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Effects of oral antihyperglycemic agents on extracellular matrix synthesis by mesangial cells

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Effects of oral antihyperglycemic agents on extracellular matrix synthesis by mesangial cells.

Background. Increased expression of the glucose transporter GLUT1 in mesangial cells (MCs) markedly stimulates glucose transport and the formation of extracellular matrix (ECM), even when ambient glucose concentrations are low. Certain antihyperglycemic agents cause GLUT1 overexpression and increase glucose transport in various tissues. However, their effects on the kidney are unknown. Because diabetic glomerulosclerosis is characterized by the accumulation of mesangial matrix, we studied the effects of antihyperglycemic agents on matrix metabolism in MCs cultured either in 8 or 20 mM glucose.

Methods. Membrane-associated GLUT1 was measured by immunoblotting. The initial rate of glucose transport was determined according to the 2-deoxy-D[^{14}C(U)]glucose uptake. Collagen metabolism was studied by metabolic radiolabeling with [^{14}C]-proline. Fibronectin in the medium was measured by ELISA. GLUT1 mRNA was estimated by Northern analysis.

Results. The sulfonylurea tolazamide increased GLUT1 protein expression by 107 and 69% in 8 and 20 mM glucose-grown cells, respectively. However, GLUT1 mRNA levels remained unchanged. Transporter-dependent deoxyglucose uptake was increased by tolazamide up to 184% in a dose-dependent fashion and was evident at both glucose concentrations after three or five days of exposure to the drug. Tolazamide significantly stimulated transforming growth factor-β1 (TGF-β1) secretion and the total synthesis of collagen and collagen and fibronectin accumulation in the medium of MCs maintained in high or low glucose concentrations. The biguanide metformin did not alter GLUT1 expression, glucose transport, fibronectin formation, or collagen metabolism, except at high concentrations.

Conclusion. Tolazamide markedly enhances ECM synthesis and accumulation in MCs probably by stimulating GLUT1 expression, glucose transport and TGF-β1 secretion, irrespective of the ambient glucose concentration. This effect was dose-dependent and minimally inducible by metformin.

Key words: diabetic nephropathy, glomerulosclerosis, sulfonylureas, metformin, collagen metabolism, TGF-β.

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Diabetic renal disease is characterized by the progressive accumulation of extracellular matrix (ECM) in the glomerular mesangium, mesangial expansion and inexorably worsening glomerulosclerosis [1, 2]. Disordered mesangial cell (MC) synthesis and catabolism of the components of ECM is the immediate cause for this glomerulopathy. Numerous studies have demonstrated that net synthesis of ECM by MCs is stimulated by increased ambient glucose concentrations in tissue culture [3–8], suggesting that poor glycemic control may play an important pathogenetic role in the mesangial matrix accumulation. The relevance of these *in vitro* observations to human disease is supported by clinical studies consistently demonstrating the beneficial effects of normalization of plasma glucose concentrations on the development and progression of glomerulosclerosis [9–14]. The effects of high glucose concentrations on ECM formation in tissue culture are persistent and independent of the osmotic contribution of the hexose [6]. There is growing evidence to indicate that the metabolism of glucose and the concurrent *de novo* synthesis of diacylglycerol by MCs is necessary to elicit changes in ECM formation [15].

In addition, there are also abundant reasons to believe that MC autocrine activation of TGF-β mediates the effects of high glucose concetration and utilization on ECM metabolism [16–18], and that this growth factor is a determinant in the development of human and experimental diabetic glomerulosclerosis [19–21].

Except for the unique case of specialized epithelial cells where glucose is concentrated by active Na^+/glucose cotransporters, this hexose enters most cells, including MCs, by passive facilitated diffusion. In this process, specific integral membrane proteins, the GLUT family of transporters, move glucose down its concentration gradient [22]. We and others have shown that rat glomeruli *in situ* express GLUTs 1, 3 and 4 [23, 24], and that GLUTs 1 and 4 are also demonstrable in rat MCs in tissue culture [24, 25]. Due to the presence of the insulin-responsive GLUT4, glucose

transport in MCs may be, at least in part, insulin-regulatable [26]. However, the functional importance of this transporter remains controversial [27]. GLUT1, constitutively expressed and primarily located on the cell surface, is the primary transporter responsible for basal uptake of glucose in most cells. Recent studies have suggested that increased glucose uptake via GLUT1 may be a major determinant of glucose utilization and ECM formation in MCs [25]. Indeed, the level of expression of this transporter is directly related to the rate of synthesis and net accumulation of ECM. When MCs overexpress GLUT1 protein following viral transduction by human *GLUT1* cDNA, the resulting increase in glucose uptake and utilization is associated with markedly enhanced net accumulation of ECM, despite the presence of low glucose concentrations. Interestingly, this increase in ECM exceeds even that observed in control MCs chronically exposed to 35 mM glucose. Because of GLUT1's potential pivotal role in MC glucose transport and thereby, ECM metabolism, factors regulating the expression of this transporter may contribute to the glomerulopathy associated with expansion of mesangial matrix.

The regulation of GLUT1 expression has not been explored in MCs, although it has been studied in non-renal tissues in which a wide range of agents acutely and chronically were shown to influence the cellular content and activity of this transporter [28]. The oral antihyperglycemic drugs commonly used in the treatment of type II diabetes affect GLUT1 expression to various degrees, depending on the tissue type and the specific agent [22]. Studies in 3T3-L1 adipocytes and L6 skeletal muscle cells have shown that chronic exposure of these to the sulfonylureas, tolbutamide and tolazamide, increases GLUT1 gene and protein expression. These changes were potentiated by insulin and closely associated with enhanced glucose transport [29, 30]. Similarly, in these cells and in fibroblasts, the biguanide metformin increased glucose transport by enhancing GLUT1 activity and/or its total cell content [31–35], and these effects were also enhanced by insulin [31, 35, 36]. Theoretically then, if the antihyperglycemics elicit similar effects in MCs *in vivo*, exposure to these agents in hyperinsulinemic forms of diabetes might potentiate MC glucose transport, with consequent expansion of mesangial matrix.

In this study, we investigated the effects of tolazamide and metformin on glucose uptake and ECM formation in cultured rat MCs, utilizing drug concentrations that have been previously demonstrated to heighten glucose transport in other cell types. These two drugs were selected because of their disparate chemical structures, metabolic effects and pharmacokinetics. Our results demonstrate that certain oral antihyperglycemic agents stimulate MC glucose uptake and ECM synthesis in a manner that is independent of the ambient glucose concentration.

