Gliclazide Decreases Vascular Smooth Muscle Cell Dysfunction Induced by Cell-Mediated Oxidized Low-Density Lipoprotein

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Accumulating evidence indicates that oxidative modification of low-density lipoprotein (LDL) plays an important role in vascular dysfunction associated with diabetes mellitus. The aim of the present study was to investigate the effect of gliclazide, a second-generation sulfonylurea with free-radical-scavenging activity, on human aortic smooth muscle cell (HASMC)-mediated LDL oxidation and HASMC dysfunction induced by oxidatively modified LDL. Incubation of HASMCs with native human LDL (100 μg/mL) in the presence of increasing concentrations of gliclazide (1 to 10 μg/mL) resulted in a dose-dependent decrease in HASMC-mediated LDL oxidation. Exposure of HASMCs to gliclazide (1 to 10 μg/mL) and native LDL (100 μg/mL) also led to a dose-dependent decrease in oxidized LDL-induced human monocyte adhesion to HASMCs. In addition, incubation of HASMCs with gliclazide dramatically reduced the ability of oxidized LDL to stimulate the proliferation of these cells. Finally, treatment of HASMCs with gliclazide resulted in a marked decrease in oxidatively modified LDL-induced monocyte chemoattractant protein (MCP)-1 and human heat shock protein 70 (hsp 70) expression, both at the gene and protein levels. These results show that gliclazide, at concentrations in the therapeutic range (5 to 10 μg/mL), is effective in vitro in reducing vascular smooth muscle cell (VSMC) dysfunction induced by oxidatively modified LDL. These observations suggest that administration of gliclazide to type 2 diabetic patients could form part of the strategy for the prevention and management of diabetic cardiovascular diseases.

BY THEIR ABILITY to generate free radicals, vascular cells oxidize low-density lipoprotein (LDL) within the subendothelial space.[1-3] Increasing evidence implicates oxidized LDL in vascular dysfunction associated with the pathogenesis of atherosclerosis. Indeed, oxidatively modified LDL enhances monocyte binding to endothelial cells,[4] favors foam cell formation,[5] induces cytokine and chemokine production by vascular cells,[6-7] and stimulates vascular smooth muscle cell (VSMC) proliferation.[8-9] The so-called oxidation hypothesis of atherogenesis provides a strong biologic plausibility for the role of antioxidants in the prevention of cardiovascular diseases. Although data from observational studies suggest that vitamin E protects against coronary heart disease,[10-11] most randomized, controlled trials did not show any efficacy of antioxidant supplements in secondary prevention of coronary heart disease.[12-15]

Increased levels of modified lipoproteins and enhanced oxidation of plasma LDL have been reported in diabetic patients.[16-17] Imbalance between free radical production and antioxidant defense mechanisms has been proposed as a key mechanism leading to increased susceptibility of LDL to oxidative modification in diabetes.[18-19] Gliclazide, a second-generation sulfonylurea used in the treatment of type 2 diabetes, has free-radical-scavenging activity.[20-21] This drug has been previously shown to inhibit LDL oxidation in vitro[22] and to reduce both endothelial and monocyte cell-mediated LDL oxidation and monocyte adhesion to endothelial cells in vitro.[23] We have shown that administration of gliclazide to type 2 diabetic patients inhibits the increased adhesiveness of monocytes isolated from these subjects to endothelial cells and lowers monocyte tumor necrosis factor alpha (TNFα) production.[24] Evidence has finally been obtained for an inhibitory effect of gliclazide on the induction of endothelial cell adhesion molecule and nuclear factor-kappa B (NF-κB) activity by glycated albumin.[25] Based on these findings and on the role of reactive oxygen species in the alterations of VSMC function,[26-27] we evaluated in the present study the effect of gliclazide on VSMC dysfunction induced by oxidized LDL.

MATERIALS AND METHODS

Reagents

Smooth muscle cell growth medium (SmGM-2) was obtained from Clonetics (San Diego, CA). Penicillin-streptomycin, phosphate-buffered saline (PBS), Hanks' Balanced Salt Solution (HBSS), sodium dodecyl sulfate (SDS), RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), and bovine serum albumin (BSA) were purchased from GIBCO (Grand Island, NY). Thiobarbituric acid (TBA) and tetraethoxypropane (TEP) were obtained from ICN Biochemicals (Costa Mesa, CA). Fetal bovine serum (FBS) was purchased from Wisent (St Bruno, Quebec, Canada). Dianisidine dihydrochloride, hexadecyltrimethylamine ammonium bromide (HTAB), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemicals (St Louis, MO). Phosphoric acid, Tween 20, ethanol, potassium bromide (KBr), methanol, and butanol were obtained from Fisher Scientific (Fair Lawn, NJ). Acetic acid and perchloric acid were purchased from Laboratoire Mat (Beauport, Quebec, Canada) and BDH (Toronto, Ontario, Canada), respectively. [Methyl-^{3}H]-thymidine was obtained from NEN Life Science Products (Mississauga, Ontario, Canada). Monoclonal antibody to human heat shock protein 70 (hsp 70) was obtained from Stressgen Biotechnologies (Victoria, British Columbia, Canada).

