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Giovanna Giannico  
*Henry Ford Health*

Pedro Cortes  
*Henry Ford Health*

Mohammed H. Baccora  
*Henry Ford Health*

Clare Hassett  
*Henry Ford Health*

David W. Taube  
*Henry Ford Health*

*See next page for additional authors*

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**Authors**

Giovanna Giannico, Pedro Cortes, Mohammed H. Baccora, Clare Hassett, David W. Taube, and Jerry Yee

## Glibenclamide prevents increased extracellular matrix formation induced by high glucose concentration in mesangial cells

Giovanna Giannico, Pedro Cortes, Mohammed H. Baccora, Clare Hassett, David W. Taube, and Jerry Yee

Division of Nephrology and Hypertension, Department of Medicine, Henry Ford Hospital, Detroit, Michigan

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**Giannico G, Cortes P, Baccora MH, Hassett C, Taube DW, Yee J.** Glibenclamide prevents increased extracellular matrix formation induced by high glucose concentration in mesangial cells. *Am J Physiol Renal Physiol* 292: F57–F65, 2007. First published August 8, 2006; doi:10.1152/ajprenal.00210.2006.—Other than stimulation of cell contractility, little is known about the potential metabolic effects induced by sulfonylureas, independently of insulin action. Previous studies from our laboratory demonstrated complete abrogation of glomerulosclerosis in an experimental model of type 1 diabetes chronically (9 mo) treated with low-dose sulfonylureas (Biederman JJ, Vera E, Pankhaniya R, Hassett C, Giannico G, Yee J, Cortes P. *Kidney Int* 67: 554–565, 2005). Therefore, the effects of glibenclamide (Glib) on net collagen I, collagen IV, and fibronectin medium net secretion and cell layer collagen I deposition were investigated in mesangial cells continuously exposed to 25 mM glucose for 8 wk and treated with predetermined increasing concentrations of Glib for the same period. Clinically relevant concentrations (0.01  $\mu$ M) of Glib fully suppressed the high glucose-enhanced accumulation of collagen I, collagen IV, and fibronectin in the medium and inhibited collagen I deposition in the cell layer. These effects occurred while transforming growth factor (TGF)- $\beta$ 1 medium concentration remained elevated and glucose uptake was increased to levels above those in 25 mM glucose-incubated cultures. The decreased collagen I accumulation occurred simultaneously with enhanced collagen I mRNA expression in concert with marked suppression of plasminogen inhibitor type-1 (PAI-1) mRNA and protein expression. This strongly suggests an accelerated matrix turnover favoring breakdown. Glib-induced effects demonstrated a biphasic pattern, being absent or reversed in cells treated with higher Glib concentrations (0.1 or 1  $\mu$ M). Therefore, chronic Glib treatment at low concentrations markedly diminishes the high glucose-induced enhanced accumulation of extracellular matrix components by suppression of steady-state *PAI-1* transcriptional activity. These results and those previously reported in vivo suggest that long-term Glib treatment may prevent glomerulosclerosis in insulin-deficient diabetes.

diabetic nephropathy; sulfonylureas; mesangial cells;  $\alpha$ -endosulfine; matrix catabolism; plasminogen inhibitor type-1

IN STUDYING THE CAUSES OF diabetic glomerulosclerosis, we and others have emphasized the altered metabolic activity of mesangial cells. The first and most characteristic glomerular lesion of diabetes is mesangial deposition of extracellular matrix that precedes interstitial disease (18, 45). Thus, it is the exaggerated mesangial cell (MC) formation of matrix that has been generally considered central to the pathogenesis of diabetic nephropathy. Numerous studies have demonstrated that MC, exposed to high glucose concentration (HG) in tissue culture, synthesize, independently of associated changes in osmolarity, in-

creased amounts of matrix material that accumulates in the culture medium (19). Based on these findings, it has been widely accepted that hyperglycemia is a crucial factor in the increased deposition of matrix and the genesis of mesangial expansion and glomerulosclerosis in diabetes.

We have recently described the complete prevention of glomerulosclerosis and increased albuminuria in a model of insulin-deficient diabetes by the administration of sulfonylureas (5). This effect occurred at dosages equivalent to those administered in humans and in the presence of unabated hyperglycemia and unaltered renal hypertrophy and hyperfunction. In support of a unique glomerular action of sulfonylureas, we have demonstrated the presence of all the needed components for the functionality of an endogenous sulfonylurea system in MC and glomeruli (2, 48).

The known site of action of sulfonylureas is the ATP-sensitive  $K^+$  channels ( $K_{ATP}$ ). The plasma membrane  $K_{ATP}$  of pancreatic  $\beta$  cells has been the most extensively studied and is considered the “classic”  $K_{ATP}$  (13). Classic  $K_{ATP}$  consist of two structurally unrelated components: a potassium pore subunit and a sulfonylurea receptor (SUR) subunit.  $K_{ATP}$  have been described in numerous cell types, including cardiomyocytes, skeletal muscle, and vascular smooth muscle cells (7). In these tissues, they control cell contractility and organ functions related to blood flow regulation (50). Pharmacological studies have also demonstrated a diversity of  $K_{ATP}$  among various tissues according to their affinity for sulfonylureas and sensitivity to  $K^+$  channel openers (17). These heterogeneous properties and functional diversity of  $K_{ATP}$  are based on unique combinations of Kir6.x and SUR isoforms and also according to their cellular distribution.

We have recently demonstrated the presence of a functional rat mesangial  $K_{ATP}$  containing a unique SUR (2, 48). MC membranes demonstrate specific binding of the sulfonylurea glibenclamide (Glib), and following exposure to it, there is increased intracellular  $Ca^{2+}$  concentration and intense MC contraction (2). Furthermore, a rat mesangial SUR2 splice variant was identified (48), mcSUR2B, which shares identity with the smooth muscle-type rSUR2B also expressed in MC. In addition to specific receptors, we have also demonstrated gene and protein expression of the endogenous counterpart of sulfonylureas,  $\alpha$ -endosulfine, in MC in tissue culture and glomeruli in situ (52). Endosulfines are members of a highly conserved family of cAMP-regulated phosphoproteins (ARPP) (14, 40). The structure of  $\alpha$ -endosulfine, encoded by the *ENSA* gene, has been well characterized and found to be ubiquitously expressed in many tissues (25). Although  $\alpha$ -endosulfine has

Address for reprint requests and other correspondence: P. Cortes, Div. of Nephrology and Hypertension, Dept. of Medicine, Henry Ford Hospital, Clara Ford Pavilion-5, 2799 West Grand Blvd., Detroit, MI 48202 (e-mail: cortes.pedro@usa.net).