METHODS

Materials

Purified rat fibronectin (Chemicon Int., Temecula, CA, USA) was used as standard in ELISA. The polyclonal antibody used for GLUT1 immunoblotting studies was generated in rabbit, and specifically reacted against a 13 amino acid carboxy-terminal peptide of this transporter isoform (East Acres Biologicals, Southbridge, MA, USA). PAGE separations were carried out on precast 10% minigels (Jule Inc., New Haven, CT, USA). An enzyme-linked alkaline phosphatase-labeled goat anti-rabbit IgG (Organon Teknika, West Chester, PA, USA) was used in ELISA. Neutralizing antibody to TGF-β1 (chicken anti-human) and recombinant human TGF-β1 were obtained from R&D Systems (Minneapolis, MN, USA). The rabbit anti-rat fibronectin polyclonal antibody (Chemicon) used in the ELISA did not cross-react with rat collagen I or laminin by immunoblotting or ELISA. All MC tissue culture media were based on a special RPMI 1640 formulation lacking glucose, glutamine and proline, and buffered with 25 mM Hepes (Life Technologies, Gaithersburg, MD, USA). Proline L-[^{14}C(U)] (286 mCi/mmol), proline L-[2, 3, 4, 5, -^{3}H] (112 Ci/mmol), hydroxyproline L-4-[^{3}H(G)] (10 Ci/mmol), and 2-deoxy D[^{14}C(U)]glucose (323 mCi/mmole), were purchased from Dupont NEN (Boston, MA, USA). The purity of the radioisotopic internal standards used in the quantitation of proline and hydroxyproline were established prior to their use by chromatographic analysis (*vide infra*). High purity collagenase VII (Sigma Chemical Co., St. Louis MO, USA) was used in the protein digestion assays. The columns used for HPLC were 4.6 mm $\times$ 25 cm Ultrasphere ODS, 5 μm particle size (Beckman Instruments Inc., San Ramon, CA, USA). Sodium tolazamide and metformin hydrochloride were generous gifts from Pharmacia Upjohn (Kalamazoo, MI, USA) and Bristol-Myers Squibb Co. (Princeton, NJ, USA), respectively.

Tissue culture

Mesangial cells were obtained from our previously characterized cloned cell line 16KC$_2$, derived from outgrowths of Fischer rat glomeruli [37]. These cells demonstrate a cytoskeleton of intermediate filaments containing vimentin, synthesize collagen types I and IV and express the Thy-1 antigen. MCs were seeded (10,000 cells/cm^2) into 59 cm^2 or 143 cm^2 plastic dishes for metabolic radiolabeling and GLUT1 protein measurements, respectively. Glucose uptake was studied in 8.5 cm^2 six-well plates (Corning, Corning, NY, USA). Cells were grown in the medium described above to which penicillin, streptomycin, 20% Nu Serum (Collaborative Research, Bedford, MD, USA), 8 mM glucose, 2.05 mM glutamine, and an amount of proline to provide a final concentration of 183 μM (including proline in Nu Serum), was added. The inclusion of Nu

Serum provided a final concentration of 4 μg/ml insulin. In studies in which the effects of high glucose concentration were investigated, cells were maintained in a 20 mM glucose-containing medium for 12 to 14 days before the experiments. Considering the high concentrations of glucose that commonly occur in the diabetic milieu (20 to 30 mM), a glucose concentration of 8 mM was considered as "normal." Indeed, lower concentrations of glucose do not support robust MC growth [3]. All experiments were terminated seven days post-seeding when cultures had just reached confluence.

Northern analysis

We used a modified standard method, obtaining total RNA from MC cultures with RNA Stat-60 (Tel-Test Inc., Friendswood, TX, USA) [25]. Twenty microgram-samples of total RNA were glyoxalated and fractionated on 1% agarose gels made in 10 mM sodium phosphate buffer. RNAs were immobilized on Genescreen membranes (Dupont NEN), prehybridized and probed with random oligomer-primed cDNAs of GLUT1 and β-tubulin in the presence of [α-^{32}P]dCTP (PRIME-1 kit; Sigma Chemical Co.). Following autoradiography at −70°C, films were analyzed by optical scanning densitometry (Scan Master 3+; Howtek Inc., Hudson NH, USA) using the NIH Image v. 1.6 software (Natl. Technical Information Service, Springfield, VA, USA). Relative quantities of GLUT1 mRNA in treated and control cells were normalized to their corresponding signal intensities for β-tubulin.

Immunoblotting of membrane-associated GLUT1

Cell layers were washed with ice-cold PBS and scraped into a 20 mM Hepes buffer, pH 7.4, containing 250 mM sucrose, 2 mM EDTA, 5 mM sodium azide, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin and 1 μM leupeptin. After homogenization in a Potter-Elvehjem tissue grinder, samples were centrifuged at 760 × g for 10 minutes to remove nuclei and unbroken cells. The resulting supernatant was centrifuged again at 142,000 × g for 60 minutes to obtain total cellular membrane particulates [38]. The membrane pellet was resuspended in 0.9 ml of 0.01 M Tris buffer, pH 7.5, containing 4 mM EDTA, and solubilized at room temperature for 30 minutes by the addition of 0.1 ml 10% (wt/vol) SDS. The solution was centrifuged at 10,000 × g for 10 minutes and an aliquot removed for protein quantitation. Immunoblotting analysis was carried out according to methods previously described for the study of GLUT1 [39]. Duplicate samples of 20 and 30 μg quantities of membrane protein were incubated for 30 minutes in 2% SDS Laemmli sample buffer at room temperature. After separation by SDS-PAGE, proteins were electrophoretically transferred to Hybond-ECL nitrocellulose membranes (Amersham Life Sciences, Arlington Heights, IL, USA). The primary antibody, polyclonal rabbit anti-GLUT1, was dissolved in 0.1 M Tris-buffered 0.9% NaCl containing 5% non-fat dry milk and incubated with the nitrocellulose membranes at 4°C for 18 hours. The secondary antibody, a horseradish peroxidase anti-rabbit-Ig conjugate (Amersham) was used in a chemiluminescence-based detection system based on the Luminol™ reaction (ECL Western blot kit; Amersham). The identification of GLUT1 bands was confirmed by preadsorption of anti-GLUT1 antiserum with 25 μg/ml of purified peptide. Quantitative analyses of the protein bands were carried out by optical scanning densitometry, as described above. All samples were analyzed in quadruplicate and the mean value of data at the two levels of electrophoresed membrane protein was considered as the final result.

