are all decreased in diabetes (1–4). There are also increased levels of plasma lipid peroxidation products, measured as substances capable of reacting with thiobarbituric acid (TBA-reactive material) (1). These changes appear to be greater in those individuals displaying diabetic complications (2–7). Although this evidence is indirect, the idea that oxidative stress contributes to diabetic pathogenesis is attractive, because individual variation in susceptibility to the complications could result, in part, from variations in individual antioxidant status (1).

If hyperglycemia is the cause of the diabetic complications, then it is reasonable to postulate that glucose is toxic and that increased plasma and tissue glucose is the proximal source of the increased oxidative stress apparent in diabetes. The demonstration that glucose can oxidize when catalyzed by trace amounts of transition metals, generating free radicals, hydrogen peroxide, and reactive ketoaldehydes, is consistent with this hypothesis (1,8; Fig. 1). The rate of glucose autoxidation is slow but is relevant to diabetic tissue damage, because it has direct bearing on studies of protein glycosylation, which is the nonenzymatic attachment of glucose to protein amino groups. Protein glycosylation is increased in diabetes and is thus used as an index of long-term glucose control and is a possible contributory factor to diabetic tissue damage (9,10).

The possible contribution of glycosylation to diabetic tissue damage is frequently examined by the exposure of proteins such as low-density lipoprotein (LDL) to high concentrations of glucose in vitro. The effect of this treatment on the physical and/or biological characteristics of the protein is then investigated. But under these circumstances, many of the changes that occur may be caused by free radical reactions. In the case of other proteins such as albumins, their exposure to glucose results in the protein becoming oxidatively damaged (8), and this appears to be responsible for many of the alterations normally ascribed to the attachment of glucose to the protein amino groups (9,10). This contribution of free radicals and peroxides to protein alterations during the glycosylation process has been referred to as autoxidative glycosylation (9,10). In the case of LDL, the situation is more complex because the lipid moiety may peroxidize when the lipoprotein is exposed to glucose. The aim of this study was thus to investigate whether glucose

From the Department of Clinical Pharmacology, University College London, London, United Kingdom.

Address correspondence and reprint requests to Dr. James V. Hunt, University College London, Department of Clinical Pharmacology, 5 University Street, London WC1E 6JJ, UK.

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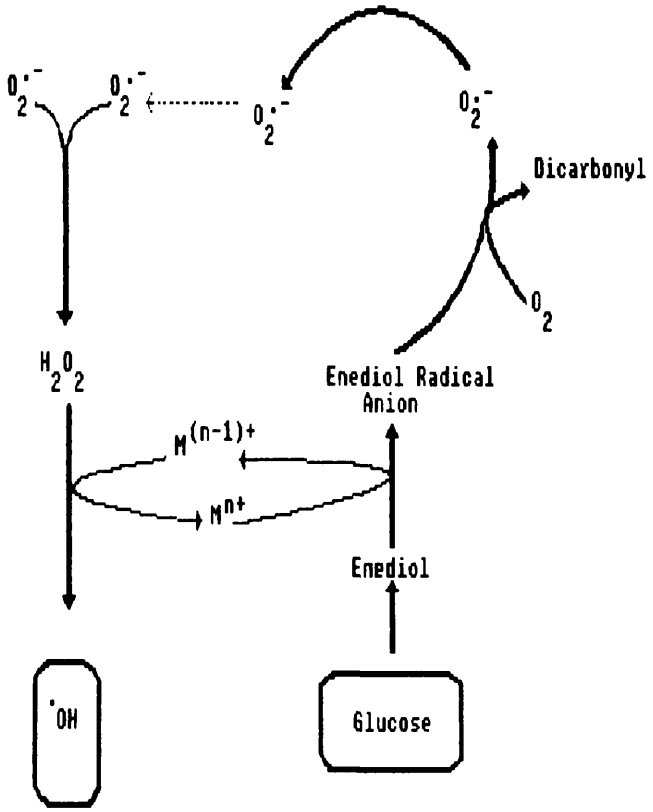

FIG. 1. Glucose oxidation and oxidant production. Oxidation of glucose to protein-reactive dicarbonyls (ketoaldehydes) is rate limited by enediol formation rather than presence of transition metal (M), because reaction is saturated at low metal concentration (7). Formation of dicarbonyl compounds is accompanied by superoxide (O_2^-) production. Superoxide free radicals undergo dismutation to hydrogen peroxide. In presence of transition metals, hydrogen peroxide leads to production of extremely reactive hydroxyl radical (8).

could stimulate peroxidation of the lipid component of LDL and to examine the effect of transition metals on glucose-stimulated oxidative damage to lipid and protein.

