High Levels of Dietary Advanced Glycation End Products Transform Low-Density Lipoprotein Into a Potent Redox-Sensitive Mitogen-Activated Protein Kinase Stimulant in Diabetic Patients

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Background—LDL modification by endogenous advanced glycation end products (AGEs) is thought to contribute to cardiovascular disease of diabetes. It remains unclear, however, whether exogenous (diet-derived) AGEs influence glycoxidation and endothelial cell toxicity of diabetic LDL.

Methods and Results—Twenty-four diabetic subjects were randomized to either a standard diet (here called high-AGE, HAGE) or a diet 5-fold lower in AGE (LAGE diet) for 6 weeks. LDL pooled from patients on HAGE diet (Db-HAGE-LDL) was more glycated than LDL from the LAGE diet group (Db-LAGE-LDL) (192 versus 92 AGE U/mg apolipoprotein B) and more oxidized (5.7 versus 1.5 nmol malondialdehyde/mg lipoprotein). When added to human endothelial cells (ECV 304 or human umbilical vein endothelial cells), Db-HAGE-LDL promoted marked ERK1/2 phosphorylation (pERK1/2) (5.5- to 10-fold of control) in a time- and dose-dependent manner compared with Db-LAGE-LDL or native LDL. In addition, Db-HAGE-LDL stimulated NF-κB activity significantly in ECV 304 and human umbilical vein endothelial cells (2.3-fold above baseline) in a manner inhibitable by a MEK inhibitor PD98059 (10 μmol/L), the antioxidant N-acetyl-l-cysteine, NAC (30 mmol/L), and the NADPH oxidase inhibitor DPI (20 μmol/L). In contrast to Db-LAGE-LD and native LDL, Db-HAGE-LDL induced significant soluble vascular cell adhesion molecule-1 production (2.3-fold), which was blocked by PD98059, NAC, and DPI.

Conclusions—Exposure to daily dietary glycoxidants enhances LDL-induced vascular toxicity via redox-sensitive mitogen-activated protein kinase activation. This can be prevented by dietary AGE restriction. (Circulation. 2004;110:285-291.)

Key Words: atherosclerosis ■ diabetes ■ endothelium ■ glycotoxins ■ glycation ■ kinases

Cardiovascular disease is the major cause of morbidity and mortality in diabetic patients.1 One of the key risk factors for cardiovascular disease in this population is increased LDL2. Aside from the quantitative alterations, chemical changes of LDL, such as glycation or glycoxidation, can delay receptor-mediated LDL clearance, contributing to a range of abnormal vascular responses.3,4 LDL, whether from type 1 or type 2 diabetic patients, is found to exhibit proatherogenic properties.4–6 Major sources that cause diabetic LDL modification are endogenous reducing sugars.7 Several advanced glyoxidation end products (AGEs) and lipoxidation derivatives, such as N-(carboxymethyl)lysine (CML)8 and 4-hydroxy-nonenal (HNE),9 have been identified as having proatherogenic properties.10–12 Involving cell surface AGE receptors, redox-dependent oxidant stress, and activation of mitogen-activated protein kinase (MAPK) signaling and nuclear factor (NF)-κB transcription factor pathways.13–15

AGE formation can be vastly accelerated as a function of degree and time of exposure to heat16 and can be introduced into the body with heat-processed foods.17 Two thirds of the absorbed AGEs (~10% of ingested) are integrated within tissue and blood components, including LDL.17 The impact of this large influx of diet-derived AGEs has come under investigation as a potentially important modulator of normal gene and protein function.18 Recently, AGE-restricted dietary intervention has been reported to be an effective inhibitor of atherogenic processes in hyperlipidemic apolipoprotein E–deficient mice, as in postinjury restenosis19 or diabetes-related severe atherosclerosis, despite persistent hyperglycemia.20 The mechanisms involved have not been defined, however.

