Overexpression of Glyoxalase-I in Bovine Endothelial Cells Inhibits Intracellular Advanced Glycation Endproduct Formation and Prevents Hyperglycemia-induced Increases in Macromolecular Endocytosis

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Abstract

Methylglyoxal (MG), a dicarbonyl compound produced by the fragmentation of triose phosphates, forms advanced glycation endproducts (AGEs) in vitro. Glyoxalase-I catalyzes the conversion of MG to S-D-lactoylglutathione, which in turn is converted to D-lactate by glyoxalase-II. To evaluate directly the effect of glyoxalase-I activity on intracellular AGE formation, GM7373 endothelial cells that stably express human glyoxalase-I were generated. Glyoxalase-I activity in these cells was increased 28-fold compared to neo-transfected control cells (21.80 ± 0.1 vs. 0.76 ± 0.02 μmol/min/mg protein, n = 3, P < 0.001). In neo-transfected cells, 30 mM glucose incubation increased MG and D-lactate concentrations approximately twofold above 5 mM (35.5 ± 5.8 vs. 19.6 ± 1.6, P < 0.02, n = 3, and 21.0 ± 1.3 vs. 10.0 ± 1.2 pmol/106 cells, n = 3, P < 0.001, respectively). In contrast, in glyoxalase-I-transfected cells, 30 mM glucose incubation did not increase MG concentration at all, while increasing the enzymatic product D-lactate by > 10-fold (18.9 ± 3.2 vs. 18.4 ± 5.8, n = 3, P = NS, and 107.1 ± 9.0 vs. 9.4 ± 0 pmol/106 cells, n = 3, P < 0.001, respectively). After exposure to 30 mM glucose, intracellular AGE formation in neo cells was increased 13.6-fold (2.58 ± 0.35 vs. 0.19 ± 0.03 total absorbance units, n = 3, P < 0.001). Concomitant with increased intracellular AGEs, macromolecular endocytosis by these cells was increased 2.2-fold. Overexpression of glyoxalase-I completely prevented both hyperglycemia-induced AGE formation and increased macromolecular endocytosis. (J. Clin. Invest. 1998. 101:1142–1147.) Key words: advanced glycation endproducts • methylglyoxal • glyoxalase-I • cell lines • endocytosis

Introduction

Chronic hyperglycemia is the primary etiologic factor in the pathogenesis of diabetic microvascular disease (1). A relation-...
Experiments were performed three times with triplicate replicates. A dose–response curve using 10, 20, 40, 80, and 100 μM of the glyoxalase-I inhibitor showed that cell viability began to decrease at a concentration of 80 μM. All experiments were performed three times with triplicate replicates.

**Methods**

**Cell culture conditions.** Transformed fetal bovine aortic endothelial cells, GM7373 cells (17), were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ). The cells were maintained in MEM containing 10% fetal bovine serum, essential and nonessential amino acids, and antibiotics, but no insulin. Confluent cells used for experiments were incubated in MEM containing 0.4% fetal bovine serum (growth arrest conditions) to mimic the state of endothelial cells in vivo, with either 5 or 30 mM glucose as described. After 10 d the cells were incubated with 100 μg/ml horseradish peroxidase type II (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C. The cells were washed thoroughly with PBS, trypsinized, lysed in PBS containing proteinase inhibitors by three cycles of freezing and thawing and sonication at 4°C, and then centrifuged at 12,000 g for 20 min. The supernatant was used as the cytosolic fraction.

**Glyoxalase-I assay.** The glyoxalase-I assay was performed according to a spectrophotometric method monitoring the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione for 2 min at 25°C (20). The standard assay mixture contained 7.9 mM MG, 1 mM glutathione, 14.6 mM magnesium sulfate, and 182 mM imidazole HCl, pH 7.0. Before initiating the reaction by adding the cytosolic fraction (10–30 μg) to the assay mixture, the mixture was allowed to stand for at least 2 min to ensure the equilibration of hemithioacetal formation. One unit of activity is defined as the formation of 1 nmol of S-D-lactoylglutathione/min/mg cell protein. Protein concentration was determined by the Coomassie plus protein assay reagent (Pierce Chemical Co., Rockford, IL).

