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Glucose-Induced Enhancement of Hemin-Catalyzed LDL Oxidation *In Vitro* and *In Vivo*

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ABSTRACT

Growing evidence indicates that oxidative modification of low-density lipoprotein (LDL) is increased in diabetes mellitus; however, the mechanism(s) of this phenomenon is still unclear. γ-Glutamyl semialdehyde (γGSA) is a product of hemin (Fe^{3+}-protoporphyrin IX)-catalyzed oxidation of apolipoprotein B-100 (apoB-100) proline and arginine residues. On reduction, γGSA forms 5-hydroxy-2-aminovaleric acid (HAVA). This report describes the application of sensitive HAVA assay, to characterize γGSA formation in LDL under normo- and hyperglycemic conditions, both *in vitro* and *in vivo*. *In vitro* studies revealed that apoB-100 proline and arginine residues are not oxidized to HAVA by HOCl or the myeloperoxidase/hydrogen peroxide (H_2O_2) oxidation system. Cu^{2+}, Cu^{2+}/H_2O_2, and Fe^{2+} induced only minor HAVA formation. In contrast, the hemin oxidation system appeared reactive toward LDL apoB-100 proline and arginine residues. The resulting significant HAVA formation was specifically inhibited by a redox-inert ferric iron chelator. Glucose further enhanced hemin-induced increase in relative electrophoretic mobility of LDL and apoB-100 HAVA formation. *In vivo* we observed elevated concentrations of HAVA in LDL apoB-100 in patients with impaired glucose tolerance and with manifest diabetes mellitus. In conclusion, glucose promotes iron-mediated oxidation of apoB-100 proline and arginine residues via a superoxide-dependent mechanism, thus rendering the LDL particles more atherogenic. The findings (a) identify a potential mechanism of enhanced atherogenesis in subjects with diabetes mellitus and (b) support the value of HAVA as a specific marker of LDL apoB-100 oxidation. *Antioxid. Redox Signal.* 7, 1507–1512.

INTRODUCTION

PATIENTS WITH DIABETES MELLITUS are at high risk to develop atherosclerotic lesions. In the atherogenic process, oxidatively modified low-density lipoprotein (LDL) particles play an important role because they are excessively taken up by macrophages via scavenger receptor pathways. These macrophages are then transformed into foam cells—the earliest morphologic substrate of atherogenesis (29). Growing evidence indicates that oxidative modification of LDL is increased in subjects with prediabetes and overt diabetes (11), thereby implicating hyperglycemia as a causative factor. However, the mechanism(s) underlying the relation between hyperglycemia and increased LDL oxidation is still unclear.

LDL particles can undergo several oxidative modifications, all of which are facilitated under conditions of high glucose-induced oxidative stress. On the one hand, the lipid constituents of the LDL particle are oxidized by reactive oxygen species. The major protein of the LDL particles, apolipoprotein B-100 (apo B-100), can also be oxidatively modified either by covalent binding to lipid peroxidation products or by direct oxidation of its amino acid side chain residues (12, 30). One *in vitro* identified pathway of direct apoB-100 oxidation involves the heme-containing enzyme myeloperoxidase and generates the oxidation products 3-chlorotyrosine and 3-nitrotyrosine (26).

Glucose itself can directly modify apoB-100 amino acid side chain residues, *e.g.,* arginine, lysine, and tryptophan, in a pro-

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cess called glycoxidation (8). However, the nature of specific modifications of protein amino acid side chain residues altering their cellular functions remains a subject of debate (6, 31).

Evidence for the important role of transition metal-catalyzed processes in diabetes-associated lipoprotein oxidation is emerging (32). However, no consensus has been achieved on whether these processes involving both copper (Cu^{2+}) and iron (Fe^{2+}) interact with the glycoxidation mechanism, or are independent. One recent study found the lack of any further stimulation of Cu^{2+}-induced lipid peroxidation or protein oxidation by glucose (8). Others describe increase of both Cu^{2+}-mediated LDL oxidation and the chemotactic activity of oxidized LDL toward proinflammatory cells by glucose in a concentration-dependent manner (18). Incubation with glucose alone, under conditions of very minor oxidation, also increased the chemotactic properties of LDL particles. In another study (20), glucose induced formation of conjugated dienes and thiobarbituric acid-reacting substances and increased LDL electrophoretic mobility, also in a concentration-dependent manner. Some of these effects of glucose have been observed in the model of iron-mediated LDL oxidation (19).