On the basis of this evidence, we hypothesized that food-derived AGEs contribute to excessive chemical and functional modification of LDL, a change that would be expected to be most obvious in diabetic patients. To test this hypothesis, we compared plasma LDL extracted from diabetic patients who were exposed to diets differing only in AGE content and evaluated its effects on cultured endothelial cells.

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Methods

Cell Culture and Preparation of Cell Lysates

Human endothelial cells (ECs) (ECV 304 cell line) from the European Collection of Cell Cultures (Wiltshire, UK) and human umbilical vein ECs (HUVECs) from American Type Culture Collection were used in these studies. ECV 304 is a human EC line that, like HUVECs, exhibits EC markers and receptors for native LDL (N-LDL) and modified LDL. 4,21,22 Quiescent cells were stimulated by different LDL preparations with or without inhibitors. After stimulation, cells were lysed in a Tris buffer containing 1% Triton X-100 and protease inhibitor cocktail. Protein concentration in supernatants was determined by Bio-Rad assay kit (Bio-Rad Laboratories).

Subjects/Clinical Protocol

Twenty-four patients with known diabetes under good metabolic control (baseline HbA1c, 7.3 ± 0.6%) and normal lipid profiles and renal function were recruited for a different study, 18 after approval by the Mount Sinai School of Medicine Institutional Review Board. No patient was taking metformin. Two diets, differing in AGE content, were designated formula (high-AGE, HAGE, or low-AGE, LAGE diet) at the beginning and end of the study. 7,18,25 In addition, native LDL (N-LDL) was prepared from freshly isolated EDTA-treated fasting plasma from nondiabetic, nonsmoking, normolipidemic volunteers who had not been receiving medications for at least the previous 6 weeks (n = 5; age range, 45 to 60 years; 3 women, 2 men).

Preparation and Characterization of Human LDL

LDL (1.019<d<1.063 g/ml) was isolated from EDTA-treated (1 mmol/L) fasting plasma samples collected from each subject (n = 24) at the beginning and end of the study. Briefly, LDL was obtained by potassium bromide density gradient ultracentrifugation in a Beckman VTI 50 rotor at 110 000g for 24 hours at 4°C in the presence of EDTA. For certain experiments, in addition to 3 individual LDL preparations, samples from 5 diabetic subjects per diet group and from 3 normal subjects were pooled for cell signaling and activation studies. To generate in vitro oxidized LDL (ox-LDL), N-LDL (0.35 mg/ml) was incubated with 5.0 μmol/L CuSO4 for 24 hours at 37°C, and the reaction was terminated by addition of 1.0 mmol/L EDTA and 100 μmol/L BHT. 7,10,14 All LDL preparations were rendered sterile by filtration (0.22-μm Millipore filter, Amicon) after endotoxin contamination had been excluded by the limulus amebocyte lysate (Bio-Whittaker). Peroxidation in all LDL preparations was determined on the basis of quantification of malondialdehyde derivatives. 26 Glycation was determined individually as AGE–apolipoprotein B–LDL on the basis of a previously described ELISA, which uses a defined CML-sensitive monoclonal antibody, 4G9; Alteon, Inc.) 15,25 (AGE and malondialdehyde content of individual or pooled diabetic HAGE-LDL or LAGE-LDL samples, as well as of normal or control samples, are shown in Table 2.)

Western Analysis for ERK1/2

Aliquots (30 μg) of cell lysates as described above were used for Western blot analysis using a rabbit phospho-specific p44/42 MAPK polyclonal antibody (1:1000 dilution, New England Biolabs) or rabbit polyclonal antibody against total p44/42 MAPK (phosphorylation-state independent) (New England Biolabs)