**Measurement of total intracellular AGE protein.** Equal amounts of cytosolic protein from cells incubated under different conditions were dot blotted onto nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH). AGEs were detected by using the monoclonal anti-AGE antibody 6D12, which recognizes carboxymethyllysine (CML) like structures, as well as carboxyethyllysine (CEL) and several unidentified AGE epitopes (21). The immunocomplexes were visualized using an enhanced chemiluminescence kit (Amersham International, Amersham, UK) and evaluated by scanning densitometry using the Ultrascan XL (LKB, Uppsala, Sweden). The assay was standardized as described previously (22). To confirm that this antibody recognized MG-modified proteins, human serum albumin minimally modified by MG was assayed by immunoblotting. The minimally modified protein was made as described previously: 0.1 mM HSA and purified MG were incubated in 0.1 M sodium phosphate buffer, pH 7.4, at 37°C for 24 h, then dialyzed extensively against Dulbecco’s modified PBS (23). Immunoblots with mAb 6D12 showed a linear dependence on both MG-modified protein concentration and on MG concentration when incubated with an invariant amount of protein (data not shown). To determine whether the mAb 6D12 was in fact detecting changes in intracellular CML, MG-derived CEL, or an unidentified MG-derived epitope, CML and CEL were quantitated in 1.5 mg of cytosol protein by gas chromatography/mass spectrometry as described previously (24, 25) in both neo- and glyoxalase-I–transfected cells.

**Measurement of macromolecular endocytosis.** The endocytosis assay was performed as described previously (16). Briefly, confluent neo and glyoxalase-I–expressing cells were exposed to either 5 or 30 mM glucose as described. After 10 d the cells were incubated with 100 μg/ml of lipofectin (GIBCO BRL, Gaithersburg, MD) for 4 d with media changed twice a day. A preliminary dose–response curve using 10, 20, 40, 80, and 100 μM of inhibitor showed that cell viability began to decrease at a concentration of 80 μM. All experiments were performed three times with triplicate replicates.

**Cell cytosol preparation.** Cytosolic fractions of confluent cells were prepared as described previously (19). Briefly, the cells were trypsinized, lysed in PBS containing proteinase inhibitors by three cycles of freezing and thawing and sonication at 4°C for 1 min, and then centrifuged at 12,000 g for 20 min. The supernatant was used as the cytosolic fraction.

**Glyoxalase-I Prevents Intracellular Advanced Glycation Endproducts**

Figure 1. Schematic representation of potential pathway leading to AGE formation. AGES arise from autoxidation of glucose to glyoxal, decomposition of the Amadori product to 3-DG, and the glyceraldehyde fragmentation product MG, which all react with amino groups of proteins. MG is metabolized to the unreactive D-lactate by the glyoxalase system (see Introduction for details).

has been shown to reduce hyperglycemia-induced increases in macromolecular endocytosis, the effect of glyoxalase-I overexpression on this phenomenon was also investigated (16).
both Western blotting using an antibody against human glyoxalase-I (gift of Dr. Kenneth D. Tew) and by measuring the enzyme activity. Immunocomplexes on the blotting membrane were visualized using an enhanced chemiluminescence kit (Amersham International).

**Measurement of α-oxoaldehydes in cells.** Glyoxal (40% aqueous solution) was purchased from Sigma Chemical Co. MG and 3-DG were prepared, purified, and characterized as described previously (33, 34). Stock solutions of MG and glyoxal were calibrated by end point enzymatic assay with glyoxalase-I (35) using molar changes of the extinction coefficient for the formation of the corresponding 3,2-hydroxyacetylglutathione deduced from reference 36. The concentration of glyoxal, 3-DG, and MG were determined in endothelial cell cytosol by derivatization of the α-oxoaldehydes with 1,2-diamino-4,5-dimethoxybenzene using a previously described method (35, 37) with the following modifications: samples were acidified with 100 mM acetic acid for storage at $-80^\circ$C and transferred (on cardice) between collaborating groups before derivatization to minimize degradation of nucleotides to MG (38), the HPLC mobile phase was 20 mM ammonium formate, pH 3.4, with isotropic 30% methanol from 0 to 40 min, a linear gradient of 30-48% methanol from 40 to 60 min, 90% methanol from 60 to 70 min for washing of the column, and reequilibration with the initial mobile phase for 5 min, and the adducts and internal standard were detected by fluorescence (excitation wavelength 352 nm, emission wavelength 395 nm) with a scanning fluorescence detector (model 474; Waters Instruments Inc., Rochester, MN).