Whereas the *in vivo* availability of free copper or iron ions is under tight control under normal physiological conditions, these transition metal imbalances are well documented in diabetes mellitus. For this reason, the oxidation system containing complexed, porphyrin-bound redox-active iron is considered an experimental model of high pathophysiological relevance (7, 13, 15). Several groups have demonstrated that hemin (Fe^{3+}-protoporphyrin IX), a blood product and a physiological source of iron, actively oxidizes LDL apoB-100 (5, 14, 16, 17, 33), with the formation of apoB-100 cross-links and loss of free amino groups. Our recent *in vitro* studies revealed that hemin induces oxidation of both proline and arginine residues of apoB-100 (21, 23) with the formation of γ-glutamyl semialdehyde (γGSA), which is rapidly reduced to the stable product, 5-hydroxy-2-aminovaleric acid (HAVA). Assessment of HAVA, a highly specific and sensitive marker of protein oxidation, was used to test the hypothesis that redox-active glucose further stimulates transition metal-induced LDL apoB-100 oxidation. We performed *in vitro* studies to (a) identify potential mechanisms of HAVA formation in LDL apoB-100, using several well characterized oxidation systems, and (b) explore the effects of increasing concentrations of glucose on hemin-induced HAVA formation and LDL relative electrophoretic mobility. We have also assessed LDL HAVA concentrations *in vivo*, in subjects with normal and impaired glucose tolerance, as well as with clinically manifest diabetes mellitus.

MATERIALS AND METHODS

Chemicals

Bovine hemin chloride was purchased from Sigma (St. Louis, MO, U.S.A.). *N,N*-Bis(2-hydroxybenzyl)ethylenediamine-*N,N*-diacetic acid (HBED) was purchased from Dojindo (Gaithersburg, MD, U.S.A.). Myeloperoxidase (from human leucocytes) was obtained from Alexis Biochemicals (Gruenberg, Germany). All other chemicals were obtained from Sigma and Bio-Rad (Richmond, CA, U.S.A.).

Lipoprotein isolation and oxidation

Native, albumin-free LDL (density 1.006–1.063 g/ml) were isolated from plasma of 20 healthy, normolipidemic male volunteers by very fast ultracentrifugation as previously described (24). LDL apoB-100 was measured by immunoelectrophoresis using "ready-to-use" agarose gels (Sebia, Issy-les-Moulineaux, France). Immediately before oxidation of LDL, EDTA and salt from the density gradient were removed using a size-exclusion column (Econo-Pac 10DG, Bio-Rad) and phosphate-buffered saline (10 m*M* sodium phosphate, 150 m*M* sodium chloride, pH 7.4) as the eluent. For *in vitro* oxidation, aliquots of native LDL (1,000 μg of apoB-100/ml) were incubated with one of several well characterized oxidation systems: (a) Fe^{2+}/EDTA/ascorbate (200 μ*M*/240 μ*M*/5 m*M*), (b) hemin/hydrogen peroxide (H_2O_2) (10 μ*M*/100 μ*M*), (c) Cu^{2+} (10 μ*M*), (d) Cu^{2+}/H_2O_2 (100 μ*M*/ 2 m*M*), (e) Fe^{2+} (10 μ*M*), (f) myeloperoxidase/H_2O_2 (20 n*M*/100 μ*M*), and (g) HOCl (100 μ*M*) at 37°C for 40 h (with myeloperoxidase oxidation system for 60 min) in the dark. The samples containing the hemin oxidation systems were incubated with (a) the hemin/H_2O_2 system alone, (b) the hemin/H_2O_2 system plus the potent ferric ion chelator HBED (100 μ*M*), and (c) the hemin/H_2O_2 system and increasing concentrations of glucose, with/without HBED or manganese superoxide dismutase (MnSOD; 10 U/ml). The relative electrophoretic mobility (REM) of native LDL and the hemin/H_2O_2-oxidized LDL in the presence or absence of increasing concentrations of glucose has been measured with the help of ready-to-use agarose gels (Sebia, Issy-les-Molineaux, France) (10). On completion of the oxidation process, the 2-ml aliquots of LDL were taken from the oxidation systems and delipidated. The HAVA concentrations were determined after isolation, reduction, and enzymatic hydrolysis of apoB-100 by gas chromatography/mass spectrometry (21, 23). The HAVA content in all samples was expressed as moles per mole of apoB-100 (intraassay coefficient of variation < 4.5%; interassay coefficient of variation < 6.1%).

The *in vivo* studies were performed in 12 subjects with impaired glucose tolerance (seven males, five females; mean age 43 ± 15 years), in 10 newly detected patients with type 2 diabetes mellitus (five males, five females; mean age 42 ± 12 years), and in 10 normoglycemic controls (five males, five females; mean age 39 ± 14 years). All subjects gave written informed consent.