RESEARCH DESIGN AND METHODS

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

Human LDLs were prepared from nondiabetic subjects as previously described (11). 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 potassium bromide gradients. Centrifugations were performed at 100,000 × *g* for 18 h at 16°C in the presence of EDTA. LDL was taken as the fraction that floated at a relative density of 1.063.

Unilamellar phosphatidylcholine (Sigma) liposomes were prepared as previously described (12,13). Pure phosphatidylcholine (9:1 chloroform/methanol) was evaporated to dryness onto a sterile glass surface under a stream of N_2, then resuspended to 50 mg/ml in 100 mM filter-sterilized potassium phosphate (pH 7.2), and finally ultrasonicated for 3 min on ice to form unilamellar liposomes. Both LDL and liposomes were prepared under sterile conditions. All solutions to which the liposomes were exposed were filter sterilized.

The generation of hydroxyl radicals by glucose was assessed by monitoring the hydroxylation of benzoic acid to fluorescent products (308 nm excitation/410 nm emission) with salicylic acid as a standard (8,14). Incubations at 37°C consisted of 1 mM benzoic acid, 100 mM potassium phosphate (pH 7.2), 25 mM glucose, and increasing concentrations (from 0 to 100 μM) of copper sulfate. All incubations were performed under air within sealed sterile vessels.

Fragmentation of bovine serum albumin (BSA; 1 mg/ml) during exposure to glucose (25 mM) was used as a measure of free radical damage as previously described (8,13,15). Albumin (fraction V; Boehringer Mannheim, Mannheim, FRG) was labeled by reductive methylation of 0.1% of total lysine groups (50,000 dpm/mg protein) with [^{14}C]formaldehyde and $NaCNBH_3$ (8,15,16). Fragmentation of radiolabeled BSA during exposure to glucose was monitored by the determination of 5% trichloroacetic acid (TCA)-soluble radiolabeled fragments (8,15). All incubations contained 25 mM glucose and 100 mM potassium phosphate (pH 7.2) and were performed at 37°C.

The incorporation of D-[U-^{14}C]glucose into LDL was performed as previously described in detail (7,8). Briefly, protein was incubated with glucose (25 mM) containing 10 μCi/ml D-[U-^{14}C]glucose in the presence of 100 mM potassium phosphate buffer, pH 7.4. At appropriate intervals, samples were withdrawn and precipitated by the addition of TCA to a final concentration of 5%. The pellet was washed twice in 5% TCA and finally dissolved in formic acid before scintillation counting.

Liposomes were incubated at a final concentration of 10 mg/ml in 100 mM potassium phosphate, pH 7.2 (in the presence of suitable additions), in a shaking water bath at 37°C. At defined intervals, 100 μl of the incubation suspension was removed and dissolved by addition of 900 μl methanol. LDL was incubated at a concentration of 1 mg/ml in the same buffer at 37°C over 8 days. At the end of this time, the LDL suspension was treated in the same manner as the liposomes, except that the methanolic extract was subject to centrifugation at 12,000 × *g* for 2 min to precipitate protein.

Malondialdehyde and similar stable aldehydic products of lipid peroxidation formed in LDL or liposomes were determined with TBA (measuring TBA-reactive material) in material soluble in 5% TCA (17). Aliquots (200 μl) of the TCA extracts were incubated with 800 μl of 0.67% TBA at 100°C for 10 min. After cooling, the absorbance of the mixture was read at 532 nm. Quantitatively similar data were obtained if TBA-reactive material was determined in the methanolic extract. Lipid hydroperoxides present in the methanolic extracts were determined by their oxidation of Fe^{2+} to Fe^{3+} in 25 mM H_2SO_4 in the presence of xylenol orange (18), with butyl peroxide as standard. Before the determination of lipid peroxides, hydrogen peroxide generated during autoxidative reactions was removed by the preincubation of 1-ml samples with 100 IU catalase for 30 min.