Isolation of Human LDL

LDL was isolated from plasma obtained from healthy nonsmoker normolipidemic male and female donors. Venous blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) and

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LDL (density, 1.019 to 1.063) was isolated by sequential ultracentrifugation using KBr for density adjustements.[28] LDL was sterilized by filtration through 0.20-μm Gelman filters, stored at 4°C and used within 3 days at a final concentration of 100 μg LDL protein/mL. Before incubation with cells, LDL was extensively dialyzed for 24 hours at 4°C against 5 mmol/L Tris/50 mmol/L NaCl to remove EDTA. Minimally modified LDL (100 μg/mL), obtained by storage of EDTA-free LDL at 4°C for 3 months, was used as positive control. Protein content of LDL preparations was determined using the Bradford method[29] with BSA as standard. Endotoxin content of LDL preparations (100 μg/mL) was determined by the Limulus amebocyte lysate assay (Sigma) and was consistently found to be lower than 3 pg/mL.

Smooth Muscle Cell Culture

Human aortic smooth muscle cells (HASMCs) were obtained from Clonetics. HASMCs were grown to subconfluence in SmGM-2 at 37°C in 5% CO_2/95% air atmosphere. The cells were then trypsinized and subcultured in 24- or 96-well culture plates according to the appropriate assay conditions.

Drug Treatment

Sodium salt gliclazide was supplied by Les Laboratoires Servier (Neuilly, France). The drug was dissolved in pyrogen-free sterile water to produce a stock solution of 1 mg/mL, which was then filtered and stored at 4°C. To assess the effect of gliclazide on VSMC-mediated LDL oxidation, the cells were pretreated with increasing concentrations of gliclazide (1 to 10 μg/mL). These concentrations were chosen because they are in the therapeutic range in diabetic patients.[30]

Determination of LDL Oxidative Modification

Oxidatively modified LDL were obtained by incubating LDL preparations for 24 hours at 37°C in a cell-free system or in the presence of HASMCs in culture medium containing 3 μmol/L $CuSO_4$. At the end of the incubation period, supernatants were collected and EDTA (0.04% final concentration) was added to stop LDL oxidation. LDL oxidation was assessed by electrophoretic mobility of this lipoprotein (data not shown). The lipid peroxide content of oxidized LDL was determined by measuring thiobarbituric acid-reactive substances (TBARS) in the supernatant. TBARS values were expressed as malondialdehyde (MDA) equivalents (nmol MDA/500 μL solution) as derived by comparison to a standard curve of MDA equivalents prepared from HCl-catalyzed hydrolysis of TEP, as previously described.[31] Free 8-isoprostane levels were determined in the culture medium by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI).[32] The detection limit of 8-isoprostane with this assay is 5 pg/mL. The intra- and interassay coefficients of variation are less than 10%.

Isolation of Human Monocytes

Human monocytes were isolated from fresh heparinized blood (100 mL) collected from nonsmoker healthy male and female donors as previously described.[33] First, peripheral blood mononuclear cells were obtained by density centrifugation using Lymphoprep (Nycomed Pharma As, Oslo, Norway). The cells collected from the interface were washed 3 times with HBSS and allowed to aggregate in the presence of FBS. After further purification by rosetting technique and density centrifugation, recovery of highly purified monocytes (85% to 90%), as assessed by fluorescence-activated cell sorter analysis, was obtained. Human monocytes were resuspended in serum-free RPMI 1640 medium supplemented with 1% (vol/vol) penicillin-streptomycin.

Monocyte Adhesion Assay

On the day of the assay, the medium of confluent HASMCs was gently removed and the cells were washed twice with HBSS. A total of 230,000 highly purified human monocytes were then added to the wells and allowed to adhere to HASMCs for 2 hours. At the end of this incubation period, nonadherent monocytes were removed by washing the cells with PBS (pH 6.0). Monocyte adhesion to HASMCs was quantitated by measuring monocyte myeloperoxidase (MPO) activity as previously described.[34] The intra- and interassay coefficients of variation of this assay are 7% and 13%, respectively.