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been identified as a putative endogenous ligand for  $K_{ATP}$  channels, its physiological role remains largely unknown.

Beyond their actions on insulin secretion and cell contractility, little is known regarding direct metabolic effects of sulfonylureas. Because of the extensive expression of SUR in multiple tissues, it may be anticipated that sulfonylureas are capable of inducing metabolic changes other than those attributable to insulin action. Independently of insulin, sulfonylureas augment glucose utilization in adipocytes and myocytes in tissue culture by increasing the expression and activity of the GLUT1 glucose transporter (15, 35). Motivated by these findings, we investigated the effects of the sulfonylurea tolazamide on glucose uptake and matrix synthesis in a MC line in tissue culture (9). In cells briefly treated with high (millimolar) concentrations of tolazamide, increases in glucose uptake, transforming growth factor (TGF)- $\beta$ 1 secretion, and matrix accumulation were delineated, suggesting that this drug could aggravate diabetic glomerulosclerosis. Therefore, because these initial *in vitro* observations were contradictory to our recent findings *in vivo*, the present study was undertaken.

To ascertain a more relevant *in vitro* correlate to the setting of glomerulosclerosis occurring only after long-standing diabetes, studies on extracellular matrix metabolism were carried out in primary MC chronically exposed to HG. In addition, these cells were treated with concentrations of Glib within the nanomolar therapeutic range common in humans (41). Subsequently, it was demonstrated that under these conditions, low concentrations of Glib inhibited extracellular matrix accumulation.

## MATERIALS AND METHODS

**Materials.** Tissue culture, 75-cm<sup>2</sup> flasks, and Falcon six-well plates were from Corning (Corning, NY) and Becton Dickinson (Lincoln Park, NJ), respectively. RPMI tissue culture medium 1640 and fetal bovine serum (FBS) were obtained from GIBCO BRL (Grand Island, NY). Polyclonal goat anti-rat ( $\alpha_1$ ,  $\alpha_2$ ) collagen IV (capture antibody), anti-rat type IV ( $\alpha_2$ ) collagen, and goat anti-mouse alkaline phosphatase-conjugated antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Chemicon (Temecula, CA), and Bethyl Laboratories (Montgomery, TX), respectively. Polyclonal rabbit anti-rat collagen I antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG were purchased from Chemicon and ICN Pharmaceuticals (Aurora, OH), respectively. Anti-rat fibronectin antibody was

obtained from GIBCO BRL. Rat tail collagen type 1 and mouse collagen type IV (Upstate Biotechnology, Lake Placid, NY) or purified fibronectin (GIBCO) were used as standards. Fluorescein diphosphate and DAPI nucleic acid stain were from Molecular Probes (Eugene, OR). 2-Deoxy-D-[<sup>14</sup>C(U)]glucose (323 mCi/mmol) was obtained from DuPont NEN (Boston, MA).

**Cell culture.** Primary cultures of MC were obtained from outgrowths of isolated glomeruli from Munich-Wistar rats (48). Cells were grown in RPMI medium 1640, pH 7.4, containing 5 mM glucose, 2 mM L-glutamine, 23.8 mM NaHCO<sub>3</sub>, 15% FBS, 5 U/ml penicillin G, and 5 U/ml streptomycin. Cells were identified according to their stellate appearance, formation of cell hillocks in postconfluent cultures, expression of vimentin and  $\alpha$ -smooth muscle actin but not cytokeratin, and ability to grow in medium containing D-valine. Cells at passages 3–6 at the initiation of the experiments were used. Eight weeks before the termination of the experiments, the medium in the experimental group was changed to a similar one containing 25 mM glucose and predetermined amounts of Glib, while the control group was continuously grown in 5 mM glucose. At the end of the experimental period, the last cell passage was in six-well culture plates, seeded at  $40 \times 10^3$  cells/well. Except where indicated, 48 h before the end of the experiments, experimental and control media were changed to media of the same composition but containing 1% FBS and 210  $\mu$ M sodium ascorbate. Cultures were studied when 80–90% confluent.

**ELISA.** The concentration of collagen I, collagen IV, and fibronectin in the 48-h conditioned media was determined by an indirect, antibody sandwich ELISA (26) using an alkaline phosphatase-*p*-nitrophenylphosphate chromogenic system. Purified rat collagen type I (1–2,000 ng/well), mouse collagen type IV (32–2,000 ng/well), and rat fibronectin, (0.5–500 ng/well) were used as standards. Unknowns and standards were diluted in the same medium used in tissue culture. All samples were tested in triplicate. Color intensity was measured with a Titertek Multiscan MCC/340 (Flow Laboratories, McLean, VA), and the results were analyzed using curve-fitting software (Interactive Microware, State College, PA). The concentration of total TGF- $\beta$ 1 and rat plasminogen inhibitor type-1 (PAI-1) in aliquots of conditioned media was determined using commercially available ELISA kits (for TGF- $\beta$ 1: Quantikine R&D Laboratories, Minneapolis, MN; for PAI-1: Aniaira, Mason, OH) according to the manufacturer's instructions. The PAI-1 assay reacts with latent, active, and inactive PAI-1. Results were factored by number of cells according to DNA content in the cell layer of the corresponding sample.

