Gliclazide Decreases Low-Density Lipoprotein Oxidation and Monocyte Adhesion to the Endothelium

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Increasing evidence implicates oxidized low-density lipoprotein (LDL) and advanced glycation end products (AGE) in the atherogenesis associated with diabetes mellitus. In the present study, we examined the in vitro effects of gliclazide on LDL oxidation and monocyte adhesion to endothelial cells induced by oxidized LDL and glycated albumin. To assess the clinical relevance of our in vitro findings, we also measured the effect on monocyte adhesion of gliclazide administration to type 2 diabetic patients. Incubation of human monocytes and endothelial cells with increasing concentrations of gliclazide (0 to 10 μg/mL) and native LDL (100 μg/mL) induced a dose-dependent diminution of cell-mediated LDL oxidation. Pretreatment of endothelial cells with gliclazide (0 to 10 μg/mL) before addition of native LDL (100 μg/mL) or glycated albumin (100 μg/mL) resulted in a dose-dependent diminution of oxidized LDL- and glycated albumin-induced monocyte adhesion to endothelial cells. In type 2 diabetic patients, administration of gliclazide inhibits the increased adhesiveness of monocytes to levels similar to those observed in control subjects. These results indicate that gliclazide is an antioxidant and suggest a beneficial effect of this drug in the prevention of atherosclerosis associated with type 2 diabetes.
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DIABETIC PATIENTS SHOW increased levels of circulating modified lipoproteins[1] and enhanced oxidation of plasma low-density lipoproteins (LDLs).[2] Increasing evidence suggests that oxidative modification of LDL plays a significant role in atherogenesis associated with diabetes mellitus.[3] LDL, once oxidized by resident vascular cells in the subendothelial arterial space, is taken up by macrophages leading to foam cell formation.[4-6] Oxidatively-modified LDL is also cytotoxic to endothelial cells,[7] induces local vascular cells to produce chemokines and growth factors,[8,9] and has direct chemotactic activity for monocytes,[10,11] stimulating monocyte binding to endothelium.[12]

Besides LDL oxidation, one biochemical mechanism involved in the pathogenesis of atherosclerosis associated with diabetes is the formation and accumulation of advanced glycation end products (AGE) in the plasma and tissues of diabetic patients.[13,14] Recent data have shown that AGE-modified proteins exert, through their binding to specific cell receptors, several atherogenic effects, including increased oxidative stress and LDL oxidation[15] and enhanced monocyte binding to endothelium.[16]

Gliclazide, a second-generation sulfonylurea, is widely used in the treatment of type 2 diabetic patients. Independently of its metabolic effects,[17] gliclazide possesses some nonmetabolic effects specifically related to vascular disease in diabetes. Among them, free radical scavenging activity of gliclazide has been recently documented.[18] Based on this finding, we anticipated that gliclazide may be effective in vitro in reducing both LDL oxidation and monocyte adhesion to endothelium induced by oxidized LDL and AGE. To assess this clinically, we also evaluated the effect of gliclazide administration to type 2 patients on monocyte adhesion to cultured endothelial cells. The present report summarizes our recent findings on the in vitro and in vivo antioxidant effects of gliclazide.

MATERIALS AND METHODS

Reagents

RPMI-1640 was purchased from Gibco (Grand Island, NY), and penicillin-streptomycin and fetal calf serum (FCS) were obtained from Flow Laboratories (McLean, VA) and Hyclone Laboratories Inc (Logan, UT), respectively. Dulbecco's minimal essential medium (DMEM) and L-glutamine were obtained from ICN Biochemicals Inc (Costa Mesa, CA). Thiobarbituric acid (TBA) and tetraethoxypropane (TEP) were purchased from ICN Biochemicals. Dianisidine dihydrochloride and hexadecyltrimethyl-amine ammonium bromide (HTAB) were purchased from Sigma Chemical Co (St Louis, MO). Phosphoric acid and butanol were obtained from Fisher Scientific (Nepean, Montreal, Canada). Gliclazide was kindly provided by Les Laboratories Servier (Neuilly, France).

