# Benefits of Gliclazide in the Atherosclerotic Process: Decrease in Monocyte Adhesion to Endothelial Cells

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**Atherosclerotic cardiovascular disease is the leading cause of premature death in patients with diabetes. Atherosclerosis is a chronic immune-mediated disease, the initiation, progression, and destabilization of which is driven and regulated by inflammatory cells. One critical event in the initiation of this vascular inflammatory disease is the adhesion of leukocytes to the activated endothelium and their migration into the vessel wall. These processes are mediated by the upregulation of adhesion molecules on endothelial cells (ECs) and an increased expression in the vascular wall of chemotactic factors to leukocytes. Monocyte binding to ECs is increased in diabetes. One major determinant of this alteration could be oxidative stress. Given the free-radical scavenging activity of gliclazide, we determined the ex vivo and in vitro effects of this drug on human monocyte binding to ECs and the molecular mechanisms involved in this effect. Our results demonstrate that short-term administration of gliclazide to patients with type 2 diabetes normalizes the levels of plasma lipid peroxides and monocyte adhesion in these subjects. Gliclazide (10 $\mu$g/mL) also reduces oxidized low-density lipoprotein (oxLDL)– and advanced glycation end product (AGE)–induced monocyte adhesion to ECs in vitro. The inhibitory effect of this drug on AGE-induced monocyte adhesion involves a reduction in EC adhesion molecule expression and inhibition of nuclear factor $\kappa$B (NF-$\kappa$B) activation. In addition, gliclazide inhibits oxLDL-induced monocyte adhesion to cultured human aortic vascular smooth muscle cells (HASMCs) in vitro and reduces the production of monocyte chemotactic protein-1 (MCP-1) by these cells. Taken collectively, these results show that gliclazide, at concentrations in the therapeutic range, inhibits ex vivo and in vitro monocyte adhesiveness to vascular cells. By doing so, this drug could reduce monocyte recruitment into the vessel wall and thereby contribute to attenuating the sustained inflammatory process that occurs in the atherosclerotic plaque. These findings suggest that treatment of diabetic patients with this drug may prevent or retard the development of vasculopathies associated with diabetes.**



IT IS NOW FIRMLY established that atherosclerosis is an immune-mediated inflammatory disease, the initiation of which is driven by the intimal recruitment of inflammatory cells, including monocytes and T lymphocytes, into the arterial wall.[1] This process requires the adhesion of these cells to the endothelium and their migration into the vessel wall. Mechanisms responsible for monocyte binding and recruitment include upregulation of endothelial cell (EC) adhesion molecules[2,3] and increased expression in the vessel wall of various chemotactic factors, including oxidized low-density lipoprotein (oxLDL)[4,5] and monocyte chemotactic protein-1 (MCP-1).[6,7] Diabetes is closely associated with the development of accelerated atherosclerosis.[8] Previous studies have demonstrated that patients with type 2 diabetes have elevated levels of circulating modified lipoproteins[9-11] and adhesion molecules,[12-17] and that monocytes isolated from these subjects exhibit enhanced adhesiveness to ECs.[18,19] One potential key determinant of increased monocyte–EC interactions in type 2 diabetes could be oxidative stress. Indeed, patients with diabetes are under increased oxidative stress[20,21] and many metabolic alterations associated with diabetes, including oxLDL and advanced glycation end products (AGE), enhance monocyte binding through an oxidant-sensitive mechanism.[22-24] Previous studies have demonstrated that gliclazide has free-radical scavenging activity[25] and that this drug effectively inhibits LDL oxidation in vitro[26,27] and reduces plasma lipid peroxide levels in patients with type 2 diabetes.[19,28] Given the potential key role of oxidative stress on monocyte–endothelium interaction, these data suggest that this drug may be effective in reducing monocyte adhesion to vascular cells. This review summarizes the results of our ex vivo and in vitro studies on the effect of gliclazide on monocyte binding to ECs and human aortic smooth muscle cells (HASMCs) and provides an insight into the molecular mechanisms involved in the effect of this drug on AGE-induced monocyte adhesion to endothelium.