**ELISA for adherent cells (cell-ELISA).** The cell layer-associated collagen I was measured by an innovative strategy based on the indirect cellular ELISA. Cells were plated at 5,000 cells/well in 96-well Falcon 3945 Microtest (BD Biosciences, Franklin Lakes, NJ)

Table 1. Primers, probes, and experimentally determined optimal annealing temperature used in duplex analysis with  $\beta$ -actin as a housekeeping gene

|                | $\beta$ -Actin                          | PAI-1                                 | $\alpha_1(I)$ Collagen                    |
|----------------|---|---------------------------------------|---|
| Forward Primer | ACCCACACTGTGCCCA<br>TCTA                | CCATCTCCGTGCCCATG<br>AT               | GCCCCTCTGAGGCTTCT<br>G                    |
| Reverse Primer | GGCCATCTCTTGCTCG<br>AA                  | GTCATGTTGCTCTTCCA<br>TTGTCT           | AGACTTCTCTTGAGGTG<br>GCTGA                |
| Donor Probe    | GCCACGCTCGGTCAG<br>GATCTTCAT X<br>LC705 | GCTGAGGGTTTCGCCGT<br>GGTAG X<br>LC640 | CCAGGGAGACCGTTGA<br>GTCCATCTTT X<br>LC640 |
| Acceptor Probe | AGGTAGTCTGTCAGGT<br>CCGGGCCA P          | CAGTTCAGGATGTCGT<br>ACTCGTGC P        | CCAGGAGAACCAGCAG<br>AGCCAGG P             |
| T <sub>A</sub> |   | 57°C                                  | 58°C                                      |

PAI-1, plasminogen inhibitor type-1; T<sub>A</sub>, annealing temperature.

and fixed in 3.7% paraformaldehyde in PBS at predetermined intervals between 24 and 96 h. After blocking with 3% fat-free dry milk in PBS, samples were incubated at 4°C overnight with rat anti-collagen I as the primary antibody. Following washing of the wells, samples were successively incubated for 3 h at 4°C with phosphatase-conjugated goat anti-rabbit IgG, as the secondary antibody, and for 15–30 min in 4  $\mu$ M fluorescein diphosphate. Fluorescence was read at 485-nm excitation/527-nm emission in a Fluoroskan, Ascent FL (Thermolabs Systems, Franklin, MA) microplate reader. Finally, after washing of the wells, samples were incubated with 14.3  $\mu$ M of the DNA-binding DAPI in PBS for 30 min for specific staining of nuclei, washed, and placed in PBS, and read at 355-nm excitation/460-nm emission. Fluorescein and DAPI fluorescence was compared with that obtained in standard curves set up within the same plate using 0.01–0.62  $\mu$ g/ $\mu$ l collagen I and  $5 \times 10^3$ – $5 \times 10^4$  cells/well, respectively. Results were expressed as micrograms collagen I per  $10^4$  cells.

**DNA content.** Following removal of media, cell layers were rinsed twice with ice-cold PBS and cell layers were scraped in a 10 mM sodium phosphate lysis buffer, pH 7.5, containing 150 mM NaCl, 2 mM EDTA, 1% Igepal, and 1% sodium deoxycholate. Samples were homogenized in the cold in a glass-glass tissue grinder with five strokes at 10 rpm and centrifuged at 26,000 g for 20 min at 4°C. The resulting supernatants were used for the measurement of DNA in 96-well plates by a fluorescence method using the PicoGreen dsDNA kit (Molecular Probes) according to the manufacturer's directions. Fluorescence was determined at 480-nm excitation/520-nm emission. DNA standards yielded linear regressions between 5 and 200 ng/ml DNA. Analyses were done using a Fluoroskan Ascent FL microplate reader (Thermo Electron).

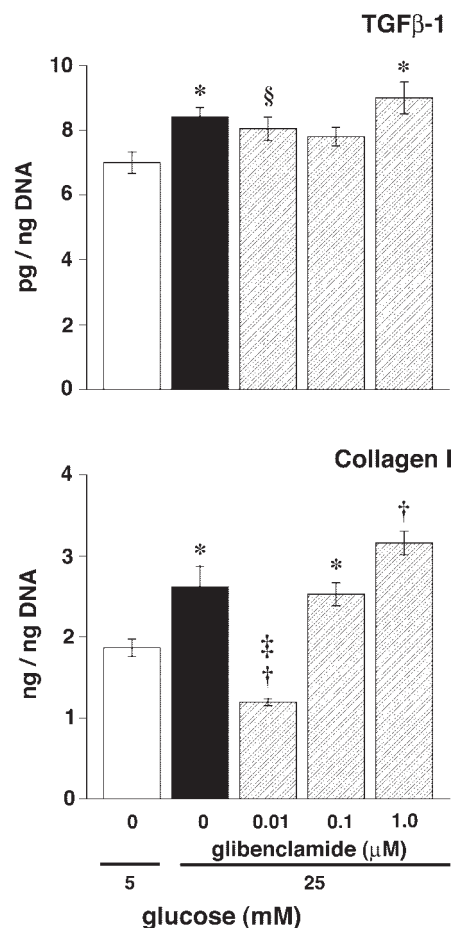
**Quantitative, real-time gene expression.** Total RNA was extracted from cell layers using RNeasy minikits (Qiagen, Valencia, CA) after DNase treatment. The approach for the measurement of gene expression was a one-step RT-PCR with specific FRET probes using a multiplex mode (gene of interest and housekeeping gene expression measured simultaneously in the same reaction capillary) in a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany). Primers and probes for  $\alpha_1(I)$ collagen and *PAI-1* quantification were obtained from Roche Pharmaceuticals (Indianapolis, IN) and TIB Molbiol LCC (Adelphia, NJ), respectively. Reactions were done using the QuantiTect Probe (Qiagen) and SuperScriptIII Platinum (Invitrogen, Carlsbad, CA) kits for  $\alpha_1(I)$ collagen and *PAI-1*, respectively. Analyses were done in triplicate. As reference we used  $\beta$ -actin expression because recent published studies from our laboratory have demonstrated this gene to be one of the most stable in MC cultured in HG conditions and in isolated glomeruli of diabetic rats (4). In addition, since our previous studies demonstrated that total RNA was also a reliable reference for gene expression in cultured MC (4), the stability of  $\beta$ -actin expression was also documented by factoring for the sample's RNA content. Total RNA was quantified using a RiboGreen RNA kit (Molecular Probes) following the manufacturer's instructions. The primers, probes, and experimentally determined optimal annealing temperature for the multiplex conditions used are presented in Table 1.