Human LDL Isolation

LDL was isolated from plasma obtained from healthy, normolipidemic, human subjects. Venous blood was drawn into tubes containing ethylene diaminetetraacetic acid (EDTA), and LDL was isolated at a density of 1.019 to 1.063 after sequential centrifugation in KBr according to the method of Hatch.[19] LDL was extensively dialyzed for 24 hours at 4°C against 5 mmol/L Tris-50 mmol/L NaCl to remove EDTA, stored at 4°C, and used within 2 days at a final concentration of 100 μg LDL protein/mL. Protein content in the LDL preparations was measured according to the Bradford method[20] using a colorimetric assay (Bio-Rad, Mississuaga, Ontario).

Oxidative Modification of LDL

Cellular modification of LDL was obtained by incubating 100 μg LDL protein for 20 hours at 37°C in the presence of human control monocytes in serum-free RPMI-1640 containing 3 μmol/L $CuSO_4$. At the end of the incubation period, supernatants were removed and EDTA (0.04% final concentration) was added to stop LDL oxidation.

Determination of Serum Lipid Peroxides and LDL Oxidation

The lipid peroxide content of oxidized LDL and serum was determined by measuring in the serum or supernatants the TBA-reactive substances (TBARS) expressed as malondialdehyde (MDA) equivalents (nmol/500-μL medium).[21-22] Samples (500 μL) were mixed with 3 mL of phosphoric acid (1%) and 1 mL of TBA (0.6%).

The mixture was heated to 95° for 45 minutes in a hot water bath.

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Supported by Les Laboratories Servier.

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After cooling, the MDA-TBA complex was extracted by adding butanol, and the optical density was read at 532 nm. Freshly diluted TEP, which produces MDA after hydrolysis, was used as standard.

Preparation and Characterization of Glycated Albumin

Immunoglobulin-free bovine serum albumin (BSA) (Sigma Chemical Co) was glycated in vitro by incubation at 37°C for 4 weeks in the presence of 0.5 mol/L glucose in 0.4 mol/L sodium phosphate buffer containing 0.5 mmol/L EDTA. Incubation was performed in the absence of oxygen after sterilizing the solution by passage through a 0.2-μm filter. Nonglycated albumin was obtained by incubating BSA in the same reaction mixture in the absence of glucose. At the end of the incubation period, samples were extensively dialyzed against 10 mmol/L phosphate-buffered saline (PBS) (pH 7.4). Glycation of albumin was confirmed by the typical absorption and fluorescent spectra patterns of this protein.[23] Endotoxin content of the nonglycated and glycated albumin preparations (100 μg/mL) was determined by the limulus amebocyte lysate assay (E-toxate, Sigma Chemical Co) and was consistently found to be less than 6 pg/mL. Protein concentrations were determined by the Bradford method.

Human Monocyte Isolation

Fresh heparinized blood (100 mL) was obtained from healthy men and women nonsmoker donors. Peripheral blood mononuclear cells were isolated by density centrifugation using Ficoll (Gibco-BRL, Grand Island, NY),[24] allowed to aggregate in the presence of FCS, then further purified by the rosetting technique. After density centrifugation, recovery of highly purified monocytes (85% to 90%) as assessed by fluorescence-activated cell sorting analysis was obtained. Monocytes were resuspended in serum-free RPMI-1640 medium with 2 mmol/L glutamine supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin and used immediately for adhesion assay.

Endothelial Cell Culture

Subcultured (17th passage) bovine aortic endothelial cells (BAE) were kindly provided by Dr R. Sauvé (Montreal University, Montreal). Cells were plated in tissue culture flasks at 37°C in 5% CO_2/95% air atmosphere and grown in DMEM supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L glutamine. After 6 days, cells were trypsinized and cultured for an additional 48 hours in 96 multiwells (Falcon, Franklin Lakes, NJ), at which time cell confluence was reached. In all experiments, cells were used between the 18th and the 23rd passage.