VSMC Proliferation Assay

HASMCs were trypsinized and cultured at a density of 7,500 cells/cm^2 in SmGM-2. After 24 hours, cultured cells were washed with PBS and growth-arrested for 48 hours by serum deprivation. HASMCs cultured in serum-free DMEM containing 3 μmol/L $CuSO_4$ were then treated with native LDL in the presence or absence of gliclazide for 48 hours at 37°C, with inclusion of sterile [methyl-^{3}H]-thymidine (5 μCi/mL) during the last 24 hours of incubation. Nonincorporated [^{3}H]-thymidine was removed by washing the cells with cold PBS. Cells were then fixed with cold ethanol-acetic acid solution (3:1) for 10 minutes at 4°C, washed twice, and incubated with perchloric acid 0.5 N for 15 minutes at 4°C. After further washing, HASMCs were incubated with perchloric acid 0.5 N for 30 minutes at 80°C and allowed to detach. The level of [^{3}H]-thymidine incorporation was determined by scintillation counting (Packard, Meriden, CT). The intra- and interassay coefficients of variation of this assay are less than 10%.

RNA Isolation and cDNA Preparation

Subconfluent HASMCs were treated or not with native LDL in the presence or absence of gliclazide for 24 hours at 37°C. Supernatants were then collected and added to a culture of growth-arrested HASMCs for a 4-hour incubation period. At the end of this incubation time, the cells were lysed with Trizol reagent, and total cytoplasmic RNA was extracted by the acid-phenol technique of Chomczynski,[35] precipitated, and resuspended in diethyl pyrocarbonate water. cDNA was synthesized from RNA by incubating total cellular RNA with 0.1 μg oligodT (Amersham Pharmacia, Piscataway, NJ) for 5 minutes at 98°C. The mixture was then incubated for 60 minutes at 37°C and for 10 minutes at 99°C in reverse transcription mixture (Boehringer Mannheim, Laval, Quebec).

Measurement of Monocyte Chemoattractant Protein-1 and hsp 70 mRNA Expression

The levels of monocyte chemoattractant protein (MCP)-1 and hsp 70 mRNA in untreated and LDL-treated HASMCs in the presence or absence of gliclazide were assessed by polymerase chain reaction (PCR). cDNA was amplified by using 2 synthetic primers specific for human MCP-1 (5′-TGCTCATAGCAGCCACCTTC-3′) (5′-GCTTGTCCAGGTGGTCCATG-3′) and hsp70 (5′-TGCCGGCCTACTTCAACGAC-3′) (5′-CCAGCCTGTTGTCAAAGTCC-3′) in separate PCR reaction mixtures (Boehringer Mannheim). A 268-bp human MCP-1 and a 275-bp human hsp 70 cDNA fragment were amplified enzymatically by 35 repeated cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes in a programmable thermal controller (PTC-100; MJ Research, Watertown, MA). A 456-bp of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was also amplified by 30 amplification cycles at 98°C for 40 seconds, 60°C for 40 seconds, 72°C for 90 seconds using 2 synthetic primers specific (5′-CCCTTCATTGACCTCAACTACATGG-3′) (5′-AGTCTTCTGGGTGGCAGTGATGG-3′) for human GAPDH. The reaction products were visualized by electrophoresis on a 1% agarose gel containing 1 μg/mL ethidium bromide. A 100-bp DNA ladder (Fermentas, Flamborough, Ontario, Canada) was run to generate size markers. The integrated absorbance of the bands was measured with an image analysis scanning system (Alpha Imager 2000, Packard).

Measurement of MCP-1 Protein

Subconfluent HASMCs were treated or not with native LDL in the presence or absence of gliclazide for 24 hours at 37°C. Supernatants were then collected and added to a culture of growth-arrested HASMCs for 24 hours. At the end of this incubation, the amount of human MCP-1 secreted by HASMCs in the culture medium was measured using a double-sandwich enzyme-linked immunosorbent assay (ELISA) (R & D Systems, Minneapolis, MN). The minimum detectable concentration of MCP-1 with this assay was typically less than 5.0 pg/mL. The intra- and interassay coefficients of variation of this assay are less than 0.5% and 10%, respectively.

Measurement of hsp 70 Protein

Hsp 70 protein levels in HASMCs were detected by immunoblotting. Briefly, subconfluent HASMCs were treated or not for 24 hours at 37°C with native LDL in the presence or absence of gliclazide. Supernatants were then collected and added to a culture of growth-arrested HASMCs for 24 hours. At the end of this incubation, cells were lysed in Tris buffer containing 3% SDS, 1 mmol/L PMSF, and 2% β-mercaptoethanol. Ten micrograms of total cellular proteins were separated by electrophoresis through a 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane using a trans blot cell system (Bio-Rad, Mississauga, Ontario, Canada). The membrane was blocked for 1 hour at room temperature with PBS containing 3% BSA. After 3 washes with PBS/Tween 20 0.1%, the membrane was incubated for 1 hour at room temperature with a human monoclonal antibody to hsp 70 (1:1,000) in PBS/Tween. The membrane was next washed with PBS/Tween and incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated goat antimouse IgG (1:5,000). Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham Pharmacia, Baie d'Urfe, Quebec, Canada).