## MATERIALS AND METHODS

### *Subjects*

Eight patients (4 women and 4 men) with type 2 diabetes and 8 healthy control subjects were studied. The patients were selected for poor diabetes control (glycated hemoglobin $\geq$ 9%), glibenclamide treatment, no decompensated cardiac or renal conditions, and absence of smoking. Their mean $\pm$ SD age (range) was 61 $\pm$ 5 years (55 to 70 years), body mass index (BMI) 29 $\pm$ 3 kg/m$^2$ (26 to 34 kg/m$^2$), duration of diabetes 10 $\pm$ 9 years (3 to 30 years), glycated hemoglobin 12% $\pm$ 1% (9.2% to 15%), and daily glibenclamide dose 16.5 $\pm$ 5.8 mg (5 to 20 mg). All were also treated with metformin. None of the patients was primarily insulin-dependent. Four patients were hypertensive and treated with angiotensin-converting enzyme (ACE) inhibitors, 4 were hypertriglyceridemic, 2 had macroangiopathy, and 5 had microangiopathy (retinopathy or microalbuminuria). Control subjects were matched with patients for sex and BMI. Subjects with infectious or inflammatory conditions or treated by anti-inflammatory or antioxidant drugs were excluded from the study. In this pilot study, the patients were switched for 3 months from glibenclamide to an equivalent hypoglycemic dose of gliclazide (5 mg of glibenclamide equivalent to 80 mg of gliclazide). Blood samples were obtained from

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control subjects and from patients before and after 3 months of treatment with gliclazide.

### *Reagents*

Dulbecco's minimal essential medium (DMEM), penicillin-streptomycin, RPMI-1640, and phosphate-buffered saline (PBS) were purchased from Gibco (Grand Island, NY), Fetal bovine serum (FBS) was obtained from Wisent (St Bruno, Quebec, Canada). Immunoglobulin-free bovine serum albumin (BSA) was obtained from Sigma chemicals (St Louis, MO). Gliclazide and glibenclamide were provided by Les Laboratoires Servier (France) and Hoechst (Canada), respectively.

### *LDL Isolation and Oxidation*

LDL was isolated from plasma of healthy normolipidemic subjects by sequential centrifugation according to the method of Hatch[29] and used within 2 days at a final concentration of 100 $\mu$g LDL protein/mL. Protein content in the LDL preparations was measured according to the Bradford method.[30] Oxidation of LDL was obtained by incubating native LDL (100 $\mu$g protein/mL) for 20 hours at 37°C in the presence of ECs or HASMCs in serum-free culture medium containing 3 $\mu$mol/L $CuSO_4$. The lipid peroxide content of oxLDL was determined by the thiobarbituric acid-reactive substances (TBARS) assay.[31]

### *Preparation and Characterization of Glycated Albumin*

Immunoglobulin-free BSA was glycated in vitro by incubation at 37°C for 4 weeks in a reaction mixture containing 0.5 mol/L glucose as described previously.[32]

### *EC and HASMC Culture*

Bovine aortic endothelial (BAE) cells were used to determine ex vivo adhesion of human monocytes. BAE cells, human aortic and umbilical vein endothelial cells (HUVECs), and HASMCs (Clonetics, San Diego, CA) were used to assess in vitro monocyte adhesion.

### *Human Monocyte Isolation and Monocyte Adhesion Assay*

Highly purified (85% to 90%) human monocytes were isolated from heparinized blood as described previously.[19,27] Quantification of adherent monocytes was undertaken by measuring monocyte myeloperoxidase (MPO) activity.[33]

### *Determination of EC-Associated Adhesion Molecule Expression*

EC surface expression of endothelial leukocyte adhesion molecule (ELAM-1), intercellular adhesion molecule (ICAM-1), and vascular cell adhesion molecule (VCAM-1) was determined by cellular enzyme-linked immunosorbent assay (ELISA) method.[32]

### *Reverse Transcription-Polymerase Chain Reaction*

The levels of ELAM-1, ICAM-1, and VCAM-1 mRNA expression were measured by semiquantitative polymerase chain reaction (PCR) as described previously.[32] PCR products were analyzed on a 1% agarose gel containing 1 $\mu$g/mL ethidium bromide.