Adhesion Assay

On the day of the experiment, the medium of confluent monolayers of endothelial cells was removed and replaced by fresh serum-free RPMI-1640 medium. One hundred microliters of a monocytic cell suspension (2.3×10^6 cells/mL) was then added to each well. After a 30-minute incubation period, nonadherent monocytes were removed by washing twice with PBS without calcium or magnesium (PBS-A). Adherent cells were lysed in 50 μL of HTAB (0.5%) in PBS-A at pH 6.0 for 30 minutes. Quantification of adherent monocytes was made by measuring monocyte myeloperoxidase (MPO) activity.[25] Briefly, MPO activity was determined by the addition to each well of 250 μL of dianisidine dihydrochloride (0.2 mg/mL in PBS-A) warmed to 37°C and mixed with hydrogen peroxide (0.4 mmol/L final concentration). After 2 to 5 minutes of incubation, the optical density of the plate wells was read at 450 nm using a Titertek multiscan (Flow Laboratories, McLean, VA) spectrophotometer.

Subjects

The study group comprised 8 type 2 diabetic patients and 8 healthy control subjects. The patients, 4 women and 4 men, gave written consent to participate in this study, which was approved by the Notre-Dame Hospital Research and Ethics Committees. All patients were selected, from our diabetic outpatient clinic, on the basis of poor diabetes control caused by lack of compliance with dietary regimes (glycated hemoglobin of 9% or more), glyburide treatment, no decompensated cardiac or renal conditions, and because they were nonsmokers. Their mean ± SD (range) age was 61 ± 5 years (55 to 70), body mass index (BMI) 29 ± 3 kg/m^2 (26 to 34), duration of diabetes 10 ± 9 years (3 to 30), glycated hemoglobin 12 ± 1% (9.2 to 15) and daily glyburide dose 16.5 ± 5.8 mg (5 to 20). All were treated with metformin. One patient was a candidate for insulin therapy and was switched at the end of this study from gliclazide to insulin. Four patients were hypertensive and treated with ACE inhibitors, 4 were hypertriglyceridemic, 2 had macroangiopathy, and 5 had microangiopathy (retinopathy or microalbuminuria). Control subjects matched with patients for gender and BMI were recruited from the hospital staff and relatives. Subjects with infectious or inflammatory conditions or treated by anti-inflammatory or antioxidant drugs were excluded from the study. In this pilot study, diabetic patients were switched for 3 months from glyburide to an equivalent hypoglycemic dose of gliclazide (5 mg of glyburide equivalent to 80 mg of gliclazide). Venous blood samples were obtained from control subjects and from diabetic patients before and after 3 months of treatment with gliclazide.

Statistical Analysis

Statistical analysis of the results was performed by one-way analysis of variance followed up by the Tukey test. The Spearman rank correlation test was used to evaluate correlation between lipid peroxide levels and monocyte adhesion to cultured endothelial cells. Results were expressed as mean ± SEM.

RESULTS

In Vitro Experiments

Effect of gliclazide on the oxidative modification of LDL mediated by human monocytes and endothelial cells. Incubation of native human LDL (100 μg protein/mL) in medium containing 3 μmol/L Cu^{2+} in the presence of freshly isolated human monocytes for 20 hours at 37°C significantly enhanced oxidative modification of LDL as measured by lipid peroxidation products in the supernatants. Medium TBARS content in the presence of monocytes was 4.9 ± 0.2 MDA equivalents/500 mL medium, while TBARS production in the absence of cells was 2.3 ± 0.3 MDA equivalents/500 mL medium ($P < .005$). To evaluate the effects of gliclazide or glyburide on the degree of LDL oxidation mediated by human monocytes, these cells were incubated for 1 hour in the presence of increasing concentrations (1 to 10 μg/mL) of gliclazide or glyburide (0.05 to 5 μg/mL) before the addition of native LDL. As shown in Fig 1A the addition of gliclazide resulted in a dose-dependent inhibition of the degree of monocyte-induced LDL oxidation. The maximal decrease was about 20% and was observed at a concentration of 10 μg/mL of this drug. In contrast, glyburide had no significant effect on the monocyte-induced LDL oxidation (Table 1).