Production of extracellular matrix components, metabolic radiolabeling and enzyme-linked immunosorbent assay

The metabolism of total collagen was studied as previously described [25, 40]. In brief, the culture medium was changed 24 hours before the start of the radiolabeling period to a medium lacking proline. Radiolabeling was carried out by incubation for 72 hours in an identical medium that also contained 0.15 mM β-aminopropionitrile, 210 μM ascorbic acid and 183 mM [^{14}C]-proline (82.3 mCi/mmole). In previous experiments we had demonstrated that [^{14}C]-proline incorporation into collagen increases linearly during 72 hours of radiolabeling [40]. At the termination of radiolabeling, the medium was rapidly aspirated, the plate placed on ice, and then 2 to 6 ml of ice-cold 0.2 N perchloric acid was poured onto the cell layer. Total protein in media samples was precipitated in 75% ethanol at −5°C. Following the addition of 89 μCi [^{3}H]-proline as an internal standard, supernatants were filtered in Centricon™-3 filters (Amicon Co., Danvers, MA, USA) and amino acids separated by solid phase extraction using Poly-Prep® AG50W-X8 (H^+) columns (Bio Rad Laboratories, Hercules, CA, USA). Purified amino acids were resuspended in 0.1 N HCl for subsequent determination of [^{14}C]-hydroxyproline, [^{14}C]-proline, total proline, and the calculation of proline specific radioactivity.

Net collagen accumulation in the medium was estimated by two independent methods that have been previously described in detail [25]. In the first, the rate of net collagen accumulation was measured according to ^{14}C incorporation into protein-associated hydroxyproline. In addition, quantitation of catabolic rates was also carried out in the same sample by determining ^{14}C incorporation into newly formed free hydroxyproline in the medium. The validity of these measurements of collagen metabolism has been well established [41, 42]. After cold ethanol precipitation, proteins were hydrolyzed and amino acids separated, as above, by solid phase extraction after the addition of 3.32 μCi [^{3}H]-hydroxyproline as an internal standard. These purified amino acids were subsequently analyzed for measurement of ^{14}C incorporation into hydroxyproline. In the second

method, the amount of total ^{14}C incorporated into collagenase-digestible protein was determined [43]. Following completion of metabolic radiolabeling, an aliquot of medium was mixed with a proteinase inhibitor solution (providing per milliliter: 3 μmole PMSF, 0.1 mmole EDTA, 40 μmole N-ethylmaleimide). Proteins were precipitated with ice-cold 10% TCA, and the pellet was extensively washed and resuspended in 1 N NaOH. After neutralization and adjusting the pH to 7.5 with 1 N Tris buffer solution, PMSF and N-ethylmaleimide were added in the same amounts as before, and $CaCl_2$ was added to provide a final concentration of 5 mM. For enzymatic digestion, the sample was divided into two equal portions and 140 U/ml of collagenase added to one of them while the other was used as a control. After incubation, the undigested protein was removed by precipitation and the ^{14}C radioactivities in the supernatants and protein precipitates were measured. Respective radiolabel incorporation into collagenase-digestible and collagenase-resistant protein was determined from the difference between treated and non-treated samples. To measure total collagen deposited in the cell layer, immediately after addition of cold 0.2 N perchloric acid, the cell layer was scraped, briefly homogenized in the cold and the precipitate separated by centrifugation. This precipitate was then, lipid-extracted and consecutively subjected to alkaline and acid hydrolysis for the measurement of total RNA, DNA [44] and the separation of protein. The final protein precipitate was hydrolyzed as described above, [^3H]-hydroxyproline internal standard added, and amino acids purified and separated for the quantitation of ^{14}C incorporation into proline and hydroxyproline as described for the medium protein.

Amino acids were analyzed as their precolumn-dansylated derivatives by reverse-phase high pressure liquid chromatography (HPLC) [40]. The column effluent was monitored for fluorescence (Spectroflow 980 fluorescence detector; Applied Biosystems, Ramsey, NJ, USA) at 350 nm excitation and 470 nm emission wavelengths, and 0.3 ml fractions were collected for measurement of ^3H and ^{14}C content. The recoveries of [^3H]-labeled proline and hydroxyproline were 43 to 77% and 70 to 96%, respectively.

The fibronectin that had accumulated in the culture medium was quantified by ELISA, using a procedure previously described [25], except that the Nu Serum contained in the medium was not removed. The amount of fibronectin-reacting material detected in the Nu Serum used for tissue culture was subtracted from all samples.

2-Deoxyglucose transport

The initial rate of glucose transport was determined, according to a modification of the method of Bashan et al, by using the non-metabolizable analog, 2-deoxy-D-glucose [45]. MC layers were rinsed and incubated for 10 minutes in a medium similar to that used for cell growth, but lacking glucose and Nu Serum (thus, without insulin) and containing 10 mg/ml BSA. After removal of the medium and washing the cell layers with 1 mg/ml BSA in Dulbecco's buffer (Gibco 14040-206; Life Technologies) at room temperature, cultures were incubated for three minutes in 1.5 ml/well of a solution containing a total of 10 μM 2-deoxy-D-glucose and 1 μCi/ml of 2-deoxy-D[^{14}C(U)]glucose in Dulbecco's buffer. Incubations were terminated by the addition of 1.5 ml ice-cold PBS containing 0.3 mM phloretin and 20 μM unlabeled 2-deoxy-D-glucose, and the rapid removal of the incubation solution from the culture well. After rapidly rinsing cultures three times with this solution, 1 N NaOH was added to the wells for incubation at room temperature for 60 minutes with gentle agitation to remove cell layers. This was followed by the transfer of the pooled contents of two wells to form one sample and completion of the protein digestion by an additional 60 minute incubation at 80°C. A portion of this final solution was used for measurement of protein and the remainder used for the determination of ^{14}C content. The amount of extracellular radioactivity present in the sample and the transporter-independent glucose uptake were determined in parallel culture wells in which rinsing with BSA-Dulbecco's buffer and incubation with radiolabeled 2-deoxyglucose were carried out in the presence of 10 μM of cytochalasin B, an inhibitor of glucose transporters.

Transforming growth factor-β bioassay

The production of bioactive TGF-β from mesangial cells was determined in a mink lung epithelial cell responder line, stably transfected with the human plasminogen activator inhibitor (PAI-1) gene fused to a luciferase reporter gene (kindly provided by Dr. D.B. Rifkin, New York University, New York, NY, USA). The promoter is responsive to active TGF-β. The assay was carried out as described by Abe et al [46]. In brief, transfected mink lung cells were seeded in a 96-well tissue culture dish (1.8×10^4/well) and allowed to attach for three hours at 37°C in a 5% CO_2 incubator. The medium was then replaced with 100 μl of test sample. Samples, both untreated for active TGF-β, and heat treated (80°C for 10 min) for total TGF-β (latent plus active), were used. Following overnight incubation at 37°C, the cells were washed twice with phosphate-buffered saline and extracted with 100 μl of lysis buffer (Promega, Madison, WI, USA). Twenty microliters of the lysate was transferred to a test tube and analyzed using a Optocomp 1 Luminometer (MGM Instruments Inc., Hamden, CT, USA) with 100 μl injections of luciferase reagents (Promega, kit E1500). Specificity was determined by the ability of TGF-β1 antibodies (R&D Systems) to block luciferase stimulation. All samples were analyzed in triplicate. Concentrations were determined from a standard curve, generated in each experiment using recombinant TGF-β1 (R&D Systems), and expressed as picograms per microgram of DNA.

