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Inhibiting low-density lipoprotein glycation ameliorates increased cholesteryl ester synthesis in macrophages and hypercholesterolemia and aortic lipid peroxidation in streptozotocin diabetic rats

Margo P. Cohen*, Elizabeth A. Shea, Van-Yu Wu

Glycadia, Inc, Philadelphia, PA 19103, USA

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Abstract

Increased nonenzymatic glycation of apolipoprotein (apo) B–containing lipoproteins impairs uptake and metabolism by the high-affinity low-density lipoprotein receptor and is one of the postsecretory modifications contributory to accelerated atherosclerosis in diabetes. The present study evaluated in vitro and in vivo effects of 2,2-chlorophenylaminophenylacetate to probe the influence of glycated lipoprotein on cholesterol homeostasis. This compound prevented the increased formation of glycated products in low-density lipoprotein incubated with 200 mmol/L glucose and the increased cholesteryl ester synthesis in THP-1 macrophages induced by apo B–containing lipoproteins preincubated with high glucose concentration. The elevated circulating concentrations of glycated lipoprotein and cholesterol and higher vascular levels of lipid peroxidation products observed in streptozotocin diabetic rats compared with nondiabetic controls were significantly reduced in diabetic animals treated for 6 months with test compound. These results are the first to demonstrate that inhibiting nonenzymatic glycation of apo B–containing lipoproteins ameliorates abnormalities contributory to hypercholesterolemia and atherogenic risk in diabetes.

1. Introduction

Increased nonenzymatic glycation of apolipoprotein (apo) B is one of the postsecretory modifications of low-density lipoproteins (LDLs) contributory to the increased susceptibility to atherosclerosis in patients with diabetes, in whom elevated levels of glycated LDL have been consistently demonstrated [1-6]. Plasma concentrations of glycated LDL show positive associations with serum cholesterol and other markers of cardiovascular disease [7] and with the incidence of myocardial infarction [8]. Glycation diminishes the uptake and degradation of LDL by the high-affinity LDL receptor [9-14] and promotes uptake and metabolism by alternative pathways in monocyte-macrophages that give rise to cholesterol-laden foam cells [15-18]. Glycated LDL has reduced ability to regulate hydroxymethylglutaryl–coenzyme A reductase and acyl–coenzyme A: cholesterol transferase activities [14,16], induces functional changes in smooth muscle and endothelial cells and in monocytes [19-26], binds to arterial wall proteoglycans [6], and accelerates free radical production and lipid peroxidation that can generate glycooxidized and oxidized LDL and exaggerate cellular responses elicited by glycated LDL [27-31]. Glycated LDL–induced stimulation of arterial smooth muscle cell proliferation is mediated by phosphorylation of extracellular signal–regulated kinase and involves activation of protein kinase C, phospholipase C, and mitogen-activated protein kinase [26].

The above considerations suggest that reducing the formation of glycated LDL could decrease LDL uptake by alternative pathways, improve regulation of cholesterol synthesis, and decrease oxidative stress. The present study probed this hypothesis by evaluating the in vitro and in vivo effects of 2,2-chlorophenylaminophenylacetate (CAP22), a small molecule that is structurally related to the nonsteroidal

Conflict of interest: The authors are employed by Glycadia, a funding source for this study, and are inventors on a relevant patent application. Dr Cohen is a stockholder in Glycadia.

Institutional approval: The Institutional Care and Use Committee approved the protocol and procedures for this research on experimental animals.

* Corresponding author. Tel.: +1 215 557 8021; fax: +1 215 557 8053.
E-mail address: drmpcohen@aol.com (M.P. Cohen).

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anti-inflammatory agent diclofenac but lacks the 2, 6-dichloro substitution responsible for inhibition of cyclooxygenase enzymes [32,33]. However, CAP22 shares the anionic nature and diphenylamino structure of diclofenac, which has been shown to saturably bind to the hydrophobic core of LDL [34], suggesting that this property could affect Amadori glucose modification of LDL and thereby provide an opportunity to explore physiologic consequences of reducing the formation of glycated LDL. We report that CAP22 prevents the accelerated formation of glycated products in vitro in LDL incubated with high glucose concentration and the increased cholesterol ester synthesis in THP-1 macrophages that is induced by lipoprotein preincubated with high glucose. Long-term administration of CAP22 to streptozotocin diabetic rats decreased circulating glycated lipoprotein and serum cholesterol concentrations, and reduced aortic levels of lipid peroxidation products.