Incubation of endothelial cells with LDL (100 μg protein/mL) for 20 hours led to a significant increase in the medium TBARS content (3.9 ± 0.2 MDA equivalents/500 mL medium) compared with that observed in the absence of cells (1.8 ± 0.3 MDA equivalents/500 mL medium) ($P < .005$). Preincubation of endothelial cells with gliclazide (1 to 10 μg/mL) for 1 hour before the addition of native LDL resulted in a significant

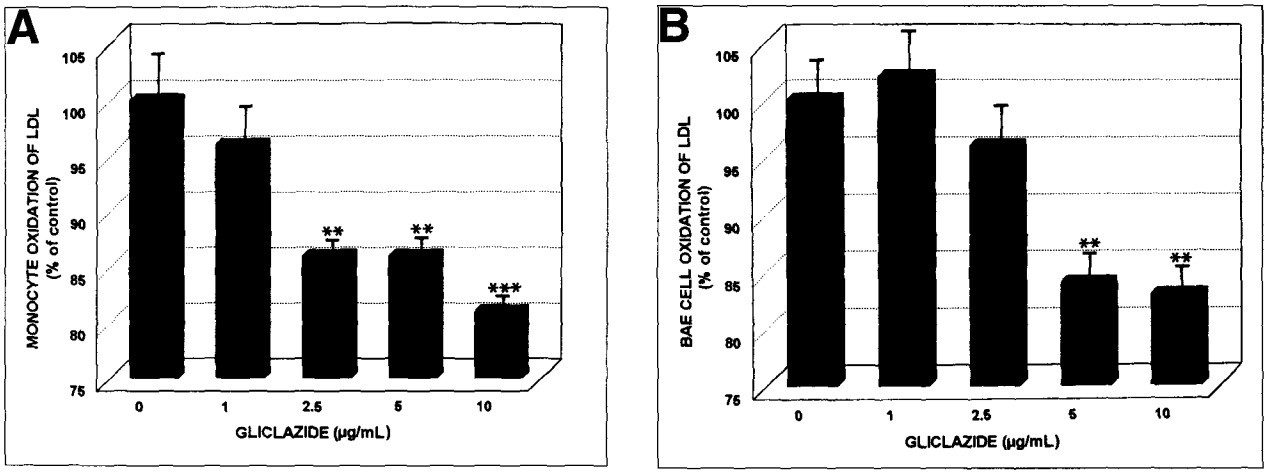

Fig 1. (A) Gliclazide decreases in a dose-dependent manner LDL oxidation mediated by human monocytes. Native LDL (100 μg/mL) were incubated for 20 hours with human monocytes in RPMI containing 3 μmol/L $CuSO_4$ in the presence of increasing concentrations of gliclazide (0 to 10 μg/mL). At the end of the incubation period, TBARS content was measured in the medium. Results were expressed as % TBARS content observed in the absence of gliclazide. Data represent the mean ± SEM of 7 different experiments. $^{**}P < .02$, $^{***}P < .005$ versus control. (B) Gliclazide decreases in a dose-dependent manner LDL oxidation mediated by endothelial cells. Native LDL (100 μg/mL) were incubated for 20 hours with bovine aortic endothelial (BAE) cells in RPMI containing 3 μmol/L $CuSO_4$ in the presence of increasing concentrations of gliclazide (0 to 10 μg/mL). At the end of the incubation period, TBARS content was measured in the medium. Results were expressed as % TBARS content observed in the absence of gliclazide. Data represent the mean ± SEM of 7 different experiments. $^{**}P < .02$ versus control.

decrease in the endothelial cell-mediated oxidation of LDL (Fig 1B). The inhibition of LDL oxidation by gliclazide was dose-dependent and the maximal effect (18% decrease) was observed with 10 μg/mL of this drug. Preincubation of endothelial cells with glyburide (0.05 to 5 μg/mL) was found to be ineffective in reducing LDL oxidation in the presence of endothelial cells (Table 1).

Effect of gliclazide on monocyte adhesion to endothelial cells. Preincubation of endothelial cells with native LDL (100 μg protein/mL) in the presence of 3 μmol/L Cu^{2+} for 20 hours at 37°C resulted in a 2.8-fold increase in the number of adherent monocytes to the endothelium as assessed by the MPO assay (Table 2). In the presence of LPS (10 ng/mL), used as a positive control, monocyte adhesion to endothelial cells was enhanced 2.3-fold (Table 2). Pretreatment of endothelial cells with gliclazide (1 to 10 μg/mL) for 1 hour dramatically decreased the ability of oxidized LDL to stimulate endothelium cell adhesiveness (Fig 2). Gliclazide-induced inhibition of monocyte adhesion to the endothelium was dose-dependent, and at the highest concentration of gliclazide (10 μg/mL) monocyte adhesion to endothelial cells was decreased by 43% (Fig 2). Pretreatment of endothelial cells with glyburide (0.5 μg/mL) did not affect the LDL-induced enhancement of monocyte adhesion to the endothelium (data not shown).