For 3-DG, glyoxal and MG: the retention time of derivatized adducts was 8.5, 22.4, and 38.3 min (retention time of the internal standard was 53.3 min), the recoveries were 52, 70, and 59%, the limits of detection (anlyte/concentration equivalent to 2 SDs of the intercept on the analyte/internal standard peak area ratio) were 21, 11, and 14 pmol, and the interbatch coefficients of variation were 15, 11, and 10% ($n = 9$), respectively. For application of the α-oxoaldehyde assay using 1,2-diamino-4,5-dimethoxybenzene as a derivatizing agent for the assay of 3-DG, the 3-DG adduct 2-(2,3,4-trihydroxybutyl)-6,7-dimethoxyquinoxaline (TDQ) was prepared, purified, and characterized. 3-DG (72 mg, 0.4 mmol) and 1,2-diamino-4,5-dimethoxybenzene dihydrochloride (48 mg, 0.2 mmol) were dissolved in hydrochloric acid (1.5 M, 1.75 ml) and stirred under nitrogen at room temperature for 6 h. The product mixture was neutralized to pH 7.4 by the addition of 2 M potassium bicarbonate (~1.3 ml), filtered (0.2-mm pore size), and TDQ purified by preparative reversed-phase HPLC. The column was a 2.5 × 8 cm ODS cartridge with a 2.5 × 1 cm precolumn, the flow rate 9.9 ml/min, the sample loading 1 ml of neutralized crude product, and the mobile phase 20 mM acetic acid, 10% methanol (0-12 min) with a linear gradient of 10-50% methanol (12-32 min). The eluate absorbance was monitored at 352 nm. The TDQ-containing fraction, retention interval 4-6 min, was collected from three runs, pooled, and lyophilized to dryness. TDQ was characterized: melting point 94–95°C (decomposed); 1H NMR (50% vol/vol CD3CN-D2O) 8.15 (s, 1H, H-1), 7.33 (s, 1H, H-8), 7.19 (s, 1H, H-5), 3.97 (s, 6 H, 6,7-MeO), 3.78 (dd, 1H, H-3), 7.04 (s, 2H, H-2), 6.94 (t, 1H, J = 9.9 Hz, H-6), 6.74 (s, 1H, H-3). The acetol derivative formed with 2,4-dinitrophenylhydrazine was separated using a 5 μm, C-18 column (Spherex; Phenomenex, Inc.), 250 × 4.6 mm, with a linear gradient elution with water and methanol (initial conditions 55% methanol, 1 ml/min, final conditions 100% methanol, 2 ml/min) and detected with a UV/visible detector (Dynamax; Rainin Instruments Co.).

**Results**

**High glucose (30 mM) does not affect glyoxalase-I activity in GM7373 cells.** To evaluate the effect of high glucose on glyoxalase-I activity, the GM7373 cells, cells were incubated in media containing either 5 or 30 mM glucose for 7 d. Then, cytosolic fractions were extracted and glyoxalase-I activity was determined. No significant difference in enzyme activity was found between cells exposed to 5 and 30 mM glucose (0.69 ± 0.01 vs. 0.66 ± 0.1 μmol/min/mg protein, $n = 3$, $P = NS$).