RESULTS

The ability of glucose to generate highly reactive oxidizing substances and the implications of this for models of diabetic

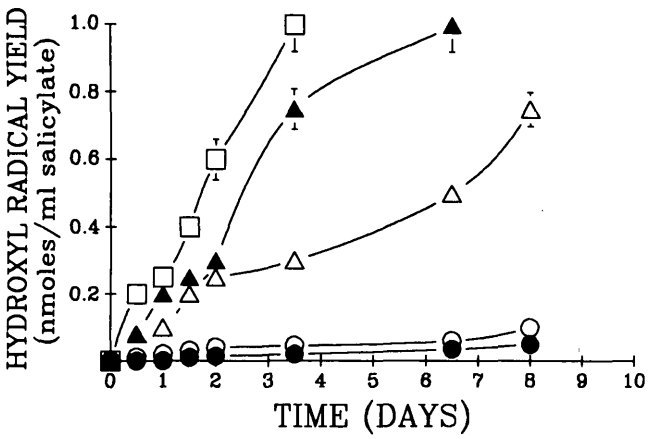

FIG. 2. Production of free radicals by glucose. Generation of fluorescent benzoic acid (308 nm excitation/410 nm emission) hydroxylation products was measured for 8 days. Incubations at 37°C consisted of 1 mM benzoic acid, 100 mM potassium phosphate (pH 7.2), 25 mM glucose, and increasing concentrations (from 0 to 100 μM) of copper sulfate with 0 (○), 10 (△), 50 (▲), or 100 (□) μM copper sulfate added. Pentetic acid (1 mM; ●) is included as control. Values are expressed as salicylate equivalents and are means ± SD.

tissue damage are poorly appreciated. Glucose, under the physiological conditions of temperature and pH used for studies of protein glycosylation, generated hydroxyl radicals (Fig. 2), which were detected by their ability to hydroxylate benzoic acid. This production of free radicals was dependent on the presence of transition metals (exemplified by the stimulatory effect of Cu^{2+}) and inhibitory effect of the metal chelator pentetic acid (DETAPAC) and was slow (Fig. 2). Over the time course of glycosylation experiments, however, these highly reactive substances were formed in quantities that approached the concentration of protein, leading to profound protein structural alterations such as frank fragmentation (Fig. 3).

Exposure of albumin (15 μM) to glucose (25 mM) in the presence of transition metal produced a steady increase in protein fragmentation (Fig. 3), which was almost completely inhibited by the addition of DETAPAC and by EDTA (data not shown) and proceeded also in the absence of added metal. Previous work has shown that this glucose-mediated protein fragmentation is associated with the production of distinct peptides similar to those produced by the exposure of protein to hydroxyl radicals generated by γ-radiolysis or peroxides and can be inhibited by free radical scavengers and catalase (8).

The oxidative ability of glucose was not restricted to its effect on protein. When liposomes prepared from phosphatidylcholine were exposed to glucose, there was a steady increase in the extent of lipid peroxidation, measurable as both peroxide accumulation and the generation of malondialdehyde (Fig. 4). Although Cu^{2+} stimulated this process in isolation, the effect was greater when Cu^{2+} and glucose were included in the incubation together. In particular, note that glucose abolished the lag phase (presumably due to the presence of small amounts of contaminating antioxidant in the liposomes) observed in the presence of Cu^{2+} but with no further additions. DETAPAC inhibited liposome peroxidation totally when added together with Cu^{2+} and glucose. Also note that the concentration of measurable peroxide was much greater than the concentration of malondialdehyde, the major stable product of lipid peroxidation and major reactant with TBA.