Incubation of cultured endothelial cells in the presence of glycated albumin (100 μg/mL) for 1 to 48 hours resulted in a time-dependent increase in human monocyte adhesion to these cells (Fig 3). The maximal effect of glycated albumin on monocyte adhesion to endothelial cells was observed from 4 to 12 hours. This effect gradually declined towards baseline after 24 hours of stimulation by glycated albumin (Fig 3). Nonglycated albumin (100 μg/mL), used as a control, did not significantly affect monocyte adhesion to endothelial cells (data not shown). Pretreatment of endothelial cells with gliclazide (10 μg/mL) for 1 hour before the addition of glycated albumin (100 μg/mL) significantly reduced the stimulatory effect of glycated albumin on monocyte adhesion to endothelial cells (Fig 3).

Table 1. Effect of Glyburide on the Generation of TBARS by Monocytes and Endothelial Cells

Glyburide (μg/mL)	Monocytes + 3 μmol/L $CuSO_4$	Endothelial Cells + 3 μmol/L $CuSO_4$
0	100 ± 2.5	100 ± 4.4
0.05	101 ± 4.2	115 ± 5.8
0.13	104 ± 1.8	105 ± 3.8
0.25	96 ± 8.6	103 ± 3.5
0.5	104 ± 2.6	103 ± 1.4
5	96 ± 4.3	112 ± 6.9

Data represent the mean ± SEM of 3 experiments.

Ex Vivo Experiments

Effect of gliclazide on diabetic monocyte adhesion to cultured endothelial cells. To assess the adhesiveness of diabetic monocytes to the endothelium, we measured the adhesion of control and diabetic monocytes to endothelial cells. A marked increase in the adhesion of diabetic monocytes to endothelial cells was observed before gliclazide treatment (163 ± 24% over control values, $P < .005$) (Fig 4). This increase was positively correlated ($r = 0.74$, $P < .01$) with enhanced serum

Table 2. Monocyte Adhesion to Endothelial Cells

	Adhesion (% over basal values)
Control (with LDL)	100 ± 10
LDL + 3 μmol/L $CuSO_4$	280 ± 20*
LPS (10 ng)	230 ± 20*

Data represent mean ± SEM of 6 experiments.
*$P < .01$ versus control.

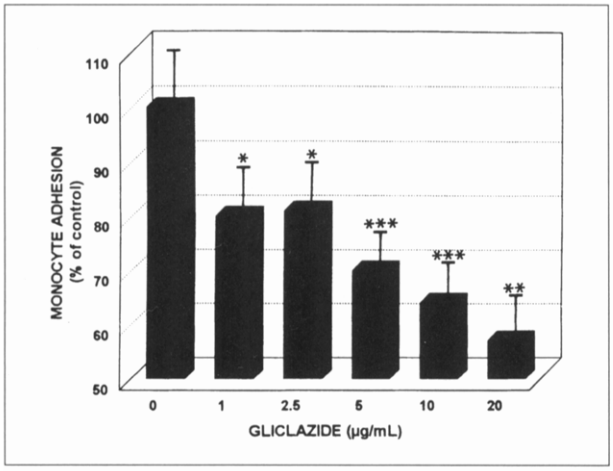

Fig 2. Gliclazide decreases in a dose-dependent manner monocyte adhesion to endothelial cells induced by oxidized LDL. Endothelial cells were treated for 20 hours with native LDL (100 μg/mL) in the presence of increasing concentrations of gliclazide (0 to 20 μg/mL). Monocyte adhesion to endothelial cells was measured by the MPO assay. Results were expressed as % of control. Data represent mean ± SEM of 7 different experiments. *$P < .05$, **$P < .01$, ***$P < .005$ versus control.