A glyoxalase-I inhibitor, HCCG, enhances hyperglycemia-induced intracellular AGE formation in GM7373 cells. To evaluate the effect of glyoxalase-I inhibition on intracellular AGE formation in GM7373 cells, cells were incubated in either 5 or 30 mM glucose, or 30 mM glucose containing 40 μM of a glyoxalase-I inhibitor, HCCG, for 4 d. The media were changed every 12 h. Intracellular AGE formation in the cells exposed to 30 mM glucose was increased 5.4-fold compared to 5 mM (1.40 ± 0.08 vs. 0.26 ± 0.02 total absorbance units, $n = 3$, $P < 0.001$). This hyperglycemia-induced AGE formation was enhanced an additional 2.4-fold in the presence of the glyoxalase-I inhibitor (3.20 ± 0.03 vs. 1.40 ± 0.08 total absorbance units, $n = 3$, $P < 0.001$).

**Stable expression of human glyoxalase-I in GM7373 cells increases glyoxalase-I activity.** To evaluate the effect of glyoxalase-I on intracellular AGE formation, a GM7373 endothelial cell line stably expressing human glyoxalase-I activity was generated as described in Methods. The expression of human glyoxalase-I protein was demonstrated by Western blotting using a polyclonal antibody against human glyoxalase-I. As shown in Fig. 2 (lanes 3 and 4), a single band around 20 kD was detected, which corresponds to the molecular mass of the monomeric subunit of human glyoxalase-I (20,774 D) (26). To evaluate glyoxalase-I activity in transfected cells, glyoxalase-I activity of cytosolic fractions was determined. The enzyme activity was increased about 28-fold in glyoxalase-I-transfected
Table I. Effect of Glyoxalase-I on Glyoxal, MG, D-lactate, Acetol, and 3-DG Content in the neo- and Glyoxalase-I–transfected cells compared with neo-transfected cells. (21.8 ± 0.1 vs. 0.76 ± 0.02 μmol/min/mg protein, n = 3, P < 0.001).

Glyoxalase-I expression in GM7373 endothelial cells reduces hyperglycemia-induced intracellular AGE formation, but does not affect CML or CEL content. Both neo cells and glyoxalase-I–expressing cells were incubated in either 5 or 30 mM glucose, to evaluate the effect of glyoxalase-I on hyperglycemia-induced intracellular AGE formation. AGE concentration in the cells was measured by dot blotting using an mAb against AGE. After the cells were exposed to 5 and 30 mM glucose for 7 d, cytosolic fractions were used for the measurement. Intracellular AGE formation in neo-transfected cells exposed to 30 mM glucose was increased 13.6-fold compared with the cells exposed to 5 mM glucose (2.58 ± 0.15 vs. 0.19 ± 0.03 total absorbance units, n = 3, P < 0.001), whereas intracellular AGE in glyoxalase-I–transfected cells was not increased by 30 mM glucose (0.25 ± 0.03 vs. 0.21 ± 0.08 total absorbance units, n = 3, P = NS). Analysis of two epitopes recognized by the antibody 6D12, CML and CEL, showed no differences between neo- and glyoxalase-I–transfected cells (CML: neo cells, 0.0126 ± 0.0010 mmol/mmol lysine; glyoxalase-I–transfected cells, 0.0201 ± 0.0047 mmol/mmol lysine, n = 6, P = NS; CEL: neo cells, 0.0387 ± 0.0093 mmol/mmol lysine; glyoxalase-I–transfected cells, 0.0286 ± 0.0056 mmol/mmol lysine, n = 6, P = NS).

Glyoxal and 3-DG levels in endothelial cells are low and are not increased significantly by 30 mM glucose. Glyoxal has been identified as a key intermediate in the autoxidative modification of proteins by glucose (11). 3-DG also has been thought to be an important intermediate in AGE formation in vivo (41, 42). We evaluated both glyoxal and 3-DG levels in both neo- and glyoxalase-I–transfected cells incubated in either 5 or 30 mM glucose. In both cell types exposed to either glucose concentration, both glyoxal and 3-DG levels were below the statistical limit of detection (glyoxal; < 11 pmol/10⁶ cells and 3-DG; < 21 pmol/10⁶ cells) (Table I).