Determination of Cell Viability

To evaluate a possible cellular toxicity of gliclazide at the maximal concentration used in the study, cell viability was determined by trypan blue exclusion. It was consistently found to be higher than 90%.

Statistical Analysis

Statistical analysis of the results was performed by 1-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Differences were considered to be of statistical significance at $P < .05$. Results are expressed as the mean ± SEM.

RESULTS

Effect of Gliclazide on the Oxidative Modification of LDL Mediated by HASMCs

Incubation of HASMCs with native human LDL (100 μg protein/mL) in the presence of 3 μmol/L Cu^{2+} for 24 hours at 37°C induced oxidative modification of LDL, as assessed by the significant increase in TBARS content and 8-isoprostane levels in the incubation medium. Medium TBARS content and 8-isoprostane levels in the presence of HASMCs was 9.0 ± 3 MDA equivalents/500 μL medium and 212 pg/mL, respectively, whereas TBARS production and 8-isoprostane levels in a cell-free system was 5.28 ± 1 MDA equivalents/500 μL medium and 66 pg/mL, respectively ($P < .05$). Pretreatment of HASMCs with increasing concentrations of gliclazide (1 to 10 μg/mL) before addition of native LDL to the culture medium decreased TBARS formation and 8-isoprostane levels in a dose-dependent manner (Fig 1A and 1B). The maximal decrease in these parameters was observed at a concentration of 5 μg/mL of the drug.

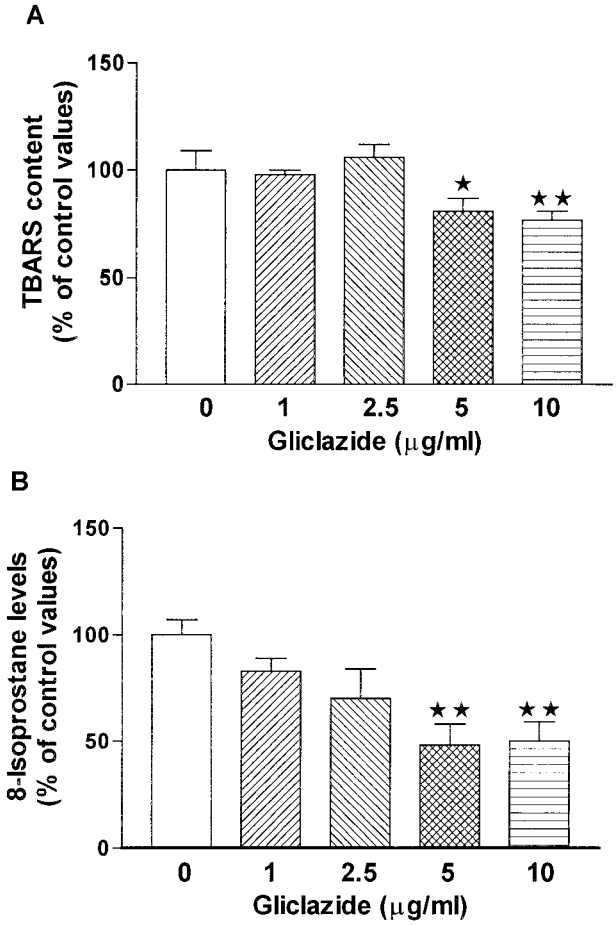

Fig 1. Gliclazide decreases LDL oxidation mediated by HASMCS. HASMCs were incubated for 24 hours at 37°C with native human LDL (100 μg/mL) in medium containing 3 μmol/L Cu^{2+} in the presence of increasing concentrations of gliclazide (0 to 10 μg/mL). At the end of the incubation period, supernatants were collected and oxidation of LDL was stopped by addition of EDTA (0.04% final concentration). Oxidative modification of LDL was assessed by measuring TBARS content (A) and 8-isoprostane levels (B) in the supernatant. Data represent the mean ± SEM of 6 separate experiments. *$P < .05$ and **$P < .01$ *v* control.