Cell Transfection and Luciferase Assay

NF-κB-luc reporter plasmid (0.2 μg NF-κB-luc reporter plasmid/ well) (Stratagene) was transfected by use of LipofectAMINE PLUS reagent (Gibco/BRL). pFc-MEKK (the catalytic domain of MEKK, Stratagene) was used as a positive control in NF-κB assays. Twenty-four hours after transfection, N-LDL, ox-LDL, Db-HAGE-LDL, or Db-LAGE-LDL from 3 individuals/group and a pooled sample (n = 5/group) was added, as indicated, for 3 hours, with or without inhibitors. After reporter lysis buffer (Packard Instrument Company) had been added to each well for 10 minutes, luminescence
Human Soluble VCAM-1 Assay

ECV 304 cells were incubated in serum-free medium with different LDL preparations (50 μg/mL) for 6 hours at 37°C. Cell supernatants were concentrated by 10-fold by use of Centricon-10 filters (Amicon) and assayed for soluble human vascular cell adhesion molecule (sVCAM)-1 by an ELISA kit (R&D Systems, Inc) as described previously.27

Statistical Analysis

Data are expressed as mean±SD. Statistical significance between groups was determined by Student’s t test or 1-way ANOVA with Bonferroni correction, depending on the number of groups compared. A probability value of P<0.05 was considered significant.

Results

LDL From Diabetic Patients on an HAGE Diet Is More Extensively Modified Than if Patients Consumed an LAGE Diet

The clinical characteristics of the diabetic patients exposed to either a high- or a low-AGE diet are shown in Table 1. There were no significant differences between the baseline and end of the study within each group with regard to body mass index, HbA1c, fasting plasma glucose, and lipid profile, although significant changes were noted in total serum AGE levels between the baseline and the end of the study (Table 1).18 LDL from diabetic patients on an HAGE diet (Db-HAGE-LDL), measured either individually or as a pooled sample, was significantly more glycated (~4-fold, P<0.01) and oxidized (~7-fold) compared with normal LDL (N-LDL, P<0.05) (Table 2).18 In contrast, LDL from diabetic patients who were fed a low-AGE diet (Db-LAGE-LDL) was less glycated (by 50%) and less oxidized (by ~80%) compared with Db-HAGE-LDL. LAGE-LDL AGE and malondialdehyde content were ~1.4- and 2.0-fold above N-LDL, respectively (Table 2).

Diabetic LDL Is a Potent Activator of ERK1/2 When Derived From HAGE-Fed Patients but Not When Derived From LAGE-Fed Patients

After incubation of ECV 304 cells with Db-HAGE-LDL for 10 minutes, a significant increase of ERK1/2 phosphorylation was detected in a scintillation TopCount microplate (Packard Instrument Company).

Diabetic high AGE diet LDL (Db-HAGE-LDL)‡ 11 (5) 214±51 (192±25§) 5.7±2.3]
Diabetic low AGE diet LDL (Db-LAGE-LDL)‡ 13 (5) 106±23 (92.4±14) 1.5±0.5
Normal LDL (N-LDL)‡ 5 (3) 64±15 (45±8) 0.8±0.2
OxLDL‡ 1 (1) 76±4 (76±5) 10.3±2.8

All values represent the mean±SD of 3 independent measurements. Statistical analysis was performed using ANOVA.

*Based on competitive AGE ELISA (4G9 mAb).25
†Malondialdehyde equivalents by thiobarbituric acid–reactive substances.26
‡Denotes individual values of plasma LDL samples from diabetic patients on test diets. N-LDL was derived from nondiabetic subjects on regular (equivalent to HAGE) diet. 18 OxLDL was generated from N-LDL by exposure to CuSO4 as described in Methods. Numbers in parentheses indicate pooled samples.
§P<0.0006 for AGE-LDL among groups (Db-HAGE-LDL, Db-LAGE-LDL, and N-LDL).
||P<0.007 for OxLDL among groups (Db-HAGE-LDL, Db-LAGE-LDL, and N-LDL).

Diabetic HAGE-LDL–Induced Phosphorylation of ERK1/2 Is Prevented by Antioxidant NAC and NADPH Oxidase Inhibitor DPI

ECV 304 cells were preincubated with the antioxidant NAC or the NADPH oxidase inhibitor DPI for 30 minutes and then stimulated with Db-HAGE-LDL (5 μg/mL) for 10 minutes. Both NAC and DPI effectively prevented HAGE-LDL–induced ERK1/2 activation (Figure 2C), suggesting that reactive oxygen species generation was involved.