### *Preparation of Nuclear Extracts*

Nuclear extracts were prepared as previously described.[32] Briefly, ECs were trypsinized and lysed. After centrifugation, the nuclei were washed, lysed, and collected by centrifugation. Supernatants were harvested and the protein concentration was determined.

### *Electrophoretic Mobility Shift Assay*

Nuclear extracts (5 $\mu$g) were incubated for 15 minutes in the presence of a binding buffer. Double-stranded oligonucleotides representing a portion of the human ELAM-1, ICAM-1, and VCAM-1 promoters containing an NF-$\kappa$B site were synthesized.[32] Oligonucleotides were end-labeled with $\gamma$-[$^{32}$P]adenosine triphosphate (ATP) and added to the samples. Samples were analyzed on a nondenaturating 4% polyacrylamide gel. The specificity of the nuclear protein binding was assessed by incubating the nuclear proteins with a labeled DNA probe in the presence of a 100-mol excess of nonlabeled DNA probe.

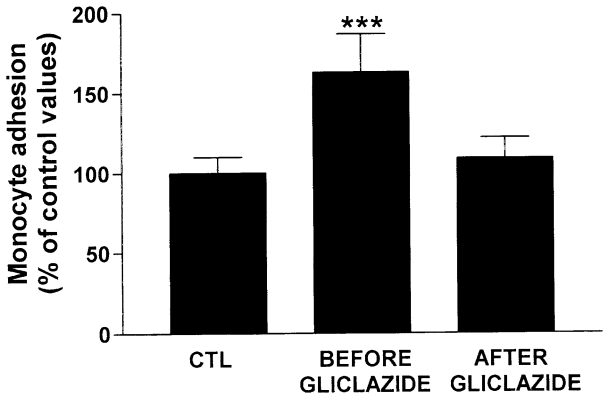


**Fig 1. Gliclazide administration to patients with type 2 diabetes decreases monocyte adhesion to cultured BAE cells. Results are expressed as % adhesion of control (CTL) values. Data represent the mean ± SEM. ***$P < .005$ *v* controls.**

### *Measurement of MCP-1 Protein*

The amount of human MCP-1 secreted by HASMCs was measured using a double-sandwich ELISA (R & D Systems, Minneapolis, MN).

### *Determination of Cell Viability*

Cell viability was estimated using trypan blue exclusion and was consistently found to be higher than 95%.

### *Statistical Analysis*

Statistical analysis of the results was performed by 1-way analysis of variance (ANOVA) followed by the Tukey's test. The Spearman rank correlation test was used to evaluate the correlation between lipid peroxide levels and monocyte adhesion to cultured ECs. Results were expressed as mean ± SEM.

## RESULTS

## Ex Vivo Studies

### *Effect of Gliclazide Administration in Patients With Type 2 Diabetes on Monocyte Adhesion to Cultured ECs*

Before gliclazide treatment, adhesion of monocytes of diabetic patients to BAE cells was markedly increased (Fig 1). This increase was positively correlated ($r = 0.74$, $P < .01$) with the levels of serum lipid peroxides measured in diabetic patients before gliclazide administration (nmol malondialdehyde/500 $\mu$L serum: diabetic patients, 9.6 ± 1.1; controls, 5.8 ± 0.6; $P < .005$). Gliclazide administration totally reversed these anomalies, lowering serum lipid peroxide levels (data not shown) and adhesion of monocytes isolated from the diabetic patients to levels identical to those observed in control subjects (Fig 1). In contrast, this treatment did not modify glycemic

control ($HbA_{1c}$: before gliclazide, 12.0% ± 1.0%; after gliclazide, 12.3% ± 1.0%).