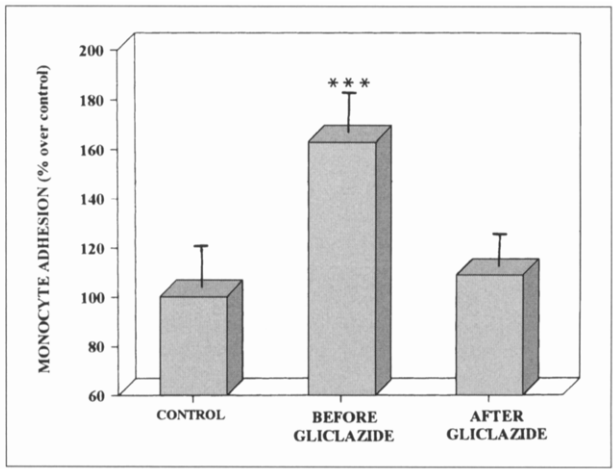

Fig 4. Monocyte adhesion in type 2 diabetic patients before and after gliclazide treatment. Results were expressed as % of adhesion over control values. Data represent the mean ± SEM. ***$P < .005$ versus controls.

lipid peroxide levels observed in diabetic patients (lipid peroxide levels [mmol/mL]): diabetic subjects: 9.6 ± 1.1; control subjects: 5.8 ± 0.6, $P < .005$). Gliclazide administration totally reversed these anomalies, reducing lipid peroxide levels (data not shown) and monocyte adhesion (Fig 4) in type 2 diabetic patients to levels identical to those observed in control subjects.

DISCUSSION

Monocyte adhesion and migration into the arterial wall is one of the earliest events in atherogenesis. Increased adhesiveness of diabetic monocytes to endothelium[26] may contribute to the accelerated atherosclerosis associated with type 2 diabetes. Metabolic alterations that may account for the enhanced diabetic monocyte binding include oxidized LDL and AGE.[12,16] In vitro and in vivo studies have shown that gliclazide possesses antioxidant effects. We and other investigators have reported that gliclazide inhibits in vitro copper-induced and cell-mediated LDL oxidation and increases the resistance of LDL to oxidation.[27,28] The in vivo antioxidant properties of this drug have also been shown in two independent, randomized, glibenclamide-controlled trials in type 2 diabetic patients.[18,26] In these studies, administration of gliclazide was found to effectively reduce the levels of plasma lipid peroxides in type 2 diabetic subjects. Although these clinical results argue for an antioxidant activity of gliclazide in the plasma compartment, our recent findings that this drug reduces vascular cell-mediated LDL oxidation also suggest that gliclazide may exert its antioxidant effects in atherosclerotic plaques. The exact mechanisms that may account for the antioxidant action of gliclazide are presently unknown. The reduced Cu^{2+}-induced oxidation of LDL in the presence of gliclazide suggests that this drug exerts its protective effect by metal chelation or alternatively by scavenging free radicals. In the case of monocyte-induced modification of LDL, one may postulate that most of the inhibitory effects of gliclazide may be related to the ability of this drug to decrease metal-catalyzed propagation reactions. Arguing for this possibility are the observations that gliclazide reduces to a similar extent LDL oxidation mediated by copper ions and monocytes and that monocytes oxidize LDL by enhancing the redox reaction of metal ions present in the medium.[29-31] The mechanism(s) by which gliclazide might protect LDL from endothelial cell-promoted oxidation is (are) unknown. Our finding that endothelial cells exhibit less sensitivity to gliclazide than monocytes suggests that different mechanisms may be involved in LDL oxidation in the two systems. A role for endothelial cell lipoxygenase has been postulated in the oxidative modification of LDL by endothelial cells.[32-36] Because bovine aortic endothelial cells used in the present study produce little superoxide and have high levels of superoxide dismutase, one major mechanism in the inhibitory effect of gliclazide on