Hyperglycemia-induced increased MG content is normalized by increased enzymatic reduction in glyoxalase-I–expressing GM7373 endothelial cells. Although MG content in neo cells exposed to 30 mM glucose was increased by 181% compared with the cells exposed to 5 mM glucose (35.5 ± 5.8 vs. 19.6 ± 1.6 pmol/10⁶ cells, P < 0.02), there was no significant difference in MG content between 30 and 5 mM glucose in glyoxalase-I–transfected cells (18.9 ± 3.2 vs. 18.4 ± 5.8 pmol/10⁶ cells, P = NS). We next measured D-lactate to evaluate the minimal cumulative effect of high glyoxalase-I activity on MG metabolism in either 30 or 5 mM glucose for 7 d. D-lactate content in neo cells in 30 mM glucose was increased 2.1-fold compared with neo cells in 5 mM glucose (6.7 ± 1.3 vs. 10.0 ± 1.2 pmol/10⁶ cells, n = 3, P < 0.01). In contrast, in glyoxalase-I–transfected cells, the hyperglycemia-induced D-lactate formation in 30 mM glucose was markedly enhanced. D-lactate content was increased 11.4-fold compared to 5 mM glucose (107.1 ± 8.9 vs. 9.4 ± 0 pmol/10⁶ cells, n = 3, P < 0.001).

Acetol level in neo- and glyoxalase-I–transfected cells incubated in 30 mM glucose is not increased significantly. The enzyme aldose reductase catalyzes the conversion of MG to acetol (15). To evaluate the role of acetol in intracellular AGE production, we measured the level of acetol in cells incubated in either 5 or 30 mM glucose. The acetol levels in both neo- and glyoxalase-I–transfected cells in either glucose concentration were equally low and there was no significant difference between them (< 8.0 pmol/10⁶ cells).

Glyoxalase-I expression in GM7373 endothelial cells reduces hyperglycemia-induced increases in macromolecular endocytosis. Both neo cells and glyoxalase-I–expressing cells were incubated in either 5 or 30 mM glucose to evaluate the effect of glyoxalase-I on hyperglycemia-induced increases in endocytosis. The cellular endocytosis of horseradish peroxidase in neo cells exposed to 30 mM glucose for 10 d was increased 2.2-fold compared with cells exposed to 5 mM glucose (5.1 ± 0.14 AU/10⁶ cells vs. 2.13 ± 0.02 AU/10⁶ cells, n = 3, P < 0.001), whereas the endocytosis in glyoxalase-I–transfected cells was not increased (2.27 ± 0.02 AU/10⁶ cells vs. 2.29 ± 0.03 AU/10⁶ cells, n = 3, P = NS).

Discussion

In this study we found that endogenous glyoxalase-I activity was identical in GM7373 endothelial cells exposed to either 5
or 30 mM glucose. As demonstrated previously (10), intracellular AGE concentration was increased by 30 mM glucose, as was macromolecular endocytosis. This hyperglycemia-induced intracellular AGE formation was significantly increased when glyoxalase-I was partially inhibited by HCCG, providing indirect evidence that 30 mM glucose increased AGE formation by generating MG. To examine directly the role of MG in hyperglycemia-induced intracellular AGE formation, GM7373 endothelial cells were generated that stably expressed human glyoxalase-I with a 28-fold higher glyoxalase-I activity than neo-transfected cells. In neo-transfected cells, 30 mM glucose incubation increased MG and D-lactate concentration approximately twofold. In contrast, in glyoxalase-I-transfected cells, 30 mM glucose incubation did not increase MG concentration at all, while increasing the terminal product of the glyoxalase pathway, D-lactate, >10-fold. Incubation of neo cells in 30 mM glucose did not cause any change in the levels of glyoxal, 3-DG, or acetol, however, the measured levels were below the statistical detection limits. Most strikingly, overexpression of glyoxalase-I completely prevented hyperglycemia-induced AGE formation, and also prevented hyperglycemia-induced macromolecular endocytosis. These results indicate that hyperglycemia increases AGEs in GM7373 endothelial cells primarily, if not exclusively, by increasing the concentration of AGE-forming MG, and that hyperglycemia-induced increases in macromolecular endocytosis are a consequence of MG accumulation.