## In Vitro Studies

*Effect of Gliclazide on oxLDL- and AGE-Induced Monocyte Adhesion to ECs*

Incubation of BAE cells with native LDL (100 μg protein/mL) in the presence of 3 μmol/L $Cu^{2+}$ resulted in a significant increase in the number of adherent monocytes to ECs (monocyte adhesion [% over basal values]: control [without LDL], 100 ± 10; LDL + 3 μmol/L $Cu^{2+}$, 280 ± 20; $P < .01$). Pretreatment of these cells with gliclazide (1 to 10 μg/mL) dose-dependently decreased oxLDL-induced monocyte adhesion (Fig 2A). In contrast, treatment of BAE cells with glibenclamide (0.5 μg/mL) did not affect this parameter (data not shown). Incubation of human aortic (data not shown) and HUVECs (Fig 2B) in the presence of glycated albumin (100 μg/mL) for 1 to 48 hours increased monocyte adhesion to these cells in a time-dependent manner. The maximal effect was observed from 4 to 12 hours. Nonglycated albumin, used as a control, did not significantly affect monocyte adhesion to ECs (data not shown). Pretreatment of HUVECs with gliclazide (10 μg/mL) reduced the stimulatory effect of glycated albumin on human monocyte adhesion to these cells (Fig 2B).

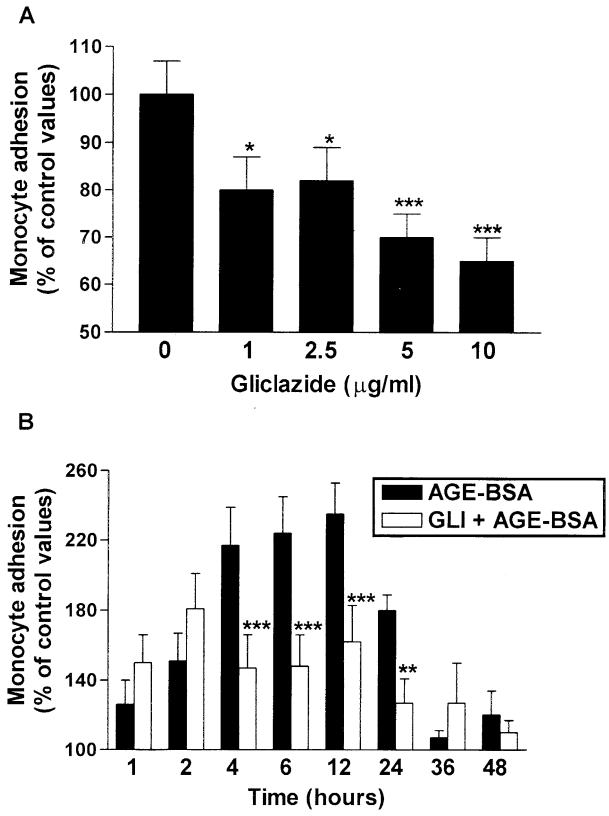


**Fig 2. Gliclazide inhibits oxLDL- and AGE-induced human monocyte adhesion to ECs. (A) BAE cells were treated for 20 hours with native LDL (100 μg/mL) in the presence or absence of increasing concentrations of gliclazide (1-10 μg/mL). Monocyte adhesion to ECs was measured by the myeloperoxidase (MPO) assay. Results are expressed as % of control values. Data represent the mean ± SEM of 7 different experiments. *$P < .05$; ***$P < .005$ *v* control. (B) HUVECs were treated with glycated albumin (advanced glycation end product–bovine serum albumin [AGE-BSA], 100 μg/mL) in the presence or absence of gliclazide (GLI, 10 μg/mL) for 1 to 48 hours. At the end of the incubation period, monocyte adhesion to stimulated ECs was measured by the MPO assay. Results are expressed as % over basal values. Data represent the mean ± SEM of 5 different experiments. **$P < .01$; ***$P < .005$ *v* AGE-BSA.**