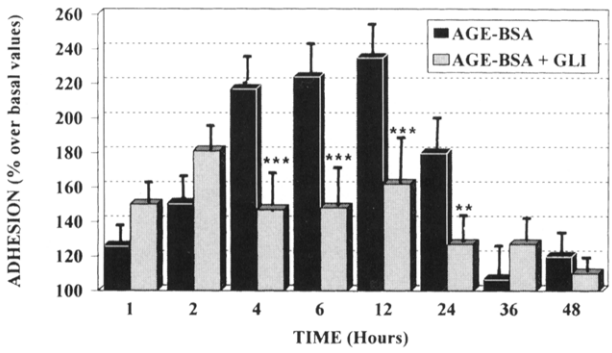

Fig 3. Effect of glycated albumin on human monocyte adhesion to endothelial cells. Inhibitory effect of gliclazide. Confluent endothelial cells were incubated with glycated albumin (AGE-BSA, 100 μg/mL) in the presence or absence of gliclazide (10 μg/mL) for 1 to 48 hours. At the end of the incubation period, monocyte adhesion to endothelial cells was measured by the MPO assay. Results were expressed as % over basal values. Data represent mean ± SEM of 3 different experiments. **$P < .01$; ***$P < .005$ versus AGE-BSA.

endothelial cell-induced LDL oxidation may therefore involve cellular lipoxygenases.

Endothelial cell-induced LDL oxidation is associated with increased monocyte adhesion to the endothelium.[37] Our results which show that gliclazide inhibits modified LDL-induced adhesiveness of endothelial cells to monocytes suggest that the antioxidative properties of this drug may be at least partly responsible for this inhibitory effect. It has been proposed that induction of adhesion molecules at the endothelial cell surface could be involved in the oxidized LDL-induced adhesiveness of endothelial cells to monocytes.[37,38] Whether the inhibitory effect of gliclazide on modified LDL-stimulated adhesion of monocytes to endothelium involves a reduction in these vascular cell adhesion molecules remains to be determined.

It has been previously shown that glycated albumin increases via an oxidant-sensitive mechanism the adhesiveness of endothelial cells for human lymphoblastic Molt-4 cells.[16] Our data provide the first evidence that gliclazide inhibits glycated albumin-induced human monocyte adhesion to cultured endothelial cells. Because interaction of AGE with their cell surface receptors induces oxidant stress, one may postulate that the inhibitory effect of gliclazide on glycated albumin-stimulated monocyte adhesion may be due to its antioxidant properties. Furthermore, because increased vascular cell adhesion molecule-1 expression occurs in glycated albumin-treated endothelial cells,[16] the suppressive effect of gliclazide on monocyte adhesion may be related to its ability to inhibit the induction of this adhesion molecule at the endothelial cell surface.

The clinical relevance of our in vitro findings is supported by our observations that short-term gliclazide administration to type 2 diabetic patients normalizes monocyte adhesion in these subjects. Our results show that monocytes isolated from type 2 diabetic patients bind to a greater extent to endothelial cells than control cells, and that lipid peroxide levels positively correlate with the degree of monocyte adhesiveness to endothelium in these patients. This suggests a key role of lipid peroxidation in the increased monocyte adhesiveness in type 2 diabetic patients. Although the antioxidant properties of gliclazide seem to be related to the presence of an aminoazabicyclooctane moiety in this sulfonylurea,[39] the molecular mechanisms by which this antioxidant drug inhibits monocyte adhesion remain to be determined. One possible mechanism would be an effect of gliclazide on some adhesion molecules expressed on the monocytic cell surface. Overexpression of CD11b/CD18 (Mac-1) has been documented in diabetic monocytes.[40] Pentoxifylline treatment reduces this enhanced antigen expression and decreases monocyte binding to endothelium.[41] Modified LDL has been identified as one major determinant of CD11b overexpression at the monocyte cell surface,[42] and gliclazide inhibits the enhancement of monocyte adhesion to endothelial cells induced by oxidized LDL.[27] Based on these findings, one may speculate that gliclazide, through its antioxidant properties, may inhibit the adhesion of diabetic monocytes by reducing CD11b expression at the monocyte cell surface.

In conclusion, our results show that gliclazide is effective in vitro in reducing both cell-mediated LDL oxidation and monocyte adhesion induced by oxidatively modified LDL and glycated albumin. They also show that short-term gliclazide administration to type 2 diabetic patients normalizes the excessive in vitro monocyte adhesion to endothelial cells. The clinical relevance of these findings remains to be determined in prospective long-term studies comparing cardiovascular events and mortality in type 2 diabetic patients treated with different compounds of sulfonylurea.

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