Intracellular glycation and AGE formation have been associated with low catalytic efficiency of aldehyde reductase in the diabetic rat kidney (43) and decreased mitogenic activity of basic fibroblast growth factor (bFGF) in cultured endothelial cells (10). After incubation of endothelial cells in 30 mM glucose for 168 h, AGE-modified bFGF was increased by 6.1-fold and bFGF mitogenic activity was reduced by 70% (10). Our finding that endogenous glyoxalase-I activity is unaffected by these incubation conditions suggests that the lysyl residue in the active site of mammalian glyoxalase-I (44) is not susceptible to modification by glucose, most likely because of its microenvironment (45).

Since MG has been associated with cellular toxicity (14), preliminary experiments were performed to establish the optimum concentration of the glyoxalase-I inhibitor HCCG. This competitive inhibitor of glyoxalase-I mimics the enediol intermediate associated with the glyoxalase-I reaction (18). At a concentration of 80 μM, GM7373 cells died after 2 d, while at 40 μM HCCG, there was no cell death as assessed by trypsin blue exclusion. Thus the observed increase in hyperglycemia-induced AGE formation reflects MG produced by a partial inhibition of glyoxalase-I. To ensure that the mAb 6D12 recognized MG-modified proteins, we demonstrated a concentration-dependent increase in the immunoreactivity of 6D12 against HSA minimally modified with highly purified MG in vitro, when either protein or MG concentration was the independent variable (46). To determine whether the mAb 6D12 was in fact detecting changes in intracellular CML, MG-derived CEL, or an unidentified MG-derived epitope, CML and CEL were quantitated using gas chromatography/mass spectrometry in both neo- and glyoxalase-I-transfected cells. Since analysis of both CML and CEL content showed no differences between neo- and glyoxalase-I-transfected cells, it is likely that the AGE formed by MG and recognized by 6D12 is a different MG protein epitope.

Our finding that incubation in 30 mM glucose increases concentrations of MG and its metabolite D-lactate in neo-transfected cells is consistent with the finding that MG concentration is increased three- to fivefold in the blood of diabetic patients (47). The lack of increase in MG concentration after incubation of cells overexpressing glyoxalase-I in high glucose and much greater increase in D-lactate are consistent with greater enzymatic conversion of MG to its metabolite, and suggest that glyoxalase-I activity is rate-limiting in hyperglycemia-induced increases in MG production. Intracellular concentrations of glyoxal and 3-DG were not detectable even after incubation in 30 mM glucose, suggesting that in these cells, glucose autoxidation and Amadori product decomposition did not occur to a significant degree. This conclusion is indirectly supported by the observation that overexpression of glyoxalase-I, which does not utilize 3-DG as a substrate, completely inhibited hyperglycemia-induced intracellular AGE formation. Elevated concentrations of 3-DG and its reduction product 3-deoxyfructose have been reported in blood from both animal and human diabetics (41, 42), however, suggesting that this intermediate may play a major role in extracellular AGE formation and/or in intracellular AGE formation in other cell types.

Acetol was also not detected even after incubation in 30 mM glucose, suggesting that conversion of MG to this alternate AGE precursor by aldose reductase (15) does not take place to any significant extent in these cells. Thus aldose reductase inhibitors are unlikely to increase intracellular AGE formation, and may even reduce MG formation by preventing the reductase-mediated inactivation of glyceraldehyde 3-phosphate dehydrogenase (5) and by preventing the hyperglycemia-induced depletion of the glutathione reductase cofactor NADPH (48).

The demonstration that hyperglycemia-induced intracellular AGE formation and increased macromolecular endocytosis in GM7373 endothelial cells result from increased MG production has important implications for the pathogenesis of diabetic vascular complications. Diabetes induces increased endothelial endocytosis in retinal capillaries in vivo, which may contribute to the capillary pathology that subsequently occurs (49).

Differences in the efficiency with which individual patients detoxify MG may explain individual differences in susceptibility to complications, while elucidation of the biochemical mechanisms by which hyperglycemia increases intracellular MG concentrations may lead to novel therapeutic strategies. Both of these possibilities deserve further exploration.

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