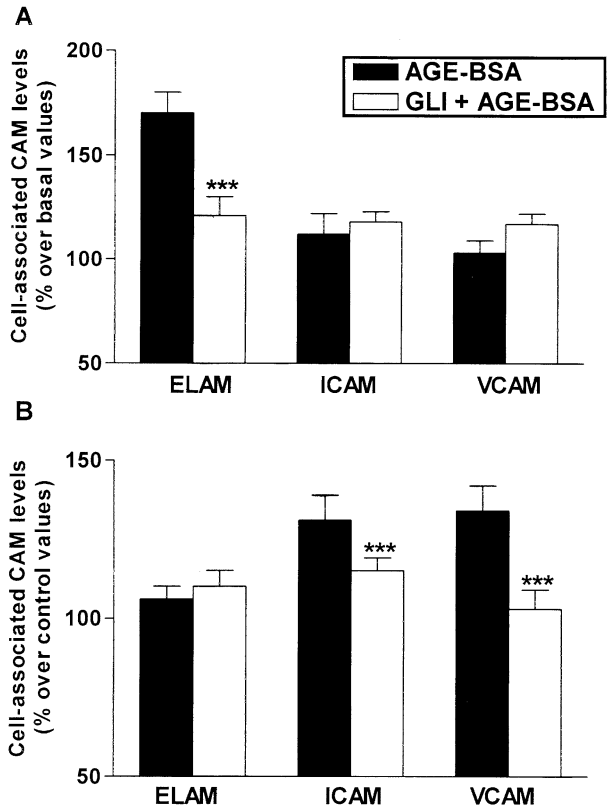


**Fig 3. Inhibitory effect of gliclazide on AGE-induced EC adhesion molecule (CAM) expression. HUVECs were treated for 4 hours (A) and 12 hours (B) with AGE-BSA (100 μg/mL) in the presence or absence of gliclazide (GLI, 10 μg/mL). At the end of the incubation period, EC-associated expression of adhesion molecules was measured. Results are expressed as % over basal values. Data represent the mean ± SEM of 3 different experiments. ***$P < .005$ *v* AGE-BSA.**

*Molecular Mechanisms Involved in the Inhibitory Effect of Gliclazide on AGE-Induced Monocyte Adhesion*

*Effect of gliclazide on EC adhesion molecule protein and gene expression induced by glycated albumin.* Studies of the time course of glycated albumin-mediated induction of ELAM-1 demonstrated an increase in cell-associated expression of this antigen after 4 hours of stimulation by glycated albumin (100 μg/mL) (Fig 3A). Induction of ELAM-1 fell to basal values by 12 hours (Fig 3B). Unlike ELAM-1, cell-associated ICAM-1 and VCAM-1 antigens were not significantly enhanced until 12 hours after treatment of HUVECs with glycated albumin (Fig 3B). Gliclazide markedly reduced the glycated albumin–mediated induction of ELAM-1, ICAM-1,

and VCAM-1 antigens (Fig 3A and B). Treatment of HUVECs with glycated albumin (100 $\mu$g/mL) for 4 hours resulted in a significant increase in ELAM-1 mRNA levels. Induction of ICAM-1 and VCAM-1 mRNA levels was observed after 12-hour exposure of ECs to glycated albumin (Fig 4A). Gliclazide (10 $\mu$g/mL) significantly decreased the induction of ELAM-1, ICAM-1, and VCAM-1 mRNA expression in HUVECs treated with glycated albumin (Fig 4A).

*Effect of gliclazide on the DNA-binding activity for NF-κB in the ELAM-1, ICAM-1, and VCAM-1 promoters induced by glycated albumin.* Incubation of HUVECs with glycated albumin (100 $\mu$g/mL) increased the binding of nuclear proteins to the NF-κB consensus sequence of the ELAM-1, ICAM-1, and VCAM-1 promoter region (Fig 4B). This effect was significantly decreased by gliclazide (Fig 4B).