Statistical analysis

Data are given as means ± SD. Mean values were compared using the Mann–Whitney test. Significance was defined at $p < 0.05$. To show an association between two variables, a correlation analysis was performed using the procedure according to Spearman.

RESULTS

In vitro *experiments*

HAVA formation in different oxidation systems. The apoB-100 HAVA concentrations in native LDL and LDL oxidized by different oxidation systems used in this study are

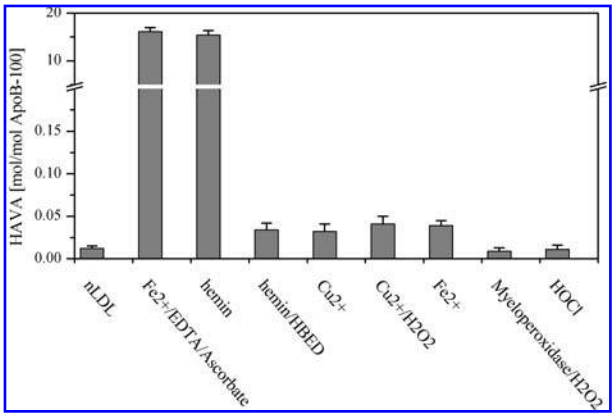

FIG. 1. LDL apoB-100 HAVA formation induced by different oxidation systems (means ± SD, *n* = 3). Hemin, hemin/H_2O_2 oxidation system; nLDL, native LDL.

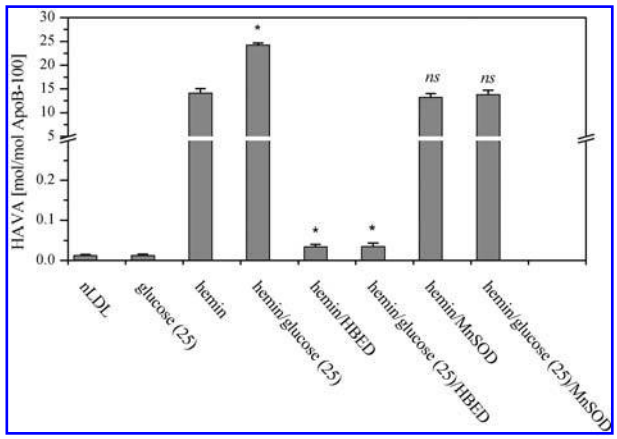

FIG. 2. HAVA concentrations in LDL apoB-100 treated with hemin/H_2O_2 with/without glucose (glucose concentration, 25 m*M*; in parentheses), HBED, or MnSOD (means ± SD, *n* = 3). Hemin, hemin/H_2O_2 oxidation system. *Significantly different compared with hemin/H_2O_2 alone ($p < 0.01$); *ns*, not significant.

shown in Fig. 1. A significant formation of HAVA was induced by the hemin/H_2O_2 and Fe^{2+}/EDTA/ascorbate oxidation systems. The addition of the redox-inert iron chelator HBED to the hemin/H_2O_2 system clearly inhibited HAVA formation. The oxidation systems containing Cu^{2+}, Cu^{2+}/H_2O_2, and Fe^{2+} induced only minor HAVA formation, whereas those containing myeloperoxidase/H_2O_2 or HOCl did not produce detectable amounts of HAVA.

Glucose effect on LDL REM. The REMs of native LDL and LDL oxidized by hemin/H_2O_2, in the presence or absence of physiological and pathophysiological concentrations of glucose, are shown in Table 1. The addition of glucose alone to native LDL did not affect their REM. The oxidation of LDL by the hemin/H_2O_2 oxidation system (without glucose) doubled this parameter. Addition of glucose to the hemin/H_2O_2 oxidation system further increased LDL REMs, and this effect appeared concentration-dependent.

Glucose effect on HAVA formation. In a similar fashion, the addition of glucose alone to native LDL did not induce any HAVA formation (Table 1, Fig. 2). Hemin/H_2O_2 increased HAVA concentration, and this effect was further enhanced by glucose, in a concentration-dependent manner. The glucose-induced HAVA formation was completely blunted by addition of MnSOD.

In vivo *experiments*

The LDL apoB-100 HAVA concentrations were significantly increased in subjects with impaired glucose tolerance and overt diabetes mellitus compared with subjects with normal glucose tolerance (Fig. 3). Only in the diabetes mellitus group was there a significant direct correlation (Spearman's rank correlation coefficient; $r = 0.869$, $p = 0.01$, $n = 10$) between plasma glucose levels and HAVA concentrations.