2. Methods

2.1. In vitro inhibition of nonenzymatic glycation

Apolipoprotein B–containing lipoproteins prepared from human plasma by dextran sulfate/$CaCl_2$ precipitation [35,36] were incubated under nitrogen for 10 days at 25°C in phosphate-buffered saline, pH 7.4, containing 5 mmol/L EDTA, without or with 200 mmol/L glucose and in the presence or absence of test compound (2:1, 4:1, and 8:1 molar ratio). For determination of the amount of glycated product, samples were delipidated and subjected to affinity chromatography on phenylboronate agarose (Helena Laboratories, Beaumont, TX) to separate the glycated from the nonglycated species. After addition of bovine serum albumin (5:1 ratio to apo B) to ensure apo B solubility [37], the samples were applied to the columns in loading buffer (0.1 mol/L glycine, 0.05 mol/L $MgCl_2$), eluting the bound (glycated) fraction with 200 mmol/L sorbitol in 0.1 mol/L glycine, desalting on Amicon Ultra-4 centrifuge tubes (Millipore, Bellerica, MA), and measuring protein content (BioRad, Hercules, CA) in the adsorbed fraction. In the absence of apo B, application of bovine serum albumin to the affinity column showed virtually no adsorption, confirming no methodological interference. For use in cell culture, apo B–containing lipoproteins were incubated for 10 days under nitrogen in 0.15 mol/L NaCl/5 mmol/L EDTA under 4 conditions: without glucose in the absence (A) or presence (B) of test compound (8:1 molar ratio), and with 200 mmol/L glucose in the absence (C) or presence (D) of test compound at the same molar ratio. The preincubated lipoprotein preparations were desalted by exchange into phosphate-buffered saline, pH 7.4, containing 5 mmol/L EDTA before use in cell cultures.

2.2. Cell culture

THP-1 acute monocytic leukemia cells (American Type Culture Collection T1B-202) were seeded into 24-well plastic plates (5×10^5 cells per well) in media containing 10% fetal bovine serum (FBS), RPMI 1640 (Mediatech, Manassas, VA), 4.5 g/dL glucose, 100 mmol/L sodium pyruvate, 0.05 mmol/L β-mercaptoethanol, 200 mmol/L L-glutamine, 10 000 U/mL penicillin, and 10 000 μg/mL streptomycin. Cells were induced to differentiate into macrophages by growing for 72 hours after making the above media 10 mmol/L in phorbol 12-myristate (Sigma-Aldrich, St Louis, MO) [38] and were fed for 18 hours with RPMI 1640 made 0.5% in lipoprotein-deficient FBS and containing the above nutrients before experimental conditions were introduced. This was accomplished by adding fresh media containing 0.5% lipoprotein-deficient FBS made 11 mmol/L in glucose, and 0-100 μg/mL of lipoprotein prepared as described above.

2.3. Experimental animals

Diabetes was induced by intravenous injection (50 mg/kg) of streptozotocin (Sigma-Aldrich) into the tail veins of male Wistar rats (Harlan, Indianapolis, IN) aged 6 weeks and weighing between 120 and 140 g. Animals with plasma glucose concentrations of at least 15 mmol/L within 1 week after the induction of diabetes were included in the study. Age-, weight-, and sex- matched Wistar rats served as nondiabetic controls. The diabetic rats were divided into 2 groups, one of which received test compound, as the potassium salt, by oral gavage at a total dose of 7.5 mg/(kg d) from age 7 weeks through age 33 weeks, with the other group serving as diabetic controls. Commercial rodent chow and water were provided ad libitum, and animals were monitored for blood glucose and weight. All diabetic rats received long-acting insulin (Lantos; Aventis, Bridgewater, NJ) every other day with adjustment of dosages to prevent ketoacidosis, improve survival, and keep animals on a positive growth curve. Animals were housed in a temperature-controlled facility, and the Institutional Care and Use Committee approved the protocol and procedures. Interim and terminal blood samples were obtained; the rats were killed at the conclusion of the experimental period; and the thoracic aorta was harvested, snap frozen, and stored at −80°C until analysis.