Effect of Gliclazide on Human Monocyte Adhesion to HASMCs Induced by Cell-Mediated Oxidized LDL

Incubation of HASMCs with native LDL (100 μg protein/mL) in medium containing 3 μmol/L Cu^{2+} for 24 hours at 37°C induced a significant increase in monocyte adhesion (170% ± 22% over control values, $P < .05$). This effect was similar to that induced by minimally oxidized LDL (169% ± 31% over control values, $P < .05$). Preincubation of HASMCs with increasing concentrations of gliclazide (1 to 10 μg/mL) for 1 hour before addition of human monocytes decreased, in a dose-dependent fashion, oxidized LDL-induced monocyte adhesion. Maximal decrease was about 50% and was observed at a concentration of 5 μg/mL of gliclazide (Fig 2). In the absence

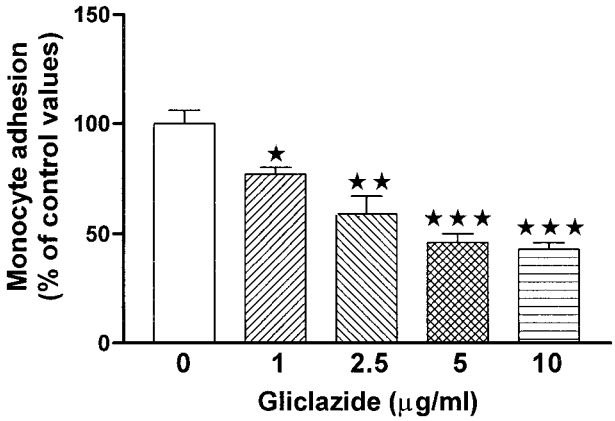

Fig 2. Gliclazide decreases monocyte adhesion to HASMCs induced by oxidized LDL. HASMCs were exposed for 24 hours at 37°C to native human LDL (100 μg/mL) in medium containing 3 μmol/L Cu^{2+} in the presence of increasing concentrations of gliclazide (0 to 10 μg/mL). At the end of the incubation period, cells were washed and freshly isolated human monocytes were added to cultured HASMCs. Monocyte adhesion to HASMCs was measured by the MPO assay. Data represent the mean ± SEM of 4 separate experiments. *$P < .05$, **$P < .01$, and ***$P < .001$ *v* control.

of LDL, no inhibitory effect of gliclazide (5 μg/mL) on monocyte adhesion was observed.

Effect of Gliclazide on VSMC Proliferation Induced by Modified LDL

Treatment of HASMCs with native LDL for 48 hours at 37°C in the presence of 3 μmol/L Cu^{2+} significantly increased VSMC proliferation (VSMC proliferation [% over basal values], 176 ± 13, $P < .01$). Exposition of cells to minimally oxidized LDL also led to an increase in VSMC proliferation (299% ± 80% over control values, $P < .05$). Preincubation of HASMCs for 1 hour with gliclazide (1 to 10 μg/mL) dramatically decreased the ability of oxidized LDL to stimulate HASMCs growth. Gliclazide-induced inhibition of HASMC proliferation was maximal at a concentration of 1 μg/mL gliclazide and was about 50% (Fig 3). Gliclazide (1 μg/mL) alone did not decrease HASMCs growth (data not shown).

Effect of Gliclazide on Oxidized LDL-Induced MCP-1 and hsp 70 mRNA Expression and Secretion in HASMCs

Incubation of HASMCs with smooth muscle cell-mediated oxidatively modified LDL increased MCP-1 mRNA levels in these cells (Fig 4A). A significant increase in MCP-1 release in the media was also observed after exposition of the cells to smooth muscle cell-mediated oxidized LDL (MCP-1 production [% over basal values], 175 ± 12, $P < .05$). This increase was similar to that observed in cells incubated with minimally modified LDL (216% ± 57% over control values, $P < .05$). Treatment of HASMCs with gliclazide led to a significant decrease in oxidized LDL-induced MCP-1 mRNA expression (Fig 4A). Reduction in MCP-1 mRNA levels was associated with a decrease in MCP-1 production, as reflected by the dose-dependent inhibitory effect of gliclazide on MCP-1 released by oxidized LDL-treated HASMCs (Fig 4B). Incubation of HASMC with smooth muscle cell-mediated oxidized LDL also enhanced hsp 70 mRNA levels in these cells (Fig 5A). A similar stimulatory effect was observed when cells were exposed to minimally oxidized LDL (data not shown). Treatment of HASMCs with gliclazide inhibited cell-mediated oxidized LDL-induced hsp 70 expression both at the gene (Fig 5A) and protein (Fig 5B) levels. Gliclazide alone did not affect MCP-1 or hsp 70 expression (data not shown).