**Glucose transport.** The initial rate of glucose transport was measured as in our prior studies (9). In brief, the 3-min transporter-specific uptake of 2-deoxy-D-[ $^{14}$ C(U)]glucose was measured in the cell layer of MC cultured to confluency. The uptake rate was determined using 10  $\mu$ M 2-deoxy-D-glucose and 1  $\mu$ Ci/ml 2-deoxy-D-[ $^{14}$ C(U)]glucose in Dulbecco's buffer. Incubations were terminated by the addition of ice-cold PBS containing 0.3 mM phloretin and 20  $\mu$ M unlabeled 2-deoxy-D-glucose. The amount of extracellular radioactivity present in the sample and the transporter-independent glucose uptake were determined in parallel culture wells in which incubation with radio-labeled 2-deoxy-D-glucose was carried out in the presence of cytochalasin B, an inhibitor of glucose transporters. Results were expressed as picomoles 2-deoxyglucose uptake per minute per unit protein in the 1 N NaOH-treated cell layer. Total protein was measured by the Folin-Ciocalteu phenol reagent method.

**Statistical analysis.** Statistical analyses were carried out using Stat-View 5.0.1 software (Abacus Concepts, Berkeley, CA). Results are expressed as means  $\pm$  SE. Results were analyzed by analysis of variance. If a significant difference was found among groups, between group comparisons were made with post hoc testing with Fisher's protected least significant difference. Statistical significance was set at the 5% level.

## RESULTS

**Effects of Glib on the extracellular matrix accumulation induced by HG concentration.** The long-term effects of HG concentration on MC matrix formation and their modification by Glib were studied in cultures treated with increasing concentrations of the sulfonylurea. The accumulation of collagen I was measured first since this is the predominant form synthesized by MC in tissue culture (1, 38). HG caused a significant 40% increase in the net accumulation of collagen I into



\*  $P < 0.005$  vs. 5 mM  
 †  $P < 0.001$  vs. 5 mM  
 §  $P = 0.036$  vs. 5 mM  
 ‡  $P < 0.001$  vs. 25 mM  
 n = 10–12 per group

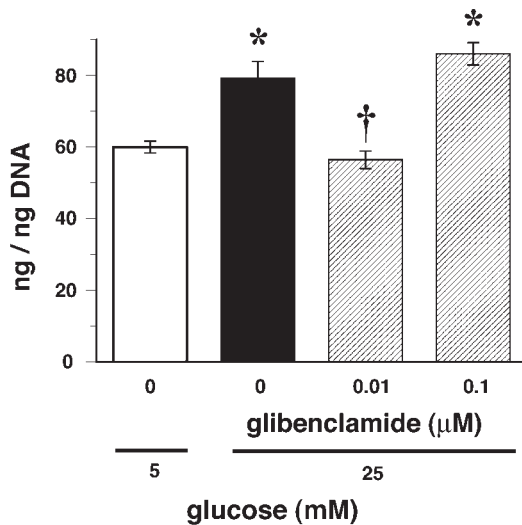
Fig. 1. Collagen I and transforming growth factor (TGF)- $\beta$ 1 medium accumulation in primary mesangial cell cultures chronically exposed to high glucose concentration and treated with glibenclamide. Mesangial cells were placed in a medium containing 25 mM glucose and increasing concentrations of glibenclamide for 8 wk. Control mesangial cells were continuously grown in 5 mM glucose. At the end of the experiment, collagen I and total TGF- $\beta$ 1 concentrations were measured in the 48-h conditioned media of 90% confluent cultures by ELISA. Results were factored by DNA in the cell layer, measured by a fluorescence-based method. Values are means  $\pm$  SE.

the medium over the values in 5 mM glucose-grown controls (Fig. 1). Continuous treatment with 0.01  $\mu\text{M}$  Glib over the entire period of exposure to HG totally prevented this alteration. Furthermore, in these Glib-treated cells collagen I net accumulation was significantly lower than in 5 mM glucose-grown controls. However, contrary to what was anticipated, increasing the medium concentration of Glib by 10-fold or higher abolished its inhibitory effect (Fig. 1). Glib treatment for periods between 6 and 10 wk or in the presence of 20 mM glucose concentration yielded similar results.

To investigate whether the changes induced in extracellular matrix formation by low concentrations of Glib were due to inhibition of TGF- $\beta$ 1 secretion, the concentration of this growth factor was measured in the same conditioned media in which collagen I accumulation was quantified (Fig. 1). TGF- $\beta$ 1 concentration was elevated in 25 mM glucose cultures without the demonstration of any significant change with Glib treatment at any of the concentrations tested. As shown in Fig. 1, there was no relationship between collagen I accumulation and medium TGF- $\beta$ 1 concentration in Glib-treated cultures.

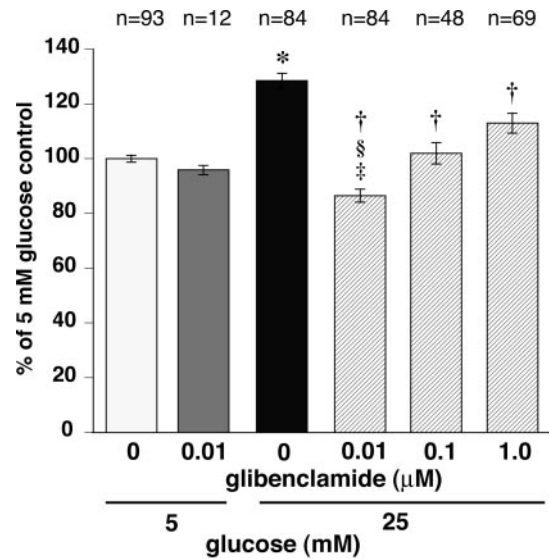
Fibronectin media net accumulation in cultures chronically exposed to HG and long-term treatment with Glib demonstrated changes similar to those for collagen I. Thus the 39% increase in media fibronectin induced by 25 mM glucose was fully prevented by 0.01  $\mu\text{M}$  Glib, although the resulting values were not lower than those in 5 mM glucose-grown controls (Fig. 2). In addition, 0.1  $\mu\text{M}$  Glib did not cause any amelioration of the 25 mM glucose enhancement in fibronectin secretion.