2.4. Analytic procedures

Glucose was measured by the glucose oxidase method. Lipid peroxide products were determined by the thiobarbituric acid assay using, as standard, the formation of malondialdehyde from 1,1,3,3-tetramethoxypropane. Aortic samples were prepared for lipid peroxide measurement by rinsing with 0.9 mol/L NaCl, grinding in a glass homogenizer, and briefly sonicating, with recovery of the solubilized tissue after centrifugation. Cholesterol was measured with commercial kits according to the manufacturer's instructions (BioVision Research Products, Mountain View, CA). For determination of glycated lipoproteins in rat serum, samples were precipitated with 10% dextran sulfate containing 0.05

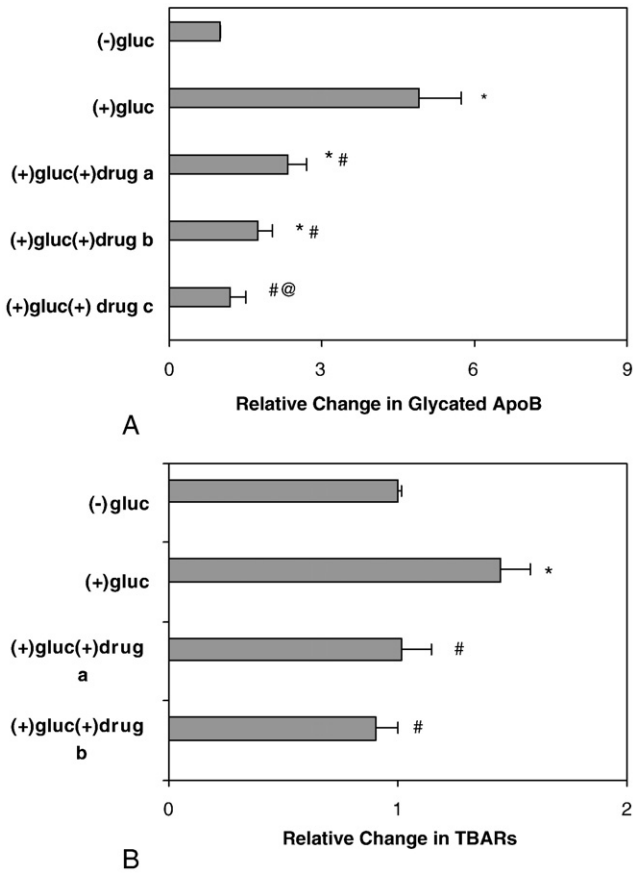

Fig. 1. Effect of CAP22 on lipoprotein glycation and peroxidation. A, Human apo B–containing lipoproteins prepared by dextran sulfate precipitation were incubated in 0 or 200 mmol/L glucose without or with test compound as described in "Methods." Results were calculated as micrograms per milligram apo B and represent mean ± SEM of 4 experiments under each condition. The amount of glycated apo B with no added glucose was assigned an arbitrary value of 1. The letters *a*, *b*, and *c* represent molar ratios of drug to apo B of 2:1, 4:1, and 8:1, respectively. $^{*}P \leq .05$ compared with (−) glucose; $^{\#}P \leq .05$ compared with (+) glucose; $^{@}P = .05$ compared with (+) glucose (+) drug a. B, Lipid peroxidation in human apo B–containing lipoprotein incubated in 0 or 200 mmol/L glucose without or with test compound as described in "Methods." Results calculated as nanomoles TBARs (thiobarbituric acid reactive substances) per milligram apo B, with 0 glucose assigned an arbitrary value of 1, and represent mean ± SEM of 4 experiments under each condition. $^{*}P = .05$ compared with (−) glucose; $^{\#}P = .05$ compared with (+) glucose.