Chemical measurements

Protein was measured by the method of Lowry using BSA as the standard. RNA was measured by the orcinol reaction for quantitation of its ribose content. In this method 1 μg of yeast RNA is equivalent to 0.6 μg of ribose [47]. DNA was measured spectrophotometrically by a two-wavelength ratio method [48] using calf-thymus DNA (Type I; Sigma Chemical Co.) as the reference standard. To calculate the amount of dansyl to be used in amino acid derivatization, total amino acids were quantified by a modified ninhydrin method [49], using L-leucine as the standard.

Expression of results and statistical analyses

Results of radiolabel incorporation, adjusted for internal standard recovery, were corrected for the specific radioactivity of the medium's proline, at the end of the incubation period, and expressed as nmole proline incorporated per milligram DNA. We previously demonstrated that calculation of the results of radiolabel incorporation according to the specific activity of proline in the endogenous pool or in the incubation media does not alter the differences between experimental groups, because changes in the endogenous pool of proline closely mirror those in the medium [40]. Net collagen accumulation in the culture medium was expressed as the proline incorporated into protein-associated hydroxyproline. In the alternate method, net collagen accumulation in the medium was measured as the total ^{14}C radioactivity incorporated into collagenase-digestible protein. Collagen catabolism was calculated as the amount of proline incorporated into newly formed free hydroxyproline in the medium. Total collagen synthesis was taken as the sum of the amounts of proline incorporated into all forms of hydroxyproline (medium free hydroxyproline and protein-associated hydroxyproline). Depending on the type of experiment, results were expressed as per unit protein in the cell layer, or as per unit DNA. With the methods utilized, the cellular content of DNA was 27.4 $\pm$ 1.74 pg. The densities of the bands in the immunoblotting and Northern analyses were expressed in arbitrary optical density units, and the final results presented as percent change from control values.

Statistical analyses were carried out using StatView® 4.0 software (Abacus Concepts Inc., Berkeley, CA, USA). Results were expressed as mean $\pm$ SEM. Differences between two groups were evaluated by Student's *t*-test for non-paired samples and the distribution of *t* in a two-tailed test. Results from experiments involving multiple groups were analyzed by analysis of variance. If a significant difference was found among groups, between group comparisons were made with *post-hoc* testing by a Fisher's protected least significant difference test. Statistical significance was set at the 5% level.

RESULTS

Expression of GLUT1 protein and GLUT1 mRNA

To investigate the influence of antihyperglycemic agents on the steady-state MC expression of membrane-associated GLUT1 transporters, the effects of a five-day exposure to tolazamide or metformin were evaluated. In addition, since the extracellular concentration of glucose could modify the action of these drugs, experiments were also carried out in cells cultured in media containing different glucose concentrations.

Compared to control MCs incubated in 8 mM glucose, prolonged exposure to a 20 mM concentration only caused a moderate, nonsignificant decrease in membrane-associated GLUT1 protein (Fig. 1). The presence of tolazamide resulted in a marked increase in GLUT1 that, upon comparison to the corresponding controls, was greater in 8 mM than in 20 mM glucose (107% vs. 69%, $P = 0.015$; Fig. 1). However, exposure to metformin at two different concentrations did not increase GLUT1 protein expression, whether MCs where incubated in 8 or 20 mM glucose (Fig. 2).

The level of GLUT1 mRNA was unaltered at the end of a two or five day period of tolazamide treatment in either 8 or 20 mM glucose, utilizing experimental conditions identical to those in which GLUT1 protein expression was elevated (Fig. 3).

Deoxyglucose transport

To determine whether the chronic changes in GLUT1 protein expression induced by tolazamide were also associated with increased glucose transporter activity, the initial rate (3 min) of 2-deoxyglucose uptake was determined in MCs previously treated for five days with increasing concentrations of tolazamide or metformin. Preliminary experiments had demonstrated that 2-deoxyglucose uptake increased linearly with time, at least for the initial five minutes of incubation. Tolazamide significantly increased 2-deoxyglucose transport by 184% over control values in a concentration-dependent manner (Fig. 4). Furthermore, the majority of this enhanced transport was due to the activity of GLUT transporters, as exemplified by the nearly complete suppression of the increased uptake in the presence of cytochalasin B. In contrast to tolazamide, metformin increased 2-deoxyglucose uptake only modestly (50%) and then, only at 500 μM, the highest concentration tested (Fig. 4). MCs chronically maintained in 20 mM glucose demonstrated a 46% greater transport of 2-deoxyglucose than cells grown in 8 mM glucose medium. Furthermore, tolazamide significantly increased glucose transport by 65 to 175% over control at either glucose concentration. This effect was maintained even after three and five days of exposure to the agent (Fig. 5). The effect of metformin on 2-deoxyglucose uptake by cells maintained in 20 mM glucose was essentially the same as for cells cultured in 8 mM