The effects of Glib on collagen I accumulation were confirmed in six additional separate experiments. The combined results obtained in these are presented in Fig. 3. These results showed similar changes in collagen I accumulation induced by



\*  $P < 0.001$  vs. 5 mM  
 †  $P < 0.001$  vs. 25 mM  
 n = 11 per group

Fig. 2. Fibronectin medium accumulation in rat mesangial cell cultures chronically exposed to high glucose concentration and treated with glibenclamide. Mesangial cells were cultured and glibenclamide-treated as in Fig. 1. At the end of the experiment, fibronectin concentration was measured in the 48-h conditioned media by ELISA. Results were factored by cell layer DNA. Values are means  $\pm$  SE.



\*  $P < 0.001$  vs. 5 mM glucose  
 †  $P < 0.001$  vs. 25 mM glucose  
 §  $P < 0.001$  vs. 5 mM glucose  
 ‡  $P < 0.001$  0.01  $\mu\text{M}$  vs. 1.0  $\mu\text{M}$  glibenclamide

Fig. 3. Collagen I medium accumulation in rat mesangial cell cultures chronically exposed to high glucose concentration and treated with the sulfonylurea glibenclamide. Studies were carried out under the same conditions as in Fig. 1. The combined data obtained in 6 separate experiments are presented with results expressed as a percentage of the corresponding 5 mM glucose-incubated controls, with the total number of samples (n) indicated in each experimental group. Values are means  $\pm$  SE.

Glib treatment in HG-incubated cells as those shown in Fig. 1. In addition, 8-wk treatment with 0.01  $\mu\text{M}$  Glib in cells continuously grown in 5 mM glucose had no effect on media collagen I net accumulation (Fig. 3).

Since initial experiments demonstrated that the quantity of collagen I deposited on the cell layer gradually decreases with increasing confluence of the cultures, the effect of Glib was studied at different periods of growth in long-term HG-incubated cells. Results were similar to those observed in the conditioned media. In cells cultured in 25 mM glucose, 0.01  $\mu\text{M}$  Glib significantly decreased collagen I deposition to levels in 5 mM glucose controls at all periods between 2 and 4 days (Fig. 4). However, in these 25 mM glucose-incubated cells, 1  $\mu\text{M}$  Glib concentration did not induce any changes over the same period of time. Contrary to the lower concentration, 1  $\mu\text{M}$  Glib transiently induced increased collagen deposition at 24 h of growth (Fig. 4).

To investigate whether changes in collagen I media accumulation were mediated by altered gene expression, cell layer total RNA was quantitatively studied for  $\alpha_1(I)$ collagen expression under the same conditions as those in Figs. 1–4. Collagen I mRNA expression was upregulated by HG, as expected (Fig. 5). Unexpectedly, however, increasing Glib concentrations resulted in changes in collagen I mRNA expression that were the reverse of its effects on protein accumulation (Figs. 1 and 5). Thus 0.01  $\mu\text{M}$  Glib enhanced the level of collagen I mRNA expression well above that induced by HG (21 vs. 69% over 5 mM controls), while the increased protein expression caused by HG (40% over 5 mM controls) was significantly diminished by Glib to values lower than controls ( $-36\%$  of 5 mM control).

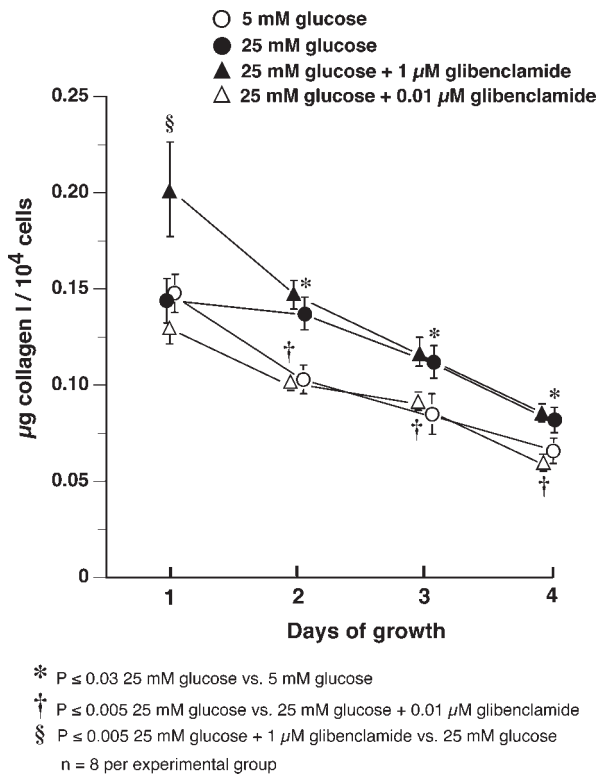


Fig. 4. Cell layer deposition of collagen I during growth of rat mesangial cell cultures chronically exposed to high glucose concentration and treated with glibenclamide. Mesangial cells were seeded in 96-well plates (5,000 cells/well) under the same experimental conditions as in Fig. 1. Separate sets of plates were processed at determined time periods during exponential growth. At each period, cell layers were fixed and cell layer-associated collagen I was measured by ELISA in a fluorogenic reaction. The number of cells in each well was assessed by reaction with the DNA-binding fluorescent dye DAPI. Results were derived from separate collagen I and cell density standard curves. The amount of collagen per well was factored by the corresponding number of cells in the same well. Values are means  $\pm$  SE.

However, high Glib concentration did not alter the HG-enhanced collagen I mRNA expression (Fig. 5).

To corroborate the above, steady-state collagen mRNA expression was also studied in undisturbed cultures without removal of FBS. In these experiments, differences between groups in mRNA expression were similar to that in 48-h serum-restricted cultures. However, the  $\alpha_1(I)collagen/\beta-actin$  ratio was markedly decreased in these non-serum-starved cultures. This was the result of suppressed  $\beta-actin$  mRNA expression during serum starvation as shown by a 58–65% decrease in  $\beta-actin$ /total RNA ratio. Thus it is important to document the stability of any housekeeping gene of choice, not only among different groups within any given experiment but also among different experiments when media composition is changed during the experimental period.