mol/L $CaCl_2$; the precipitate was collected by centrifugation, washed twice with 0.2 mol/L $CaCl_2$, and dissolved in 5% NaCl containing 0.5% EDTA [35], after which 0.9 mol/L oxalic acid (vol/vol 1:2) was added; and the samples were incubated at 85°C for 20 hours. After cooling, samples were made 10% in trichloroacetic acid; and the supernatants were assayed for glycated products by reaction with thiobarbituric acid to yield the 5-hydroxymethylfurfuraldehyde chromogen that is generated from dehydration of glucose by boiling in oxalic acid [39], calculating concentrations from the standard curve obtained with hydroxymethylfurfuraldehyde (Sigma).

For measurement of cellular cholesterol and cholesteryl esters, cells were harvested at the end of the experimental period by scraping and transferring into tubes for centrifugation at 1000 rpm for 10 minutes. The liquid was carefully removed without disturbing the cell pellet, which was resuspended into 400 μL of chloroform:isopropanol:Triton X-100 (7:11:0.1). The solvent containing lysed cells was centrifuged at 14 000 rpm for 10 minutes, and the chloroform phase was collected and air-dried at 50°C. The dried lipids were dissolved in reaction buffer provided with the assay kits for measurement of cholesterol and cholesteryl esters. Results were normalized to cell protein to correct for differences in cell number or recovery.

2.5. Statistical analysis

Differences under different experimental conditions were analyzed by means of analysis of variance.

3. Results

Incubation of human apo B–containing lipoprotein with 200 mmol/L glucose for 10 days resulted in an expected increase in glycation of apo B compared with control incubations without added glucose for the same period under the same conditions. Inclusion of test compound in high-glucose incubations reduced the amount of glycated product formed, with dose-responsive decreases in the relative proportion of glycated to nonglycated species (Fig. 1A). Lipid peroxide products also were increased after incubation with high compared with low glucose and were similarly reduced when drug was included in the high-glucose incubations (Fig. 1B).

Macrophages exposed to lipoprotein preincubated with high glucose (condition C) showed a significant increase in cholesteryl esters compared with cells exposed to control lipoprotein (condition A or B), without change in total cholesterol content (Fig. 2). In contrast, cholesteryl esters in

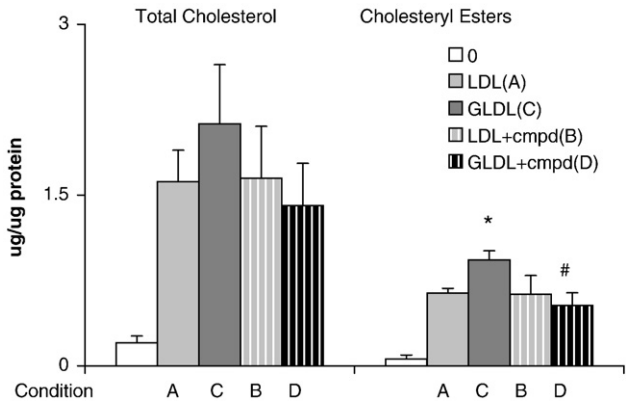

Fig. 2. Macrophage cholesteryl esters. THP-1 macrophages were incubated for 3 hours with human apo B–containing lipoproteins that had been preincubated without (conditions A and B) or with (conditions C and D) 200 mmol/L glucose in the absence (conditions A and C) or presence (conditions B and D) of test compound (8:1 molar ratio). Results are mean ± SEM of 3 experiments. $^{*}P \leq .05$ compared with conditions A and C; $^{\#}P \leq .05$ compared with condition B.

Table 1
Experimental animal data

	Nondiabetic (n = 8)	Diabetic control (n = 10)	Diabetic-CAP22 (n = 11)
Body weight (g)			
Interim	510 ± 24	330 ± 7*	346 ± 11*
Final	584 ± 17	366 ± 6*	394 ± 12*
Blood glucose (mmol/L)			
Interim	6.9 ± 0.9	27.6 ± 1.0*	24.7 ± 0.8*
Final	7.0 ± 0.9	24.7 ± 0.6*	26.9 ± 1.2*

Interim values ≈ week 17 of protocol.
* $P < .05$ compared with nondiabetic.

cells exposed to lipoprotein preincubated with high glucose in the presence of test compound (condition D) did not differ from control (Fig. 2). Macrophages incubated without added lipoprotein showed negligible amounts of cholesteryl esters, indicating that the measured cholesteryl esters represented new synthesis.