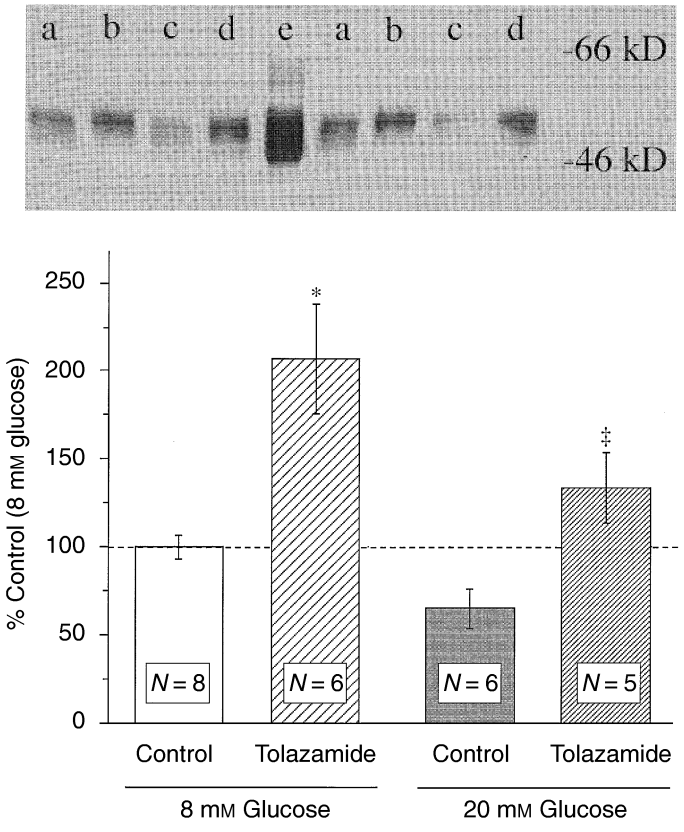

Fig. 1. Membrane-associated GLUT1 expression in mesangial cells treated with tolazamide. Mesangial cells in tissue culture were continuously grown in 8 mM glucose medium or maintained for a total of 13 to 14 days before termination of the experiment in 20 mM glucose medium. Cell layers were collected seven days post-seeding. Experimental groups were incubated with 1.5 mM tolazamide during the last 72 hours. Representative immunoblots of duplicate analyses of 20 μg cell membrane protein are presented (*top*) for each experimental group: (a), 8 mM glucose; (b), 8 mM glucose + tolazamide; (c), 20 mM glucose; (d), 20 mM glucose + tolazamide. A 2 μg protein sample of human erythrocyte membrane protein was included as a positive control (e). The final data presented (*bottom*) are the combined results obtained in three separate experiments. The number of samples is indicated, each sample having originated from the pooling of the cell membranes obtained from five culture dishes. Each individual sample was analyzed in quadruplicate and the mean taken as the final value. Results are expressed as percent change from 8 mM glucose control values. Values are mean ± SEM. $^{*}P < 0.001$ versus 8 mM control; $^{\ddagger}P = 0.022$ versus 20 mM control.

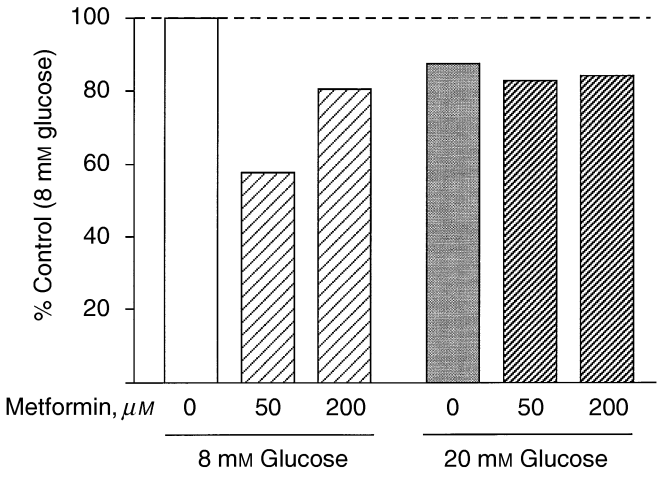

Fig. 2. Membrane-associated GLUT1 expression in mesangial cells treated with metformin. Mesangial cells in tissue culture were exposed to the concentrations of metformin noted under conditions identical to those in Figure 1. The data presented are the mean value of two samples generated in two separate experiments, each sample having originated from the pooling of the contents of five culture dishes.

glucose. That is, only at a concentration of 500 μM metformin was there a significant increase in uptake of 30% (20 mM glucose, Control, 34.0 ± 1.4; 0.05 mM metformin, 39.3 ± 1.4, $P = 0.52$; 0.5 mM metformin, 44.3 ± 2.4, pmole 2-deoxyglucose/mg protein/min, $P < 0.001$, $N = 8$ in all groups).

Production of extracellular matrix components

To analyze in detail how collagen metabolism might be altered by tolazamide or metformin, the synthesis and catabolism of collagen was studied in MCs cultured in media containing 8 or 20 mM glucose. The three day accumulation of collagenous protein and newly-formed free hydroxyproline in the medium was determined during the last three days of a total five-day exposure to the drugs. MCs exposed to 20 mM glucose demonstrated a 41% increase in collagen synthesis as compared to cells maintained in 8 mM glucose (Fig. 6). Although this enhancement of synthesis was also associated with a significant increase in the catabolic rate, the preponderance of the former resulted in a net 41% increase in the amount of collagen that had accumulated in the medium by the end of incubation. The presence of tolazamide caused a significant 40 to 42% stimulation of the collagen synthetic rate in MCs incubated in either 8 or 20 mM glucose (Fig. 6). In 8 mM glucose-incubated cells, this stimulation increased the synthetic rate to levels similar to those of non-treated MCs exposed to 20 mM glucose. Because this tolazamide-stimulated synthesis was associated only with small changes in catabolism (Fig. 6), the percentage of newly formed collagen catabolized by tolazamide-treated cells was significantly less than in controls (8 mM glucose, control, 44.8 ± 1.2%, $N = 6$; 8 mM glucose, tolazamide, 37.7 ± 0.4%, $N = 6$, $P < 0.0001$; 20 mM glucose, control, 44.9 ± 1.2%, $N = 6$; 20 mM glucose, tolazamide, 38.8 ± 1.2%, $N = 6$, $P = 0.0002$). As a consequence of this imbalance, treatment with tolazamide produced a marked net accumulation of collagen in the medium. The increments above control were 60% and 54% in 8 and 20 mM glucose, respectively (Fig. 6). Measurement of collagen accumulation at the end of incubation by another method, proline incorporation into the medium's collagenase-sensitive protein, yielded virtually identical results (8 mM glucose, control, 56.2 ± 13.6, $N = 6$; 8 mM glucose, tolazamide, 110.9 ± 19.5, $N = 5$, $P = 0.024$; 20 mM glucose, control, 153.5 ± 7.6, $N = 6$; 20