LDL exposed to glucose also experienced oxidative damage, which was detectable as a marked increase in lipid peroxidation in terms of measurable peroxide and in terms of material capable of reacting with TBA (Fig. 5). DETAPAC greatly inhibited the accumulation of peroxides within LDL. TBA-reactive material was less sensitive toward this inhibitory effect, and this may reflect the presence of preformed peroxides in the LDL, which decompose to TBA-reactive material without the intermediacy of transition metals. Again, the concentration of measurable peroxide was greater than the concentration of measurable malondialdehyde. LDL was also observed to be more resistant to peroxidation than liposomes, and this may reflect the presence of vitamin E within the LDL, which inhibits peroxidation.

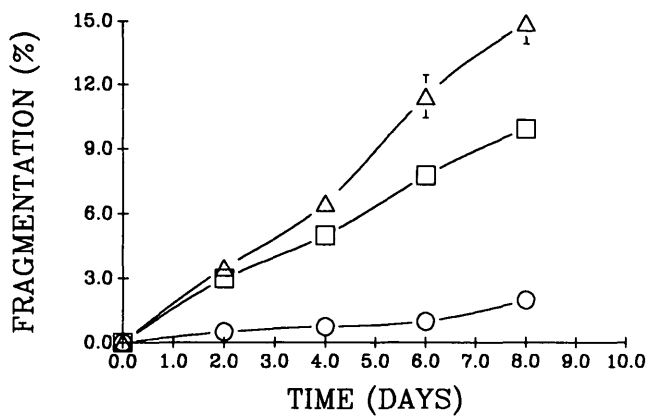

FIG. 3. Fragmentation of bovine serum albumin (BSA) by glucose. Fragmentation of radiolabeled BSA (1 mg/ml per 50,000 dpm/mg) was monitored by determination of trichloroacetic acid–soluble radiolabeled fragments. All incubations contained 25 mM glucose and 100 mM potassium phosphate (pH 7.2) and were performed at 37°C. Effect of adding 10 (□) and 100 (△) μM copper sulfate alone or 100 μM copper sulfate plus 1 mM pentetic acid (DETAPAC; ○) is shown. Values are means ± SD.

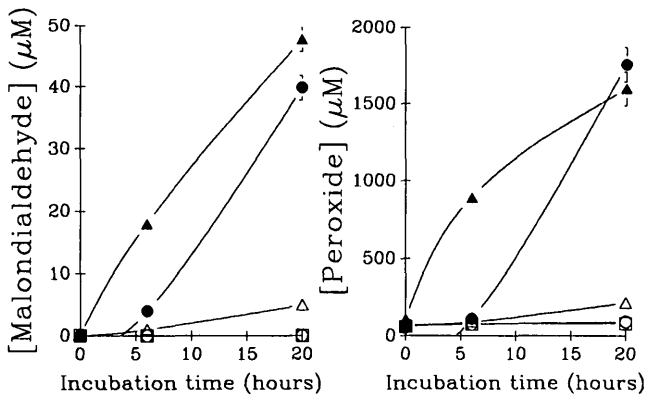

FIG. 4. Glucose-induced low-density lipoprotein (LDL) peroxidation. Production of either hydroperoxide or malondialdehyde with incubation of 1 mg/ml human LDL was monitored as described in RESEARCH DESIGN AND METHODS. Incubations were performed under sterile conditions over 8 days at 37°C, all contained 100 mM potassium phosphate (pH 7.2), and all were performed in presence or absence of 25 mM glucose and 1 mM pentetic acid (DETAPAC; □). ▲, Glucose and copper sulfate; ●, copper sulfate; △, glucose; ○, control. Values are means ± SD.