General characteristics of the experimental animals at the conclusion of the study period are shown in Table 1. Diabetic rats weighed significantly less than nondiabetic controls, but body weights and blood glucose levels did not differ in diabetic controls compared with diabetic rats receiving test compound. The growth curves and glucose levels in the animals in this study were similar to those reported by others using streptozotocin diabetic rats receiving insulin without or with coadministration of an agent that does not affect hyperglycemia [40]. Serum concentrations of glycated lipoprotein were significantly higher in diabetic compared with nondiabetic controls and were significantly reduced in diabetic rats receiving test compound, consistent with an effect on nonenzymatic glycation despite prevailing hyperglycemia (Fig. 3). Serum cholesterol concentrations were

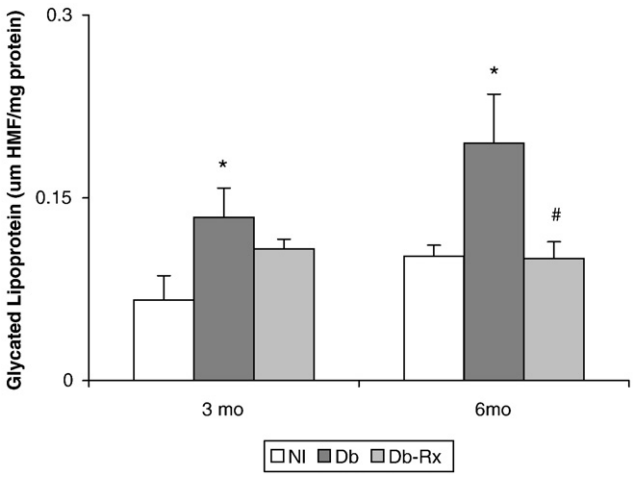

Fig. 3. Glycated lipoprotein in rat serum. Serum lipoproteins in interim and terminal samples were precipitated with dextran sulfate followed by determination of hydroxymethylfurfuraldehyde chromogen content as described in text. *$P \leq .05$ compared with nondiabetic; #$P \leq .05$ compared with diabetic control.

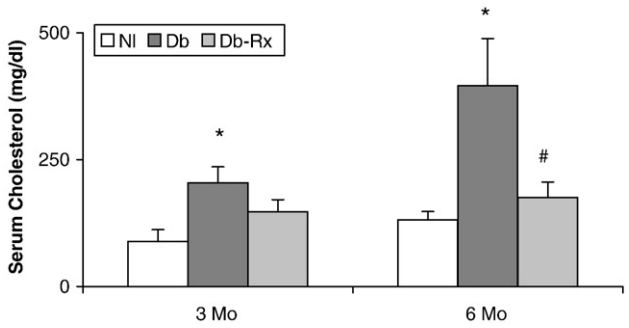

Fig. 4. Rat serum cholesterol concentrations. Samples collected at midpoint and conclusion of experimental period. Results are mean ± SEM from animals described in Table 1. *$P \leq .05$ compared with normal; #$P \leq .05$ compared with diabetic control.

about 3-fold higher in diabetic than in nondiabetic rats, but were not significantly different from nondiabetic controls in diabetic rats treated with test compound (Fig. 4). Levels of lipid peroxidation products in aortas of diabetic rats were significantly higher than in nondiabetic control and were significantly less in diabetic animals receiving test compound than in the diabetic controls (Fig. 5).