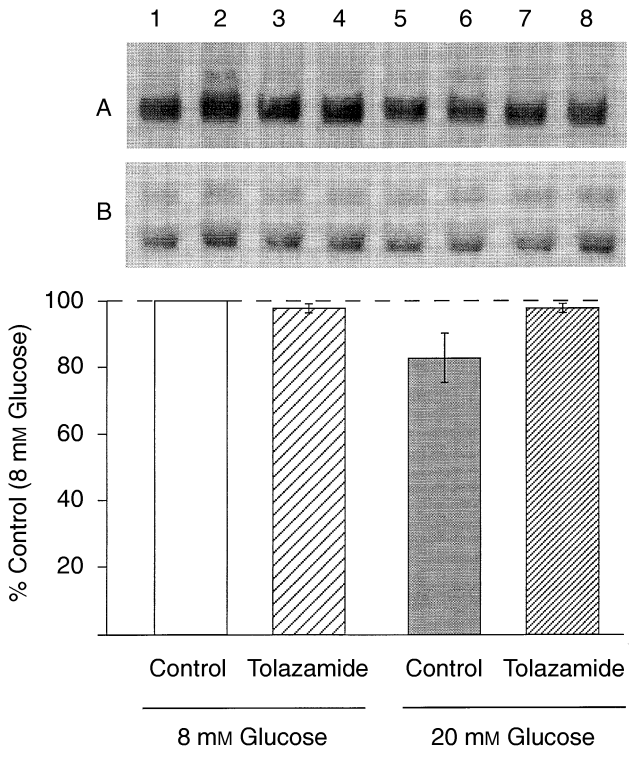

Fig. 3. Effects of tolazamide on mesangial cell GLUT1 mRNA level. Cells were grown in media with two different glucose concentrations and exposed to 1.5 mM tolazamide as in Figure 1. Total RNA was isolated for Northern analyses where GLUT1 mRNA appeared at 3.0 Kb. The autoradiograms were analyzed by optical scanning densitometry and values expressed as arbitrary units. All samples were analyzed in duplicate with normalization of the results according to the corresponding transcript levels for the housekeeping β-tubulin gene. The results presented are relative quantities of GLUT1 mRNA in treated and 20 mM glucose-incubated cells, with 8 mM glucose-incubated cells as a reference. Each value represents the mean ± SEM of three samples obtained in separate experiments. Each sample originated from the pooling of the contents of six culture dishes. A representative example of a pair of autoradiograms from the same sample is presented in panel A (GLUT1) and B (β-tubulin) in which duplicates corresponding to 8 mM glucose [1, 2], 8 mM glucose + tolazamide [3, 4], 20 mM glucose [5, 6], and 20 mM glucose + tolazamide incubated cells [7, 8] are shown.

mM glucose, tolazamide, 277.0 ± 19.1 nmole proline/mg DNA, $N = 6$, $P < 0.0001$).

Although the quantity of collagen deposited in the cell layer was only a small fraction of that accumulated in the medium (8 mM glucose-incubated cells, 6.1 ± 0.2%), the effects of high glucose and tolazamide were also evident in the cell layer, albeit the differences between groups were of lesser magnitude. The increase in the medium accumulation of collagen induced by 20 mM glucose did not achieve statistical significance (8 mM glucose, 3.34 ± 0.18, $N = 5$; 20 mM glucose, 3.79 ± 0.18, nmole proline/mg DNA, $N = 5$, $P = 0.201$). However, treatment with two different concentrations of tolazamide significantly increased collagen deposition over the levels shown in 20 mM glucose-incubated cells (1 mM tolazamide, 4.64 ± 0.45, $N = 4$, $P = 0.003$; 1.5 mM tolazamide, 4.30 ± 0.15 nmole proline/mg DNA, $N = 4$, $P = 0.018$).

The effect of tolazamide on cell growth was defined in terms of induced change in the RNA/DNA ratio. MCs incubated in a high glucose concentration demonstrated significant cellular hypertrophy (8 mM control, 219 ± 5, $N = 6$; 20 mM control, 254 ± 5, $N = 6$, $P = 0.0008$). In addition, exposure to 1.5 mM tolazamide further increased cell hypertrophy in both 8 (282 ± 9, $N = 6$, $P < 0.0001$) and 20 mM glucose (337 ± 4, $N = 6$, $P < 0.0001$).

The effect of metformin on MC collagen metabolism was studied utilizing the same experimental conditions as those described for tolazamide. MCs, cultured in 8 mM glucose and exposed to 200 μM metformin over a five-day period, did not demonstrate a change in the synthetic or catabolic rates, nor in the net accumulation of collagen in the medium (Fig. 7). In addition, this drug did not influence cell hypertrophy (RNA/DNA, control, 181 ± 15, $N = 8$; metformin, 178 ± 7, $N = 8$, $P = 0.31$). Because the metabolic effects of metformin may be only evident in cells incubated in a high glucose concentration [33], the net accumulation of total collagen in the medium was also explored in MCs grown in 20 mM glucose. Metformin, at either 200 or 500 μM concentrations, did not alter total collagen accumulation in the medium (Fig. 8). Furthermore, these results were confirmed by measurement of collagen accumulation according to the quantity of collagenase-sensitive protein in the medium (data not shown).

The effects of tolazamide and metformin on the synthesis of other ECM components were also studied by measuring fibronectin concentration in the medium at the completion of the same experiments above. As anticipated, the 72 hour accumulation of fibronectin in the medium was higher in control cultures containing 20 mM glucose than in cultures containing 8 mM glucose (Fig. 9). As demonstrated for total collagen, the addition of 1.5 mM tolazamide to the 8 mM glucose medium induced an increase in fibronectin accumulation that clearly exceeded the enhancement resulting from a high glucose concentration alone (Fig. 9). Correspondingly, the inclusion of tolazamide in the 20 mM glucose containing medium also induced a further increment in fibronectin accumulation (88% greater than in 20 mM controls), exceeding by 2.5-fold that quantity observed in 8 mM glucose controls (Fig. 9). In contrast to the effects of tolazamide, metformin only caused a 23% increase in fibronectin accumulation in cells that had been incubated in 20 mM glucose and this, as shown before for 2-deoxyglucose uptake, was only evident at a concentration of 500 μM (Fig. 8).

Finally, the dose-response relationship of the tolazamide-induced increment of ECM accumulation was studied also in MCs maintained in 8 mM glucose and exposed for five days to tolazamide in concentrations ranging from 0.1 to 1.5 mM. As shown in Figure 10, fibronectin and total collagen accumulated in the medium in parallel and in a