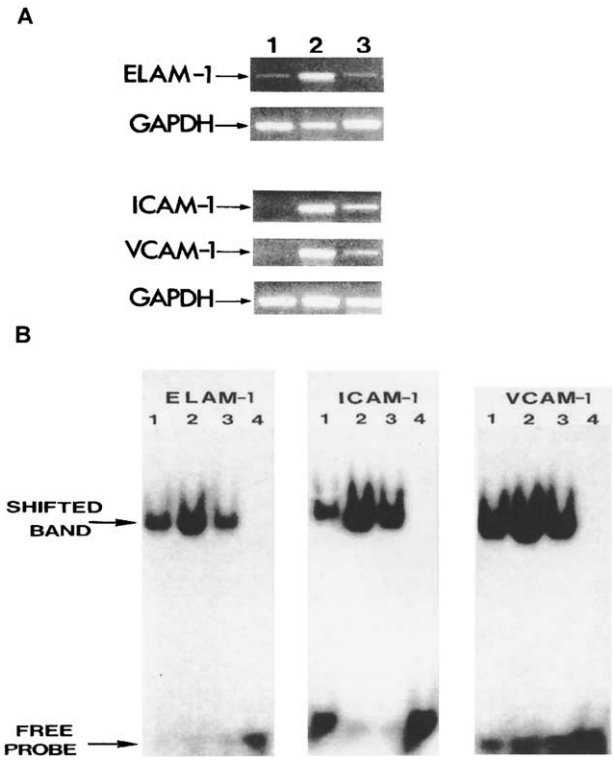


**Fig 4. Gliclazide inhibits AGE-induced EC adhesion molecule mRNA levels and NF-κB activation. (A) HUVECs were treated for 4 hours (ELAM-1) or 12 hours (ICAM-1 and VCAM-1) with nonglycated albumin (control bovine serum albumin [CTL-BSA], 100 $\mu$g/mL) or glycated albumin (AGE-BSA, 100 $\mu$g/mL) in the presence or absence of gliclazide (10 $\mu$g/mL). At the end of the incubation period, mRNA levels of ELAM-1, ICAM-1, and VCAM-1 were measured by reverse-transcription PCR. Data represent the results of one representative experiment. Lane 1: CTL-BSA–treated cells; lane 2: AGE-BSA–treated cells; lane 3: AGE-BSA + gliclazide-treated cells. (B) HUVECs were treated for 4 hours (ELAM-1) or 12 hours (ICAM-1 and VCAM-1) with nonglycated albumin (CTL-BSA, 100 $\mu$g/mL) or glycated albumin (AGE-BSA, 100 $\mu$g/mL) in the presence or absence of gliclazide (10 $\mu$g/mL). Nuclear protein binding to the NF-κB binding motif located in the ELAM-1, ICAM-1, and VCAM-1 promoters was determined by electrophoretic mobility shift assay. Data represent the result of 1 representative experiment out of 3. Lane 1: CTL-BSA–treated cells; lane 2: AGE-BSA–treated cells; lane 3: AGE-BSA + gliclazide-treated cells; lane 4: competition of the AGE-BSA–induced binding complex in the presence of 100-fold molar excess of the unlabeled NF-κB oligonucleotide.**