*Effects of Glib on changes in PAI-1 expression induced by HG concentration.* PAI-1 exerts powerful effects on extracellular matrix degradation and turnover in MC in culture (3). These effects are mediated by inhibition of plasminogen activators and plasmin-induced extracellular matrix breakdown, and the inactivation of metalloproteinases that enzymatically cleave collagenous proteins. In addition, PAI-1 also enhances renal fibrosis via stimulation of TGF- $\beta$  activity (32). Therefore, changes in PAI-1 expression were investigated as a possible cause for the contrasting effects of Glib on collagen I gene expression and protein accumulation.

The steady-state expression of PAI-1 mRNA was markedly increased (3-fold) in MC chronically exposed to HG compared with cells grown in 5 mM glucose concentration (Fig. 6). Treatment of HG-incubated cells with low concentrations of Glib fully reversed this increased expression to levels similar to those in cells kept in normal glucose (Fig. 6). In contrast, treatment with high Glib concentration induced an increased PAI-1 mRNA expression that surpassed that shown in non-treated, 25 mM glucose-incubated cells (4.5-fold over 5 mM glucose control) (Fig. 6).

To directly compare the effects of HG and Glib treatment on PAI-1 and collagen protein accumulation, these were measured in the same conditioned media in experiments carried out under

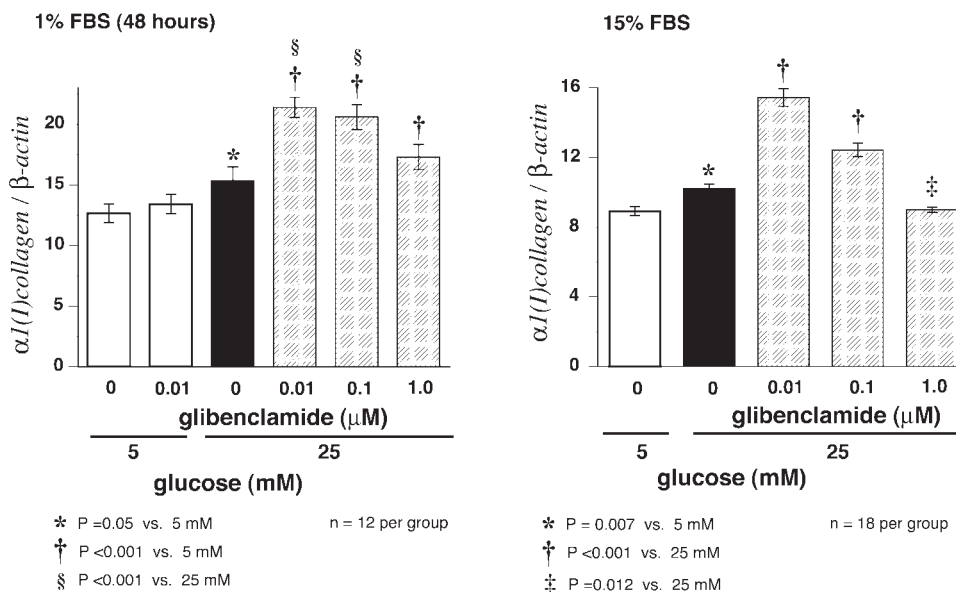


Fig. 5. Collagen gene expression in rat mesangial cell cultures chronically exposed to high glucose concentration and treated with glibenclamide. Expression of  $\alpha_1(I)collagen$  was quantified in total RNA extracted from cell layers of mesangial cells exposed to 5 or 25 mM glucose for 8 wk with or without concomitant treatment with increasing concentrations of glibenclamide. Experimental conditions were as in Figs. 1–4. Gene expression was quantified by real-time RT-PCR in a multiplex format. Results were factored per  $\beta-actin$  expression. Since the  $\beta-actin$ -to-total RNA ratio is influenced by reduced  $\beta-actin$  expression in serum-restricted cells (left), experiments were repeated in non-serum-starved cultures (15% FBS; right). Values are means  $\pm$  SE.

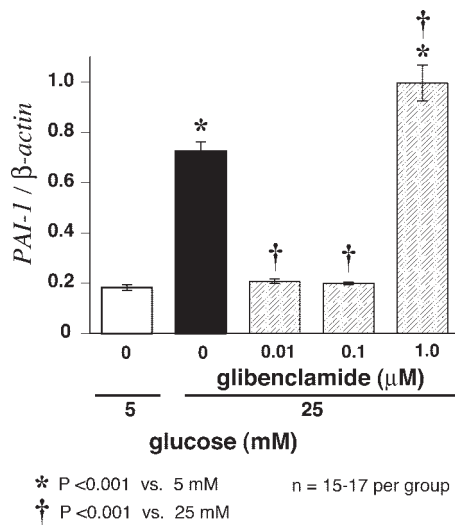


Fig. 6. Plasminogen inhibitor type-1 (PAI-1) gene expression in rat mesangial cell cultures chronically exposed to high glucose concentration and treated with glibenclamide. Studies were conducted and measurements were carried out as in Fig. 5. Cultures were not serum restricted. Values are means ± SE.

identical conditions as in the studies above. As for the gene expression, PAI-1 protein in HG-exposed cells was reduced by treatment with low Glib concentration to levels similar to those in 5 mM glucose control cells (Fig. 7). In addition, this Glib-induced inhibition was not evident in high Glib concentration-treated cultures. The changes in PAI-1 protein expression induced by HG and different concentrations of Glib were associated by parallel alterations in collagen IV and collagen I protein accumulation (Fig. 7).

*Transporter-specific glucose uptake in Glib-treated MC.* Because previous studies in mesangial and other cells demonstrated that short-term treatment with sulfonylureas may increase glucose uptake, thus enhancing collagen synthesis, the effects of long-term treatment with increasing concentrations of Glib on glucose uptake were investigated.