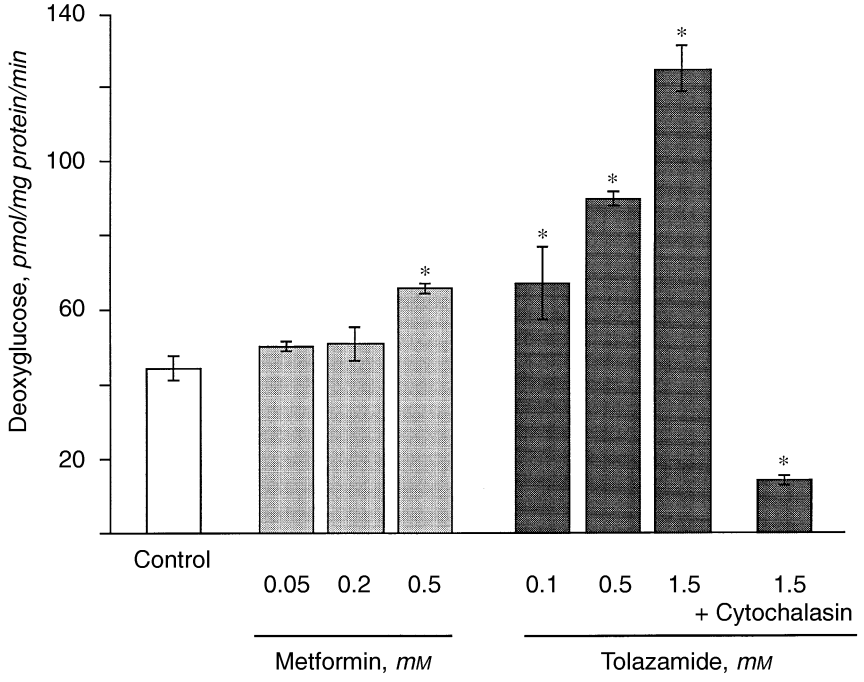

Fig. 4. Effects of metformin and tolazamide on mesangial cell 2-deoxyglucose uptake. All studies were carried out on cells continuously grown in 8 mM glucose and treated under conditions identical to those in Figure 1. Studies were carried out in cultures preincubated in a glucose-free buffer solution prior to the addition of 2-deoxy-D-[1-^{3}H]glucose. The results presented, including those for the 1.5 mM tolazamide/cytochalasin group, are values obtained after subtraction of the uptake in control cells treated with 10 μM cytochalasin (7.30 ± 0.88 pmol/mg protein/min, N = 12). Results are presented as the mean ± SEM, in control (N = 14) and in experimental groups (N = 4 to 8). *P < 0.001 versus control.

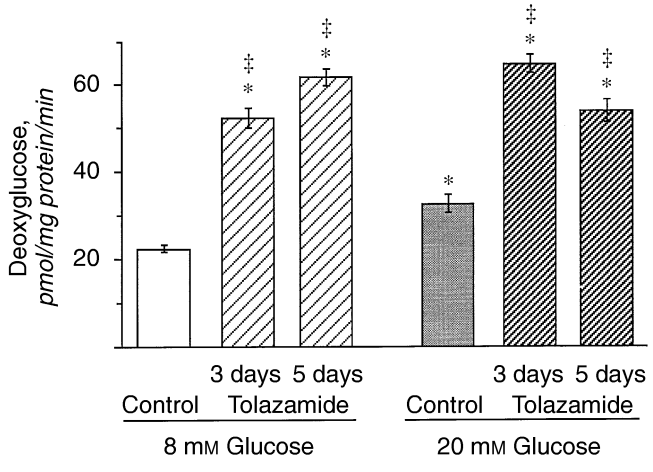

Fig. 5. Influence of medium glucose concentration and duration of exposure to tolazamide on the uptake of 2-deoxyglucose uptake by mesangial cells. Cells were grown in different concentrations of glucose as in Figure 1 and treated with 1.5 mM tolazamide during the last three or five days of the experiments. Measurements of the rate of 2-deoxyglucose uptake were carried out as in Figure 4. Results are presented as the mean ± SEM, N = 8 in all groups. *P < 0.001 versus 8 mM control; ‡P < 0.001 versus 20 mM control.

dose-dependent fashion, achieving maximal values at a tolazamide concentration of 1 mM.

TGF-β1 activity

To investigate whether the tolazamide-induced increase in glucose transport could have triggered enhanced autocrine TGF-β1 activity, even in circumstances of low glucose concentrations, the secretion of total TGF-β1 into the medium and its activation were determined in MCs continuously grown in 8 mM glucose following exposure to tolazamide. After five days of incubation in the presence of 1.5 mM tolazamide, the 24-hour secretion of total TGF-β1 (active and latent) and the medium concentration of the active form of TGF-β1 were increased by 95% and 94%, respectively (Fig. 11). Therefore, under identical experimental conditions, it was demonstrated that the long-term exposure of MCs to tolazamide induces a concomitant increase in glucose transport, TGF-β1 activity and ECM accumulation (Figs. 4, 6, 9 and 11).

DISCUSSION

Sulfonylureas, including tolbutamide, tolazamide, glipizide, glibenclamide and glimeperide are known to stimulate glucose transport in myocytes and adipocytes in tissue culture. This effect is persistent, concentration-dependent and demonstrable at drug concentrations within the therapeutic range [29, 30, 50–55]. Sulfonylureas stimulate the cellular uptake of glucose by potentiating the effect of insulin, however, they may also enhance the hexose's transport even in the absence of insulin [30, 53, 56]. The mechanism by which sulfonylureas alter glucose uptake has been attributed, in part, to increased GLUT1 activity [30, 55, 56]. In contrast to GLUT4, GLUT1 is located primarily at the cell surface [57] and demonstrates a lesser degree of cytosolic to membrane translocation following insulin stimulation. Thus, GLUT1 is generally considered hormonally insensitive [58]. However, under certain circumstances, such as exposure to stressful stimuli, augmentation of glucose transport can be accounted for by GLUT1 translocation from an intracellular pool to the plasma membrane