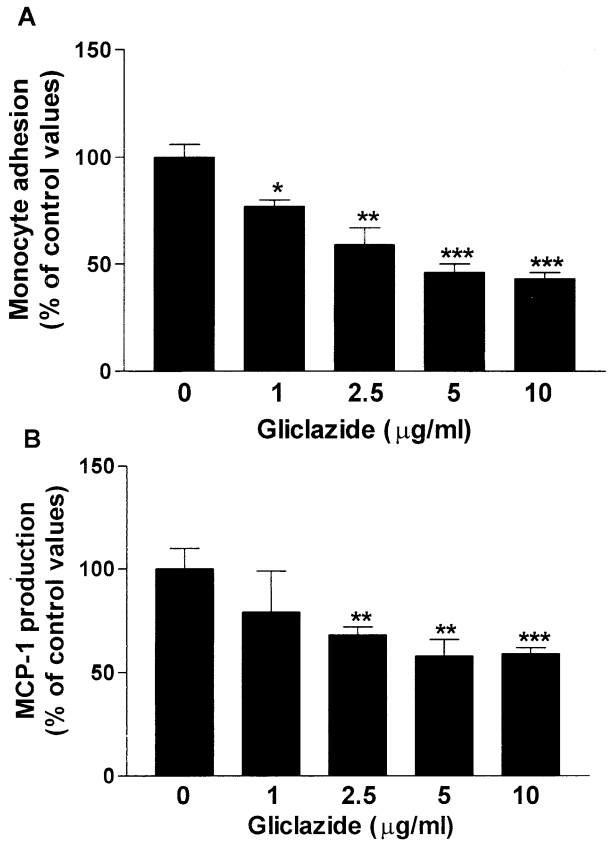


**Fig 5. Inhibitory effect of gliclazide on oxLDL-induced monocyte adhesion to HASMCs and MCP-1 secretion by HASMCs. (A) HASMCs were exposed for 24 hours at 37°C to native human LDL (100 $\mu$g/mL) in medium, containing 3 $\mu$mol/L $Cu^{2+}$ in the presence of increasing concentrations of gliclazide (1-10 $\mu$g/mL). At the end of the incubation period, human monocytes were added to cultured HASMCs. Monocyte adhesion to HASMCs was measured by the MPO assay. Data represent the mean ± SEM of 4 independent experiments. *$P$ < .05; **$P$ < .01; and ***$P$ < .001 *v* control. (B) HASMCs were incubated with native LDL (100 $\mu$g/mL) for 24 hours in the presence of increasing concentrations of gliclazide (1-10 $\mu$g/mL). At the end of the incubation period, supernatants were collected and centrifuged. MCP-1 levels in the culture media were determined by ELISA. Data represent the mean ± SEM of 4 different experiments. **$P$ < .01; and ***$P$ < .001 *v* control.**

### *Effect of Gliclazide on oxLDL-Induced Monocyte Adhesion and MCP-1 Secretion in HASMCs*

Incubation of HASMCs with smooth muscle cell–mediated oxLDL induced a significant increase in monocyte adhesion (170% ± 22% over basal values, $P < .05$) and MCP-1 production by these cells (175% ± 12% over basal values, $P < .05$). Pretreatment of these cells with gliclazide (1 to 10 $\mu$g/mL) decreased, in a dose-dependent manner, oxLDL-induced monocyte adhesion and MCP-1 production (Fig 5A and B).

## DISCUSSION

Increased monocyte–EC interactions play a crucial role in the initiation and progression of the deleterious, excessive, and chronic inflammatory responses in the arterial wall that are characteristic of atherosclerotic disease. Risk factors for atherosclerosis, including diabetes, increase monocyte adhesiveness to endothelium.[18,19] If the hypothesis that reduction of this event is associated with decreased cardiovascular risk proves to be correct in humans, then modulation of mononuclear cell recruitment may represent a suitable target for control of atherogenesis in diabetic subjects. Consistent with this possibility, a reduction in monocyte recruitment by administration of antibodies against adhesion molecules and MCP-1 has been associated with decreased atherosclerosis in experimental animal models.[34-36] Our present results demonstrate that gliclazide, a second-generation sulfonylurea with antioxidant properties, inhibits ex vivo and in vitro monocyte adhesion to endothelium and reduces oxLDL-induced secretion of MCP-1 by smooth muscle cells. Our findings that lipid peroxide levels positively correlate with the degree of monocyte adhesiveness to ECs in patients with type 2 diabetes and that gliclazide totally reverses the enhancement of both lipid peroxides and monocyte adhesion in these subjects suggest that this drug may reduce monocyte adhesiveness in these patients through its antioxidant properties. In contrast, our observation that gliclazide normalizes monocyte adhesion in patients with diabetes without changing glycemic control supports the notion that gliclazide exerts this effect independently of its metabolic effects. However, it remains to be determined whether these results obtained in subjects with poorly controlled diabetes apply to patients with better glycemic control. The molecular mechanisms by which this antioxidant drug normalizes monocyte adhesion to unstimulated cultured ECs, in diabetic patients, remain to be determined. One possible mechanism would be an inhibitory effect of gliclazide on some adhesion molecules overexpressed on the diabetic monocytic cell surface such as CD11b.[37] Alternatively, because gliclazide inhibits LDL oxidation in vitro,[26,27] this effect may be related to the ability of gliclazide to protect LDL against oxidation, a process associated with increased monocyte adhesion.[38-41]