The initial rate of transporter-specific 2-deoxy-glucose uptake was determined in MC chronically exposed to 25 mM glucose and in similar cells treated with increasing concentrations of Glib as in the studies above. Compared with 5 mM glucose-grown controls, exposure to HG induced a modest (9%) but significant stimulation of glucose uptake (Fig. 8). Low Glib concentration treatment increased glucose uptake to levels significantly above those in nontreated, HG-exposed cells (14% over control). However, 1 μM Glib did not alter the uptake demonstrated in HG-exposed cells, while a 50-fold higher Glib concentration significantly decreased glucose uptake to levels below those in 5 mM glucose controls (Fig. 8).

DISCUSSION

This study demonstrates, for the first time, the unanticipated property for low concentrations of Glib to prevent the HG-enhanced accumulation of extracellular matrix components in MC. This effect occurred in the amount secreted in the incubation medium and in the quantity deposited in the cell layer. The potential clinical significance of these observations results from the experimental conditions used that mimic the long-term effects of uncontrolled hyperglycemia. The length of

exposure to HG significantly exceeded that in previous studies of MC in tissue culture. As further support of this clinical relevance, the results obtained are in harmony with our previous observations regarding prevention of glomerulosclerosis in insulin-deficient diabetic animals, chronically treated with Glib

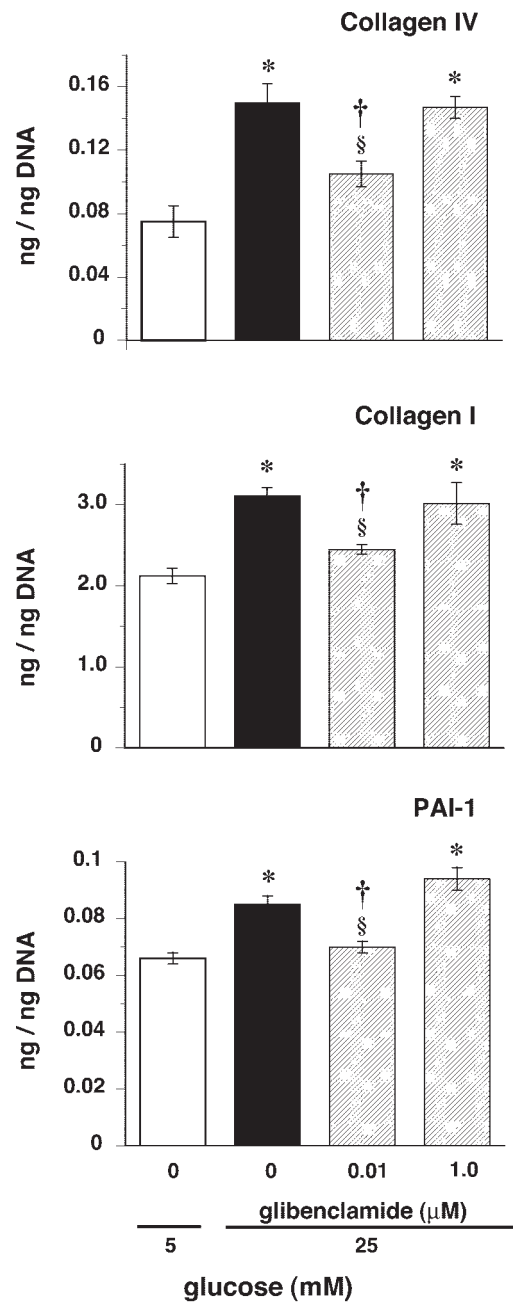


Fig. 7. Collagen IV, collagen I, and PAI-1 protein medium accumulation in primary mesangial cell cultures chronically exposed to high glucose concentration and treated with glibenclamide. Experimental conditions were similar to those used in Figs. 1–6. Quantitative analysis of collagen IV, collagen I, and PAI-1 protein was carried out in the 48-h conditioned medium by ELISA as in Fig. 1. Values are means ± SE.



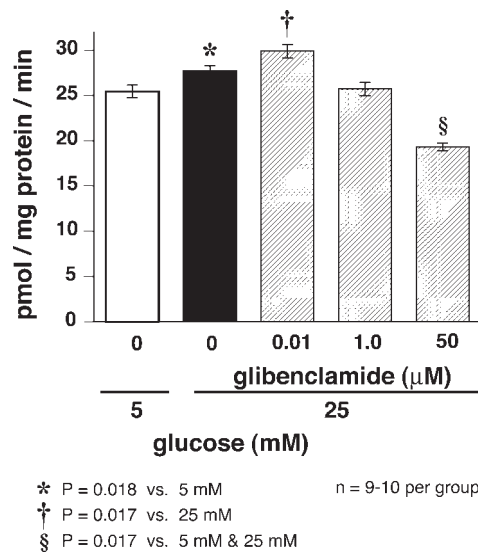


Fig. 8. GLUT transport-specific 2-deoxyglucose uptake by primary cultures of rat mesangial cells chronically exposed to high glucose concentration and treated with glibenclamide. Experimental conditions were similar to those in Figs. 1–7. Incorporation rates were corrected for transporter-independent uptake as determined in parallel cultures incubated with 10  $\mu$ M cytochalasin B. Protein-normalized incorporation rates are presented. Values are means  $\pm$  SE.

at a dosage that was therapeutically equivalent to those in humans (5). In addition, the low Glib concentration used during MC treatment in tissue culture is well within the clinical range (peak plasma level after one 5-mg oral Glib dose is 0.09–0.6  $\mu$ M; daily therapeutic dosage generally is 2.5–20 mg) (41, 49).

Glib specifically interferes with the cell response to HG concentration and does not affect the unstimulated, basal condition. This is evident by the lack of effect of 0.01  $\mu$ M Glib on collagen I mRNA expression and collagen I protein accumulation in cells maintained in 5 mM glucose. That is, irrespective of its actions on insulin secretion, Glib is not expected to directly alter MC extracellular matrix metabolism under physiological circumstances.

Low Glib concentration modestly stimulated glucose uptake, probably by increasing the expression of the GLUT1 glucose transporter, as has been reported in other cells acutely treated with Glib and other sulfonylureas (8, 15). However, the biological significance of this increased transport may be important. We have previously demonstrated the prime influence of glucose transport as a regulator of the synthesis and accumulation rate of extracellular matrix (24). Thus this increased glucose uptake may represent the stimulus for enhanced expression of collagen I mRNA and increased collagen I protein accumulation rate demonstrated in this study.