DISCUSSION

Oxidative modification of intimal lipoproteins by reactive oxygen species released by vascular cells plays an important role in the early development of atherosclerosis.[36-37] In addition, oxidized lipoproteins favor, through their effects on VSMCs, the progression of early atherosclerotic plaques to fibrotic lesions.[38] Our results show that gliclazide decreases VSMC-mediated oxidative modification of LDL and reduces VSMC dysfunction induced by modified LDL. The reduction of VSMC-mediated LDL oxidation in the presence of gliclazide is in accordance with our previous results showing that this drug inhibits in vitro both endothelial cell- and monocyte-mediated LDL oxidation.[23] Although the mechanism(s) involved in the inhibitory effect of gliclazide on VSMC-mediated LDL oxidation is (are) unknown, the similar sensitivity to gliclazide of aortic endothelial cells and smooth muscle cells suggests that similar mechanisms may operate in both systems. Because a role for both superoxide and lipoxygenase has been postulated in the oxidative modification of LDL by VSMCs,[39-41] gliclazide may reduce LDL oxidation by scavenging superoxide and/or reducing lipoxygenase activity.

Several studies have shown that VSMCs express cellular adhesion molecules[42-44] and that monocytes bind to these cells. In accordance with the results of Thorne et al,[45] our data show that oxidized LDL enhances monocyte adhesiveness to

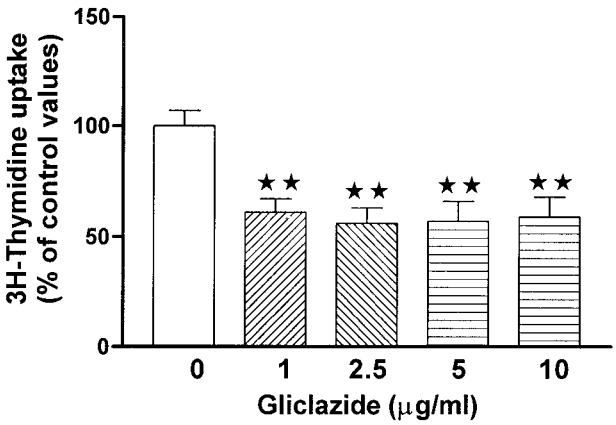

Fig 3. Gliclazide decreases HASMC proliferation induced by oxidized LDL. HASMCs cultured in serum-free medium were treated for 48 hours at 37°C with native LDL (100 μg/mL) in medium containing 3 μmol/L Cu^{2+} in the presence of increasing concentrations of gliclazide (0 to 10 μg/mL), with inclusion of sterile [methyl-^{3}H]-thymidine (5 μCi/mL) during the last 24 hours of incubation. HASMC proliferation was assessed by ^{3}H-thymidine incorporation into DNA as described in Materials and Methods. Data represent the mean ± SEM of 6 separate experiments. **$P < .01$ *v* control.