EC activation/dysfunction is unequivocally considered as a critical event in atherogenesis and is closely associated with the upregulation of specific adhesion molecules that are required for leukocyte binding to vascular endothelium. EC activation occurs in patients with diabetes, as reflected by the increased plasma concentrations of soluble adhesion molecules in these subjects.[12-17] One potential key determinant that may prime the endothelium of diabetic patients for increased adhesion of circulating monocytes is oxidative stress. Indeed, patients with diabetes are under increased oxidative stress,[20,21] a process involved in EC activation by oxLDL and AGE.[22-24,42,43] Our results demonstrating that therapeutic doses of gliclazide, but not of glibenclamide, which lacks antioxidant activity, inhibit oxLDL- and AGE-stimulated adhesion of monocytes to endothelium suggest that gliclazide may act through its antioxidant properties to reduce monocyte adhesion. This inhibitory effect of gliclazide may involve a reduction in specific EC adhesion molecules involved in oxLDL- and AGE-induced monocyte binding to ECs.[22-24] Our data showing that gliclazide reduces AGE-induced ELAM-1, ICAM-1, and VCAM-1 expression, both at the gene and protein levels, confirm this hypothesis. Our observation that gliclazide inhibits AGE-induced DNA binding activity for NF-$\kappa$B in the promoters of these antigens further suggests that the antioxidants properties of this drug are responsible for the decreased expression of these adhesion molecules. Whether other genes expressed on activated ECs that share a binding site for this oxidative-sensitive transcription factor may be downregulated by gliclazide remains to be investigated. In contrast to ECs, smooth muscle cells do not show increased expression of VCAM-1 and ICAM-1 in response to oxLDL.[44] Our results, which demonstrate that gliclazide reduces monocyte binding to these cells, clearly indicate that this drug may inhibit monocyte binding to vascular cells through different pathways. Because oxLDL is a key determinant of IG9 monocyte adhesion molecule overexpression in smooth muscle cells,[45] an effect of gliclazide on this new adhesion molecule may be postulated. Chemokine secretion by vascular cells most likely acts cooperatively with adhesion molecules to magnify the recruitment of inflammatory cells to lesion sites. Compelling evidence for the critical role of chemokines in the initiation of atherosclerosis is provided by the observation that absence of MCP-1, the most potent and specific chemotactic and activating factor for monocytes, results in dramatic protection from monocyte recruitment and atherosclerotic lesion formation in mice.[46,47] Our data demonstrate that gliclazide inhibits oxLDL-induced MCP-1 secretion by HASMCs. On the basis of the free radical scavenging activity of this drug and its ability to inhibit NF-$\kappa$B activation, it is tempting to postulate that gliclazide may exert this effect by interfering with oxidative stress and/or NF-$\kappa$B–dependent signaling pathways. Overall, these results show that gliclazide reduces monocyte–vascular cell interactions and MCP-1 secretion. By doing so, this drug may contribute to decreasing monocyte recruitment into the subendothelial space and thereby may help slow the course of the atherosclerotic process. Studies aimed at examining whether gliclazide administration in patients with type 2 diabetes may prevent or attenuate the development of atherosclerosis are currently under way.

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