DISCUSSION

Our *in vitro* and *in vivo* studies support the applicability of the γGSA-derived HAVA as a specific marker of oxidative modification of apoB-100 in human LDL. γGSA arises directly via oxidation of proline or via hydrogen abstraction and subsequent loss of the guanidine group of arginine in the presence of reactive oxygen species (Fig. 4). It is known that in the presence of hydroperoxides free transition metals, *i.e.,* iron and copper, act as strong catalysts for this type of reaction. Here we demonstrate that two free-iron-containing oxidation systems, Fe^{2+}/EDTA/ascorbate and hemin/H_2O_2, increased LDL apoB-100 HAVA concentrations >500-fold compared with those in native LDL, whereas the effects of several other systems (Fig. 1) are minor or absent.

Taking into consideration that blood-derived hemin is a physiological source of iron, the mechanism of hemin-mediated LDL oxidation deserves a thorough consideration. Upon contact with LDL, the hydrophobic part of the amphiphilic hemin molecule intercalates within phospholipids and free cholesterol in the surface monolayer of the LDL

TABLE 1. REM AND HAVA CONTENT OF LDL EXPOSED TO GLUCOSE AND TO HEMIN/H_2O_2 WITH/WITHOUT INCREASING GLUCOSE CONCENTRATIONS

Hemin/H_2O_2	—	—	+	+	+	+
Glucose (m*M*)	—	25	25	12.5	6.5	—
REM	1.0 ± 0.1	1.1 ± 0.1	4.6 ± 0.3	4.0 ± 0.2	3.2 ± 0.3	2.0 ± 0.4
HAVA (mol/mol of apoB-100)	0.011 ± 0.004	0.012 ± 0.005	24.2 ± 0.3	19.5 ± 0.2	16.6 ± 0.3	14.1 ± 0.3

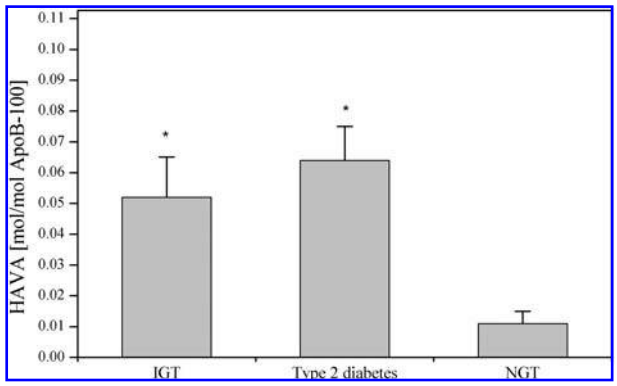

FIG. 3. LDL apoB-100 HAVA concentrations in subjects with impaired glucose tolerance (IGT), type 2 diabetes, and normal glucose tolerance (NGT). *Significantly different compared with subjects with normal glucose tolerance ($p < 0.01$); the difference between IGT and type 2 diabetes was not statistically significant.

particle (2, 9). As a result, two ionized propyl carboxyl groups of hemin appear in close contact with positively charged surface amino acid side chains of the apoB-100. Thus, hemin becomes a potent agent to specifically modify the basic amino acid arginine via the pathway involving prolonged perferryl cycles (via [Fe^{IV}=O] and [Fe^{V}-OH]) of the heme iron and hydroperoxides (1, 23, 35). Due to a frequent structural and steric clustering of arginine and proline residues in the mature apoB-100 molecule, proline side chain residues also become a susceptible target for hemin-catalyzed oxidation.

Our findings indicate that the hemin/H_2O_2-induced HAVA formation strongly depends on the presence of the complexed

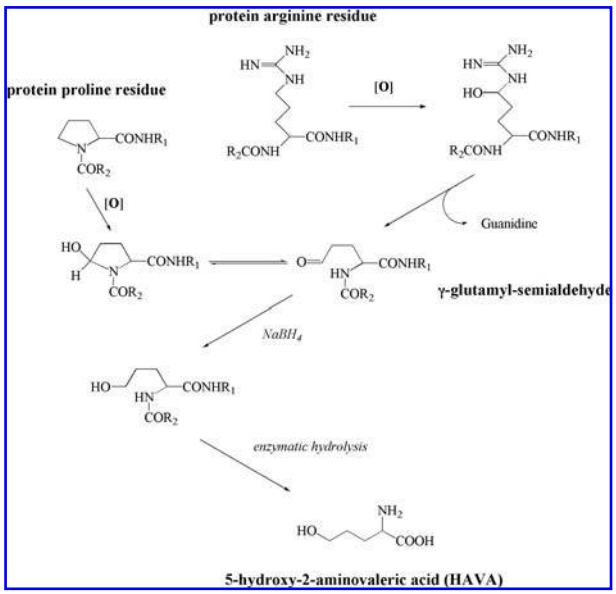

FIG. 4. Proposed reaction scheme for the formation of γGSA and its reaction product HAVA during oxidation of proline and arginine side chain residues of LDL apoB-100 according to Requena et al. (28).