NF-κB Transcriptional Activation, via ERK1/2, Is Enhanced by Diabetic HAGE-LDL but Not by Diabetic LAGE-LDL

NF-κB activity was tested in cells transiently transfected with a luciferase-coupled promoter plasmid driven by an NF-κB consensus sequence, after exposure to different LDL preparations. Transfected cells without any treatment were used as controls for basal luciferase activity. Stimulation with Db-
HAGE-LDL (5 μg/mL, 3 hours) caused a significant increase in relative luciferase activity above control values (1.8-fold), compared with the minimal response obtained from equal amounts of Db-LAGE-LDL (1.2-fold), N-LDL (1.15-fold), and ox-LDL (1.25-fold). The groups were significantly different, with a value of \( P < 0.005 \) (ANOVA) (Figure 3A).

Figure 1. Diabetic (Db)-HAGE-LDL but not LAGE-LDL stimulates EC ERK1/2 phosphorylation in a dose-dependent manner. ECV 304 cells were stimulated with different concentrations of Db-HAGE-LDL, Db-LAGE-LDL, N-LDL, and ox-LDL for 10 minutes before lysis. Cell proteins were separated by SDS-PAGE (10%) and immunoblotted by an anti-phosphorylated ERK1/2 (p-ERK1/2) or anti-total ERK1/2 (T-ERK1/2) antibody. A, p-ERK1/2 after incubation with diabetic HAGE-LDL and LAGE-LDL. B, Densitometry of data shown in A; C, p-ERK1/2 after incubation with N-LDL and ox-LDL. D, Densitometry of data shown in C; E, Total ERK1/2 after incubation with diabetic HAGE-LDL. Bars represent mean ± SD of increase (x-fold) above control values from 3 individual experiments. \( * P < 0.05 \), \( ** P < 0.01 \) vs control cells (without stimulation).

Figure 2. Db-HAGE-LDL–induced ERK1/2 phosphorylation is MAPK- and redox-dependent. A, Time course of ERK1/2 phosphorylation stimulated by Db-HAGE-LDL. ECV 304 cells were stimulated with Db-HAGE-LDL (5 μg/mL) for indicated periods of time, harvested, and lysed. p-ERK1/2 was assayed by immunoblotting cell lysates with anti–p-ERK1/2 antibody. Data are expressed as mean ± SD of 3 individual experiments. \( * P < 0.05 \), \( ** P < 0.01 \) vs time 0. B and C, Cells were preincubated with MEK inhibitor PD 98059 (10 μmol/L), antioxidant NAC (20 and 30 mmol/L), or NADPH oxidase inhibitor DPI (10 and 20 μmol/L) for 30 minutes and then with Db-HAGE-LDL (5 μg/mL) for 10 minutes. p-ERK1/2 was determined by immunoblotting as in A. Densitometric analysis of data from 3 independent experiments is shown as mean ± SD of increase (x-fold) above control values (no stimulation). \( ** P < 0.01 \) vs control cells (without stimulation). \( \# P < 0.05 \), \( \## P < 0.01 \) vs Db-HAGE-LDL.
Db-HAGE-LDL–induced NF-κB activity was further assessed in cells pretreated with PD 98059 (10 μmol/L, 30 minutes), NAC (30 mmol/L, 30 minutes), or DPI (20 μmol/L, 30 minutes), respectively. Virtually all inducible NF-κB activity was inhibited by each inhibitor: PD 98059, \( P < 0.01 \); NAC, \( P < 0.05 \); and DPI, \( P < 0.01 \) (Figure 3B).