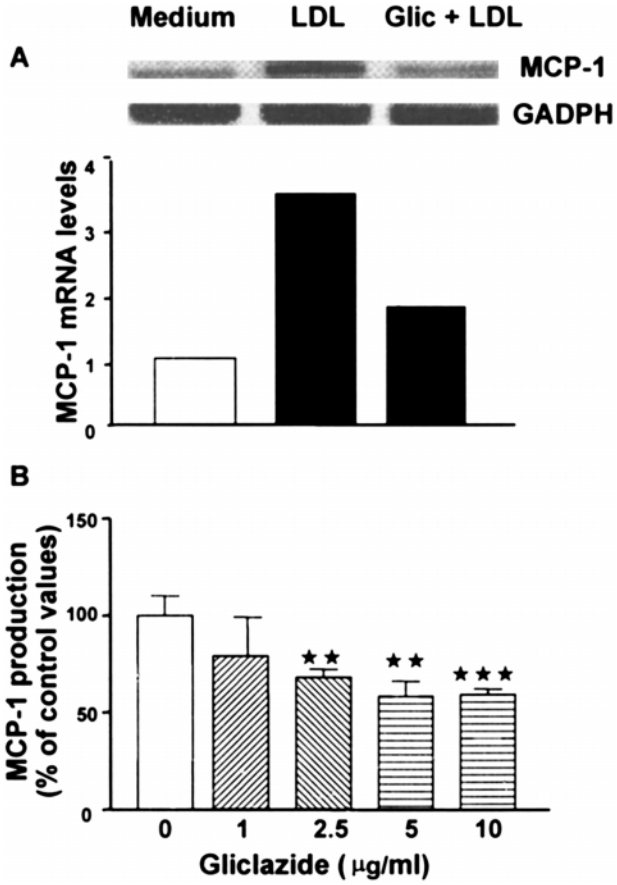

Fig 4. Effect of gliclazide on oxidized LDL-induced MCP-1 mRNA expression and secretion by HASMCs. (A) Subconfluent HASMCs were treated for 24 hours with native LDL (100 μg/mL) in medium containing 3 μmol/L Cu^{2+} in the presence or absence of 10 μg/mL gliclazide. Supernatants were then collected and added to a culture of serum-starved HASMCs for a 4-hour incubation period. At the end of this incubation period, total RNA was extracted as described in Materials and Methods, and the levels of MCP-1 and GAPDH mRNA expression were assessed by semiquantitative PCR (upper panel). MCP-1 mRNA levels (arbitrary units), normalized to the levels of GAPDH mRNA expression are presented in the lower panel. Data represent the results of 1 representative experiment of 3. (B) HASMCs were incubated with native LDL (100 μg/mL) for 24 hours in the presence of increasing concentrations of gliclazide (0 to 10 μg/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 separate experiments. **$P < .01$ and ***$P < .001$ *v* control.

VSMCs. They also show that gliclazide inhibits the ability of modified LDL to stimulate this process. These results are in agreement with our previous findings that gliclazide inhibits monocyte adhesion to endothelial cells.[23,25] The mechanisms by which oxidized LDL induces monocyte adhesion to VSMCs are unclear. We previously reported that gliclazide inhibits monocyte adhesion to endothelial cells by reducing endothelial cell-associated expression of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1).[25] Because modified LDL does not upregulate VCAM-1 and ICAM-1expression on VSMCs,[45] the inhibitory effect of gliclazide on monocyte binding to VSMCs may involve different pathways. Recently, oxidized LDL has been identified as a major determinant of IG9 monocyte adhesion molecule overexpression in VSMCs.[46] Whether gliclazide may inhibit monocyte adhesion to VSMCs by reducing the expression of this new adhesion molecule awaits further studies. Although the biological consequences of adhesion of monocytes to VSMCs are not well defined, a role of this process in monocyte accumulation in the atherosclerotic plaque and activation of mononuclear cells may be suggested.[47] Mediators released from inflammatory cells may, in turn, induce the expression of adhesion molecules on VSMCs.[42-44] Based on these data, one may propose that gliclazide, by its ability to reduce the interactions of monocytes and VSMCs, could contribute to attenuate the sustained inflammatory process that occurs in the atherosclerotic lesion.

Although the proproliferative effect of native LDL has been debated for a long time,[48-49] modified LDL has been clearly defined as a mitogen for VSMCs.[8-9,49] In accordance with these observations, our data show that exposure of VSMC to oxidatively modified LDL is associated with an increased VSMC growth. The role for reactive oxygen species in the control of

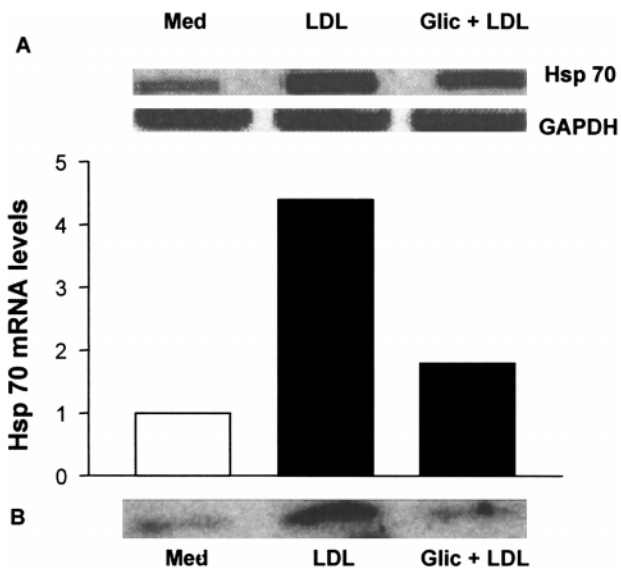

Fig 5. Effect of gliclazide on oxidized LDL-induced hsp 70 mRNA and protein expression in HASMCs. (A) Subconfluent HASMCs were treated for 24 hours with native LDL (100 μg/mL) in medium containing 3 μmol/L Cu^{2+} in the presence or absence of 10 μg/mL gliclazide. Supernatants were then collected and added to a culture of serum-starved HASMCs for a 4-hour incubation period. At the end of this incubation period, total RNA was extracted as described in Materials and Methods, and the levels of hsp 70 and GAPDH mRNA expression were assessed by semiquantitative PCR (upper panel). Hsp 70 mRNA levels (in arbitrary units), normalized to the levels of GAPDH mRNA expression are presented in the lower panel. Data represent the results of 1 representative experiment of 3. (B) HASMCs were incubated with native LDL (100 μg/mL) for 24 hours at 37°C in the presence or absence of gliclazide (10 μg/mL). Ten micrograms of total cellular proteins were separated by SDS-PAGE and subjected to Western blot analysis as described in Materials and Methods. Data represent the results of 1 representative experiment of 3.