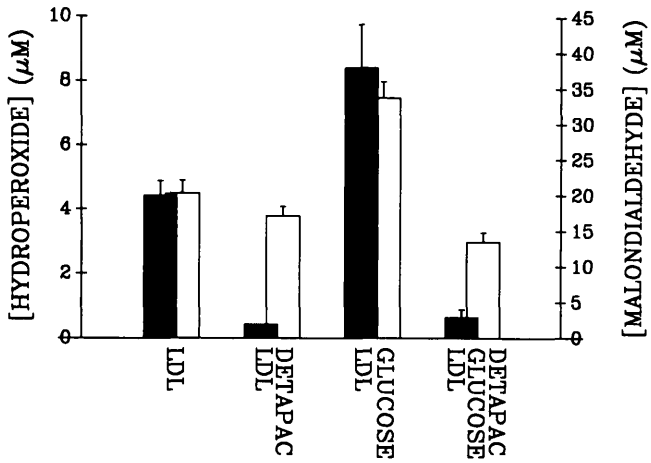

FIG. 5. Glucose-induced liposome peroxidation. Production of malondialdehyde (*open bars*; thiobarbituric acid–reactive material) and hydroperoxide (*solid bars*) in phosphatidylcholine liposomes (10 mg/ml) after incubation with glucose in presence or absence of copper, which was measured as described in RESEARCH DESIGN AND METHODS. LDL, low-density lipoprotein. Incubations were performed under sterile conditions over 20 h at 37°C. All incubations contained 100 mM potassium phosphate (pH 7.4), 200 mM glucose, 50 μM pentetic acid (DETAPAC), and/or 20 μM copper sulfate where appropriate. Values are means ± SD of 3 determinations.

Proposals have been made suggesting that glycosylation in itself can lead to the production of free radicals, because the Amadori product can autoxidize (19). This would not seem to be a necessary mechanism here, however, because liposomes prepared from phosphatidylcholine (which lacks a free amino group) were also prone to glucose-stimulated peroxidation (Fig. 4), which, like protein fragmentation and benzoic acid hydroxylation, was increased by addition of Cu^{2+}. Furthermore, the profound inhibitory effect of DETAPAC on glucose-mediated LDL peroxidation was equated with a smaller inhibitory effect of the metal-chelating agent on glycosylation over the same period (data not shown; 7,8). This does not exclude the possibility that glycosylation adducts contribute to protein oxidative damage but rather suggests that such an effect must be rather small compared with the oxidative insult given by free glucose under these conditions.

DISCUSSION

The exposure of macromolecules in vitro to hyperglycemic levels of glucose has long been considered a relevant model for the functional degeneration occurring in many tissues in diabetes mellitus (9). However, only in the past few years has it been appreciated that an oxidative component may contribute to glucose-mediated macromolecular damage and that such an effect may be important in changes frequently ascribed to glycosylation per se (1,7,8). Albumins and crystallins, ribonuclease, lysozyme, and myoglobin are all prone to oxidative fragmentation in the presence of glucose (S.P.W., J.V.H., unpublished observations). LDL is a pertinent example of a protein, which is held to be functionally and structurally modified in diabetes and is altered by exposure to glucose. For example, LDL previously exposed to glucose exhibits a decreased ability to interact with human fibroblasts in vitro and shows decreased clearance from rabbit plasma in vivo (20,21). The data shown in this study suggest that such modification could be oxidative in origin. LDL modified by products of lipid peroxidation is functionally altered in a manner similar to that which occurs on exposure to glucose (22). Glucose can hydroxylate benzoic acid, fragment protein, and cause the peroxidation of both liposomes and LDL, measurable in terms of both malondialdehyde formation and the accumulation of peroxides. Such peroxidation is detectable despite the presence of the antioxidant vitamin E in LDL (23).

There is increasing debate about the roles of glycosylation and free radical production in the complications of diabetes mellitus, and we hope that this study indicates that the two processes may be closely linked. Certainly, it is important to recognize that oxidative effects do occur in glycosylation reactions in vitro, and where these are to be avoided, it is important to include metal-chelating agents such as DETAPAC. The oxidative effects are generally small but are greatly accelerated by copper and iron, which are present at trace levels in all physiological buffers. The significance of glucose-stimulated oxidation to the in vivo situation is harder to assess. Although biological systems are replete with antioxidants such as vitamin E, there is some evidence for abnormalities in levels of catalytic transition metal in individuals with diabetes mellitus (24,25), and these, together with elevated levels of glucose, could contribute to oxidative stress, which may be apparent as an increased concentration of peroxide within LDL.

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