**Diabetic HAGE-LDL but Not Diabetic LAGE-LDL Stimulates EC Production of sVCAM-1**

Stimulation of ECV 304 cells with Db-HAGE-LDL (50 μg/mL, 6 hours) induced sVCAM-1 secretion in the supernatant (2.3-fold above control values) \( (P < 0.01) \) on the basis of ELISA. In contrast, only a slight enhancement of sVCAM production was observed in response to Db-LAGE-LDL and ox-LDL and none to N-LDL (Figure 3C). Also, as in the previous experiments, sVCAM-1 production induced by Db-HAGE-LDL was inhibited by PD 98059 (10 μmol/L), NAC (30 mmol/L), or DPI (20 μmol/L) (Figure 3C).

**Diabetic HAGE-LDL Activates HUVECs via a Similar Redox-Dependent Pathway**

To assess the reproducibility of these findings in ECs derived from normal human vessel wall, similar experiments were performed in HUVECs exposed to the same LDL preparations as used above. Db-HAGE-LDL (5 μg/mL) but not LAGE-LDL, ox-LDL, or N-LDL induced marked ERK1/2 phosphorylation in HUVECs (Figure 4, A and B) at a similar concentration with an identical time course as with ECV 304 (maximum at 10 minutes and 5 μg/mL) (Figure 4C). As in ECV 304 cells, Db-HAGE-LDL triggered marked HUVEC NF-κB activity (2.3-fold above control values, \( P < 0.05 \)) and was suppressed in the presence of identical amounts of antioxidants (NAC, DPI) or of MEK inhibitor (Figure 5).

**Discussion**

The present report demonstrates that LDL isolated from diabetic patients exposed to a diet with a high glycoxidant content is more extensively altered than LDL from diabetic subjects fed a diet with reduced AGE, both in terms of degree of chemical modification and of cell-toxic properties. The combination of endogenous hyperglycemia because of diabetes with intake of large quantities of exogenous AGE appeared to give rise to a modified LDL particle significantly more potent as a promoter of abnormal EC gene activation than LDL exposed to diabetes combined with only a fraction (1/5) of the AGE present in regular diet.

The structural and functional LDL changes found in the present studies were consistent with the differences in AGE content of the diet, with AGE levels in serum as reflected in CML and MG derivatives, but also with levels of the circulating inflammatory markers C-reactive protein, tumor necrosis factor-α, and VCAM-1 reported previously.18 These findings supported the postulated relationship between exogenous AGE and diabetic cardiovascular disease18,28,29 and opened a new window into the mechanisms involved in these events.

For instance, a significant corollary of the excessive modification of Db-HAGE-LDL was the marked increases in

![Figure 3](http://circ.ahajournals.org/)

Figure 3. Diabetic HAGE-LDL but not diabetic LAGE-LDL is a potent inducer of NF-κB activation and VCAM-1 expression via redox-dependent MAPK signaling. A, ECV 304 cells were transfected with NF-κB–luciferase reporter gene and incubated alone (control) or with different LDL preparations (at 5 μg/mL) for 3 hours. **Significance indicated as \( P < 0.005 \) on the basis of ANOVA. B, NF-κB-Luc–transfected cells were preincubated with PD 98059 (10 μM), NAC (30 mmol/L), or DPI (20 μM) for 30 minutes before exposure to Db-HAGE-LDL (5 μg/mL) for 3 hours. NF-κB-Luc activity is shown as mean±SD of 3 separate experiments, each performed in triplicate. **P<0.01 vs control cells (without stimulation); #P<0.05; ##P<0.01 vs HAGE-LDL. C, Cells were incubated with different LDL preparations (50 μg/mL, 6 hours) with or without preexposure to PD 98059 (10 μmol/L), NAC (30 mmol/L), or DPI (20 μmol/L) for 30 minutes as in A (above). Culture supernatants were concentrated (10-fold) and assayed for sVCAM-1 by ELISA. Data are shown as mean±SD of 3 separate experiments. **P<0.01 vs control cells (without stimulation); #P<0.05, ##P<0.01 vs HAGE-LDL.
MAPK phosphorylation, NF-κB activity, and VCAM production triggered on interaction of this LDL preparation with ECs. These were attributable to the greater degree of modification of the LDL by ingested reactive AGE intermediates in those patients, because they were clearly absent when LDL from the low-AGE–fed diabetic patients was used. Thus, under similar levels of hyperglycemia, diet-enhanced glycoxidation of LDL can transform it to a powerful promoter of intracellular oxidant stress–dependent changes.