VSMC proliferation has been shown both in vitro and in vivo.[26] In particular, evidence has been provided that treatment of VSMCs with the antioxidant α-tocopherol inhibits the growth response of these cells.[50] Our results provide first evidence that gliclazide decreases oxidized LDL-stimulated VSMC growth. These results and the previous observation that this drug also effectively inhibits VSMC hypertrophy induced by glycosylated human oxyhaemoglobin, a well-known inducer of oxidant stress,[51] suggest that gliclazide, by its antioxidant properties, may interfere with the response of VSMC to oxidative stress. Among the signaling pathways induced by oxidized LDL in VSMCs is the activation of the transcription factor NF-κB. The observations that NF-κB activity is essential to VSMC growth[52] and that gliclazide reduces the activation of NF-κB in glycated albumin-treated endothelial cells[25] support a role of this transcription factor in the suppressive effect of this drug on VSMC growth. Alternatively, gliclazide may inhibit VSMC proliferation via a protein kinase C (PKC)-dependent mechanism. Our present results and our preliminary data showing that 1 μg/mL gliclazide exerts a maximal inhibitory effect on both VSMC proliferation and PKC activation support this possibility.

Increased recruitment of mononuclear cells into the intima of the arterial wall is an important event in atherogenesis.[53] This process is mediated by an increased gradient of chemotactic activity in the vascular wall. MCP-1 is believed to be the most potent and specific chemotactic and activating factor for monocytes.[54,55] Modified LDL has been shown to induce MCP-1 expression by VSMCs by a mechanism involving increased generation of superoxide anion and increased activity of NF-κB.[56] Results of the present study show that gliclazide decreases the ability of VSMC-mediated LDL oxidation to induce MCP-1 expression, both at the gene and protein levels. Based on previous observations that gliclazide scavenges free superoxide radicals[57] and inhibits NF-κB activation,[25] it is tempting to postulate that this drug may inhibit modified LDL-induced MCP-1 production by scavenging free radicals and/or interfering with the NF–κB-dependent signaling pathway. Based on the chemoattractant properties of MCP-1[54,55] and on the ability of this protein to induce differentiation of VSMCs towards the synthetic phenotype,[58] the inhibitory effect of gliclazide on MCP-1 expression may be of potential value in slowing the course of the atherosclerotic process.

Hsps play an important role as molecular chaperones by facilitating the folding of nascent proteins and assisting in refolding of denaturated proteins under both normal and toxic environmental conditions.[59] Several observations suggest that hsps may contribute to the initiation and progression of atherosclerosis. First, immune response against hsp 65 has been shown to result in atherosclerotic lesions in normocholesterolemic rabbits.[60] Second, increased expression of hsps has been observed in human atherosclerotic plaques.[60-62] Third, a positive correlation has been found between antibodies against hsp 70 and different types of vascular diseases.[63] Fourth, recent evidence indicates that hsp 70 is a potent inducer of proinflammatory cytokine production by human monocytes.[64] Evidence exists that oxidation may play an important role in the regulation of hsps expression. Indeed, it has been shown that oxidative burst associated with ischemia/reperfusion increases the expression of hsps,[65] and that oxidized LDL induces the expression of hsps in endothelial cells, monocytic cells, and VSMCs.[66-68] Interestingly, actively cycling VSMCs have been found to express more hsp 70 protein than confluent cells in response to oxidized LDL.[66] Because smooth muscle cell proliferation is a crucial step in the progression of atherosclerosis, hsp 70 expression in VSMCs may play an important role in the development of atherosclerosis. Our results show that gliclazide reduces hsp 70 expression in oxidized LDL-treated VSMCs. Because hsps expression is under redox control, such an effect may be related to the antioxidant properties of gliclazide. Alternatively, because hsp 70 is a stress protein responsive to mitogenic stimulation,[69] this drug may exert its effect by inhibiting cell proliferation. The molecular mechanisms by which gliclazide reduces hsp 70 gene expression are presently unknown. Because oxidized LDL activates the activator protein-1 (AP-1) transcription factor in VSMCs,[70] it is possible that gliclazide may exert this effect by inhibiting the binding of AP-1 to the promoter of the hsp 70 gene.[71]

In conclusion, these results show that gliclazide decreases VSMC alterations induced by cell-mediated LDL oxidation. Based on the crucial role of oxidative stress in the control of VSMC function, these findings suggest that this drug may contribute, by its antioxidant properties, to reduce VSMC dysfunction associated with atherosclerosis. Further investigation concerning the biochemical and molecular mechanisms involved in the regulatory effect of gliclazide on oxidative stress-sensitive genes will provide additional insight into its potential role in the prevention and treatment of diabetic vascular disease.

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