In regard to the mechanism by which low Glib concentrations diminish HG-induced matrix accumulation, our data strongly suggest that high turnover and increased matrix breakdown are involved. While collagen I gene expression may be stimulated by increased glucose availability, there is a simultaneous repression of PAI-1 gene and protein expression, indicative of an enhanced catabolic rate, fully counterbalancing the increased synthetic activity. Thus, while Glib stimulates glucose transport, it exerts a concomitant strong suppression of PAI-1 transcriptional activity and/or enhanced PAI-1 mRNA

degradation (23). The main candidate mediator of this effect is an elevated cAMP level. Principally, the downregulation of steady-state PAI-1 mRNA content by agents that stimulate cAMP formation is a well-documented major regulatory mechanism (11, 23). Furthermore, sulfonylureas are known to activate adenylyl cyclase and inhibit the activity of phosphodiesterases, thus leading to an increase in cellular cAMP levels (43).

PAI-1 expression plays an important role in the pathogenesis of diabetic glomerulosclerosis (16). PAI-1 expression is up-regulated by HG in MC in tissue culture and by diabetes in MC in situ (21, 30, 33). Furthermore, PAI-1 activity has been demonstrated to be a pathogenetic contributor to the development of nephropathy in experimental, insulin-deficient diabetes (36). Thus PAI-1 is considered an important therapeutic target in the prevention of progressive renal fibrosis (27).

Concerning the possible relationship between Glib and cytokines stimulated by HG, Glib suppression of net collagen I accrual occurred in the setting of TGF- $\beta$ 1 enhancement, suggesting that Glib interferes with TGF- $\beta$  signaling. This is the first study reporting an inhibitory effect of PAI-1 expression in the setting of increased TGF- $\beta$  concentration. This is significant since TGF- $\beta$  is a known inducer of PAI-1 transcriptional activation via Smad signaling (34, 44), a cAMP-inhibitable reaction (11). In addition, PAI-1 activity has been also shown to be inhibited by the anti-fibrogenic cytokine bone morphogenic protein-7 (11, 51). Thus, putative mechanisms of Glib action on cytokine activity include inhibition of TGF- $\beta$  signaling and enhanced bone morphogenic protein-7 formation.

There is ample evidence that HG induces PKC activity and TGF- $\beta$ 1 and PAI-1 upregulation by generation of reactive oxygen species, following an acute exposure or chronically after formation of advanced glycosylation end-products (6, 31, 33, 44). Accordingly, the modulation of nonenzymatic glycosylation and glycoxidation could mediate the effects of long-term treatment with Glib. Concerning an antioxidative role for sulfonylureas, there is evidence that glicazide in vitro and in diabetic patients acts as a free radical scavenger (28, 37). However, Glib does not share this property (37). Besides the results presented here, there is only a single report whereby the renal effects of sulfonylureas were evaluated (39). In streptozotocin-diabetic rats fed high-cholesterol diets, glicazide decreased lipid peroxidation and urinary albumin excretion, likely through its direct radical scavenging properties. However, in this setting, Glib treatment had no effect. Thus it is highly unlikely that the Glib effects observed in this study were attributable to reductions in glycoxidative stress.

Notably, the Glib-induced decrease in extracellular matrix accumulation was only evident at low concentrations of the drug. This was particularly the case for collagen I, where 0.01  $\mu$ M Glib treatment of HG-exposed MC produced lower accumulation than in their corresponding controls cultured in 5 mM glucose (Figs. 1 and 3). This biphasic effect of a low vs. high Glib concentration was not limited to matrix accumulation. The effects on glucose transport, collagen I mRNA, and PAI-1 mRNA expression were also less evident or totally disappeared when Glib concentration was increased by 10- to 100-fold. This is in concert with our previous observations of enhanced collagen synthesis and accumulation in MC acutely treated with high concentrations (1.5 mM) of the sulfonylurea tolazamide (9). The reasons for this biphasic dose-effect are un-

clear; however, this is not an unusual biological response. For example, it has long been recognized that angiotensin II in low concentrations stimulates proximal tubule  $\text{Na}^+$  and  $\text{HCO}_3^-$  transport while high concentrations are inhibitory (22), depending on whether cytosolic  $\text{Ca}^{2+}$  or PKC is activated (12). Similarly, HMG-CoA reductase inhibitors (statins) demonstrate opposite effects on endothelial cell proliferation at low (0.01  $\mu\text{M}$ ) and high (1  $\mu\text{M}$ ) concentrations by stimulating different signaling pathways (29). Thus Glib may also enhance/repress different signaling systems depending on its prevailing concentration.

Although MC contain SUR and functional  $\text{K}_{\text{ATP}}$  channels, it is unknown whether these are required for the Glib effects described in this body of work, or whether, due to the functional diversity of  $\text{K}_{\text{ATP}}$  channels, the effects of Glib are limited to the MC. Furthermore, it is now evident that the majority (90%) of  $\text{K}_{\text{ATP}}$  are not localized on the plasma membrane, but intracellularly, in the endoplasmic (sarcolemmal) reticulum, mitochondria, and secretory granules (20, 46, 47). Thus it is also possible that the metabolic effects of sulfonylureas are independent of their well-described actions on the plasma membrane  $\text{K}_{\text{ATP}}$ . It is unknown whether the effects of Glib may be related to the presence of  $\alpha$ -endosulfine in MC and its upregulation under HG conditions (52). Thus, if upregulation of MC ENSA and  $\alpha$ -endosulfine contributes to the HG-induced increased extracellular matrix accumulation (10), Glib may displace  $\alpha$ -endosulfine from its receptors, acting as an exogenous competitive inhibitor of  $\alpha$ -endosulfine action.

In summary, this study demonstrates that chronic treatment of MC with low concentrations of the sulfonylurea Glib unequivocally prevents the extracellular matrix accumulation induced by prolonged exposure to HG via mechanisms involving inhibition of PAI-1 expression and enhanced extracellular matrix catabolism. These, and previous observations *in vivo*, raise the interesting possibility that long-term administration of low-dose Glib may prevent glomerulosclerosis in insulin-deficient diabetes, independently of the effects of glycemia.

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