As a key component of the MAPK system, ERK1/2 or p44/42 plays an important role in cell proliferation and differentiation. Activation of this kinase system is triggered by multiple stimuli, including AGE. Induction of ERK phosphorylation by in vitro modified LDL preparations has been reported in studies of cultured smooth muscle cells, mesangial cells, and macrophages. The human diabetic LDL used in this study was modified while in the circulation and contained glycation and oxidation derivatives in proportion to the dietary AGE content and not to levels of glycemia.

These derivatives prompted greater EC changes than expected on the basis of glycemic control alone. Of note, LDL derived from diabetic patients exposed to a low-AGE regimen (Db-LAGE-LDL) proved strikingly incapable of mobilizing significant activation signals, despite similar ambient hyperglycemia.

In vitro–prepared AGE can indeed activate NADPH oxidase and generate intracellular reactive oxygen species, suggesting that glycoxidation derivatives can act as second messengers, leading to MAPK activation. The events set in motion by in vivo AGE-modified LDL, however, are less well defined. The present findings provide evidence that at least reactive oxygen species and NADPH could be involved in these steps, because both an antioxidant, NAC, and an NADPH oxidase inhibitor abolished the HAGE-LDL induced ERK1/2 phosphorylation and NF-κB activity.

The identity of pathogenic AGE or lipoxidation derivatives of LDL remains largely undefined. However, a reasonable choice for making valuable correlations possible was the measurement of CML, a common end product of glucose-protein– and glucose-lipid–derived glycoxidative reactions, occurring abundantly in vivo and in heat-processed foods. Also, CML-like AGE deposits are shown to correspond to the vascular pathology and to the subinflammatory state of diabetes. Of note, circulating CML also paralleled levels of ingested CML. Whether high or low in AGE content, the diets used were equal in nutrient composition and not rich in fats or carbohydrates. Glucose and lipid levels remained likewise unchanged over the course of the study. The different levels of AGE in the diets used here were achieved by modulating cooking temperature and time. That the mode of meal preparation alone could alter the properties of circulating LDL to the extent that this would, in turn, drastically affect vascular cell signaling, nuclear factor transcription activity, and key adhesion molecule expression (sVCAM-1) was an unanticipated yet intriguing finding.

Glycoxidation of LDL involves free radical generation and fatty acid oxidation, which, together with hyperglycemia, can promote vascular injury. Given the evidence presented above, limiting AGE–apolipoprotein B–LDL formation by way of reducing dietary AGE intake could prevent atherogenic events attributed to the LDL particle. Indeed, an AGE-restrictive intervention in animals led to significant...
protection against postinjury restenosis and diabetes-
accelerated atherosclerosis in apolipoprotein E-negative mice.\textsuperscript{19,20} Moreover, in diabetic humans, manipulation of
dietary AGE led to the suppression of inflammatory markers
as well as of AGE-LDL.\textsuperscript{18} In this sense, the inflammatory
state seen in diabetic patients could be attributed to LDL
alterations caused by the high-AGE diets ingested combined
with hyperglycemia.\textsuperscript{32}

In conclusion, plasma LDL from diabetic patients can be
transformed into a powerful trigger of EC redox-dependent
activation. This may be largely a result of exogenous gly-
coxidants consumed with standard diets. The present study
provides a new mechanistic basis and the rationale for clinical
studies to establish potentially significant antiatherogenic
benefits of AGE-restricted diets in humans that protect LDL
particles from overt oxidative modification.

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