4. Discussion

The results of this study indicate that glycated lipoproteins stimulate cholesteryl ester synthesis in macrophages and that increased levels of glycated apo B–containing lipoproteins profoundly influence serum cholesterol levels and enhance the propensity for lipid peroxidation in the aorta of streptozotocin diabetic rats. The test compound was shown to inhibit the nonenzymatic glycation of LDL in vitro by reducing the formation of Amadori glucose adducts in human apo B–containing lipoproteins that were exposed to high glucose concentrations and in vivo by decreasing plasma levels of glycated lipoprotein in the face of persistent hyperglycemia in diabetic rats. The finding that lowering the elevated levels of glycated lipoprotein in streptozotocin

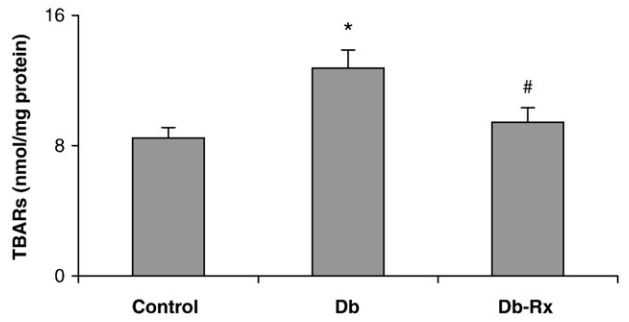

Fig. 5. Aortic lipid peroxidation products. Tissue collected at conclusion of experimental period. Results are mean ± SEM from animals described in Table 1. *$P \leq .05$ compared with normal; #$P = .05$ compared with diabetic control.

diabetic rats was accompanied by a reduction in serum cholesterol and in aortic lipid peroxidation, a proatherogenic accelerator [28-31,41,42], is interpreted to reflect improved cholesterol homeostasis consequent to decreased stimulation of cholesteryl ester synthesis and decreased oxidative stress resulting from lessening the burden of glycated LDL. The ability of CAP22 to inhibit LDL glycation is presumed to relate to lipoprotein binding properties analogous to those described for diclofenac [34], which interacts with several classes of binding sites in the lipoprotein core protein, although other mechanisms cannot be excluded. Because glycation of apo B occurs at reactive lysine-lysine or lys-x-lys sequences in proximity to a catalytic proton donor/acceptor group [4,43], one explanation for the observed inhibition of nonenzymatic glycation is that structural consequences of drug binding impede the ability of lysine amino groups to undergo the Amadori rearrangement necessary for the formation of the stable glucose adduct. Appropriately located positively charged amino acid residues promote local acid base catalysis that fosters stabilization after Schiff base condensation of glucose with protein [44], suggesting that drug binding compromises local closeness of charged amino groups that act catalytically in the Amadori rearrangement at lysine residues susceptible to nonenzymatic reaction with glucose. Direct interaction of the anionic side chain in CAP22 with potentially glycatable lysine amino groups is also possible, analogous to the direct inhibition of glycation consequent to binding of acetylsalicylic acid to albumin [45,46].

Apolipoprotein B is the protein determinant for cellular recognition of LDL by the LDL receptor, which promotes internalization, catabolism, and clearance of LDL from the plasma and regulates cellular cholesterol biosynthesis. Impaired recognition of glycated apo B by the classic LDL receptor, enhanced uptake by cells expressing alternative receptors, and faulty regulation of cellular cholesterol homeostasis favor cholesterol accumulation within the cells and compromise plasma clearance. Small dense LDLs, which associate with coronary disease, have been reported to undergo more glycation than other LDL subfractions, even in nondiabetic subjects, and to be more susceptible to glycation in vivo when exposed to high glucose concentration [47,48]. This aspect was not examined in the present study, nor was the effect of test compound on HDL glycation; but it is worth noting that nonenzymatic glycation of HDL, which is also increased in the diabetic milieu, increases the transfer of cholesteryl esters to apo B–containing lipoproteins, exaggerating cholesterol transport abnormalities contributory to hypercholesterolemia [36].

In summary, we report that inhibiting the nonenzymatic glycation of apo B–containing lipoproteins in vitro prevents increased cholesteryl ester synthesis observed in macrophages exposed to glycated LDL and reduces circulating levels of glycated lipoprotein, serum cholesterol, and vascular lipid peroxidation in streptozotocin diabetic rats. These findings encourage clinical studies of CAP22 to evaluate its potential for reducing atherogenic risk in human diabetes.

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