ferric iron because Fe^{3+} chelating by the redox-inert HBED markedly inhibited apoB-100 proline and arginine residue oxidation. The Fe^{2+}/EDTA/ascorbate-induced HAVA formation occurs via a different mechanism. This oxidation system generates H_2O_2 and is based on a Fenton-type reaction between ferrous EDTA and H_2O_2, with continuous recycling of ferric ions to the ferrous state by ascorbate. Incubation of human LDL with other well characterized oxidation systems did not cause (myeloperoxidase/H_2O_2, HOCl) or caused very minor (Cu^{2+}, Cu^{2+}/H_2O_2, Fe^{2+}) HAVA formation, consistent with the proposed mechanism for hemin-mediated oxidation of apoB-100. Strikingly different induction of HAVA formation by the oxidation systems used suggests that LDL apoB-100 oxidation to γGSA, a precursor of HAVA, is strongly dependent on a continuous redox cycling mechanism because the classical, less-selective, Fenton-type systems form only low amounts of this compound.

Our *in vitro* studies have also demonstrated that glucose markedly enhanced the hemin-mediated oxidation process in a concentration-dependent manner. This effect is probably mediated via acceleration of the redox cycling of coordinatively bound ferric iron due to enhanced reduction of iron by glucose. The stimulating effect of physiological and pathophysiological concentrations of glucose on the hemin-catalyzed LDL apoB-100 oxidation appeared superoxide-dependent, and was completely inhibited by MnSOD. Thus, glucose mediates reduction of complex-bound iron via a superoxide-dependent mechanism. In contrast, in the absence of glucose the hemin-catalyzed LDL apoB-100 oxidation was not affected by the MnSOD, thus pointing to the fact that the redox cycling of coordinatively bound ferric iron is superoxide-independent.

Our *in vivo* studies revealed markedly elevated LDL apoB-100 HAVA concentrations in patients with impaired glucose tolerance and clinically manifest diabetes mellitus compared with healthy normolipidemic and normoglycemic individuals. The latter is consistent with the important role of hyperglycemia in LDL apoB-100 oxidation and increased atherogenesis in the diabetic condition. The LDL HAVA levels in patients with diabetes mellitus in the present study (0.064 ± 0.011 mol/mol of apoB-100) appeared remarkably similar to those in circulating LDL of hypercholesterolemic subjects with a high risk for atherosclerosis (0.063 ± 0.02 mol/mol of apoB-100) (25). Recently, γGSA formation has also been demonstrated in LDL apoB-100 in atherosclerotic lesions (22), thus providing additional information implicating oxidative modification of LDL apoB-100 proline and arginine residues in the development of atherosclerosis.

In conclusion, our *in vitro* studies provide evidence of superoxide-mediated glucose enhancement of the hemin/H_2O_2-induced LDL apoB-100 oxidation. The latter is consistent with the *in vivo* evidence of enhanced iron-mediated LDL oxidation in the diabetic and prediabetic state. Our findings suggest that elevated plasma glucose concentrations provide a major contribution to increased formation of circulatory atherogenic LDL particles in subjects with impaired glucose tolerance and overt diabetes mellitus. Glucose-enhanced LDL apoB-100 oxidation may represent one mechanism of increased atherosclerosis in subjects with prediabetes and diabetes mellitus. However, further studies are needed to under-

stand both the nature of the original oxidative insult and the specific consequences of γGSA formation for the metabolic fate of apoB-100-containing lipoproteins *in vivo*.

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ABBREVIATIONS

apoB-100, apolipoprotein B-100; γGSA, γ-glutamyl semialdehyde; HAVA, 5-hydroxy-2-aminovaleric acid; HBED, *N,N*-bis(2-hydroxybenzyl)ethylenediamine-*N,N*-diacetic acid; H_2O_2, hydrogen peroxide; LDL, low-density lipoproteins; MnSOD, manganese superoxide dismutase; REM, relative electrophoretic mobility.

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