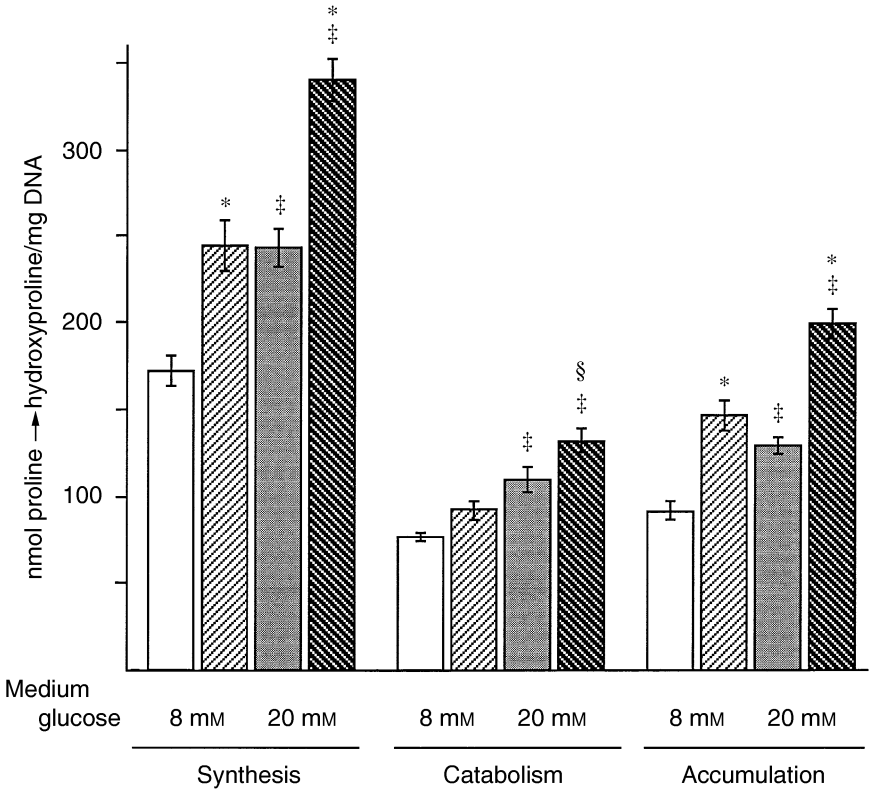

Fig. 6. Effects of medium glucose concentration and exposure to tolazamide on mesangial cell collagen metabolism. Mesangial cells were incubated in media containing two different glucose concentrations with and without the addition of 1.5 mM tolazamide as in Figure 1. Metabolic radiolabeling with [^{14}C]-proline was carried out during the last 72 hours of the experiment. Collagen catabolism and accumulation were measured according to the amount of proline incorporated into the medium's newly formed free hydroxyproline and protein-associated hydroxyproline, respectively. Total collagen synthesis was calculated from the sum of the amounts catabolized and accumulated. Each value represents the mean ± SEM of six samples. $^{*}P < 0.001$ versus corresponding 8 or 20 mM untreated control; $^{\ddagger}P < 0.001$ versus 8 mM control; $^{\S}P = 0.01$ versus 20 mM control. Symbols are: (□, ▨) control; (▨, ▧) 1.5 mM 1.5 mM tolazamide.

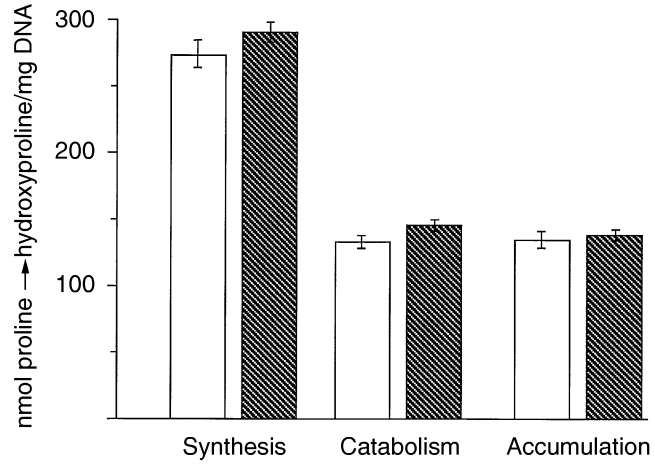

Fig. 7. Effects of metformin on mesangial cell collagen metabolism. Collagen metabolism was studied in the presence of 200 μM metformin in the medium following methods identical to those in Figure 6, except that all cultures were carried out in 8 mM glucose. Values are mean ± SEM, $N = 6$. Symbols are: (□) control; (▧) 200 μM metformin.

[59]. Conversely, increased cell GLUT1 content, in response to either inhibition of oxidative phosphorylation or exposure to calcium ionophore, has been attributed to enhanced gene transcription of GLUT1 and specific stabilization of its message [60, 61]. In adipocytes and myocytes, sulfonylureas have been shown to effectively increase GLUT1 expression and promote its membrane translocation [30, 56, 62]. Tolazamide specifically increases GLUT1 protein in a dose-dependent fashion at 0.3 to 1.8 mM concentrations in L6 skeletal muscle cells [29]. This effect is associated with increased levels of GLUT1 mRNA and is cycloheximide-inhibitable, implying that there is a stimulated synthesis of the transporter, rather than augmentation of its intrinsic activity [29].

Our study demonstrates that tolazamide concentrations between 0.1 and 1.5 mM increase glucose transport in MCs. In addition, the enhanced transport is equally evident in cells cultured in media containing either 8 or 20 mM glucose. This effect of tolazamide is sustained over prolonged periods and is equally prominent following three or five days of exposure to the drug. Because this change is inhibited by cytochalasin B and is also accompanied with increased membrane-associated GLUT1, the effect of tolazamide is most likely mediated through augmented transporter activity. Notably, the increase in GLUT1 protein induced by tolazamide, as with glucose transport, is similarly demonstrable after prolonged exposure to the agent and evident in cells grown at widely different glucose concentrations. In contrast to other cell types, the increased amount of transporter in MCs is not associated with altered steady-state levels of GLUT1 mRNA, suggesting that stabilization of GLUT1 transcripts or a decrease in catabolic rate of the GLUT1 protein may be responsible for this change [63]. Nevertheless, an enhanced membrane translocation, without an associated increase in transporter

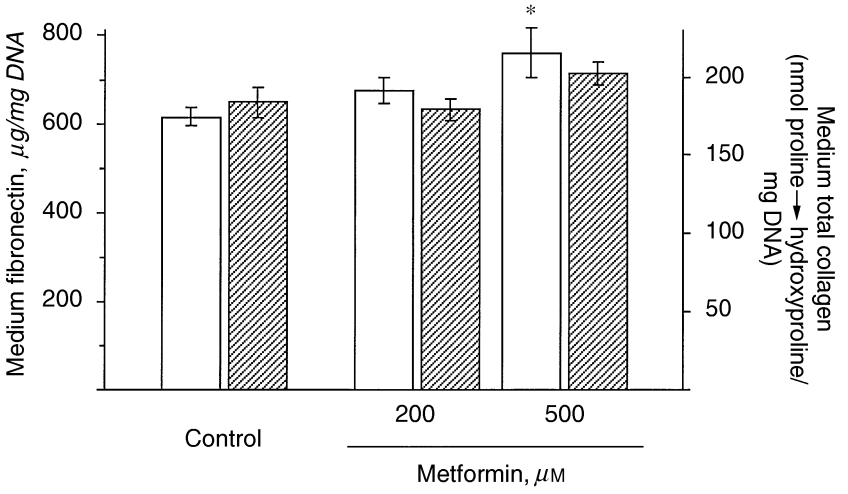

Fig. 8. Effects of metformin on the formation of fibronectin and collagen by mesangial cells cultured in 20 mM glucose concentration. The accumulation of total collagen in the medium was studied as in Figure 7. In addition, in the same experiment, the 72 hour formation of fibronectin was also evaluated according to its medium concentration. Cultures were exposed to the amounts of metformin noted during the last five days of the experiment. Each value represents the mean ± SEM of eight samples. *P = 0.016 versus 8 mM control. Symbols are: (□) fibronectin; (▨) collagen.

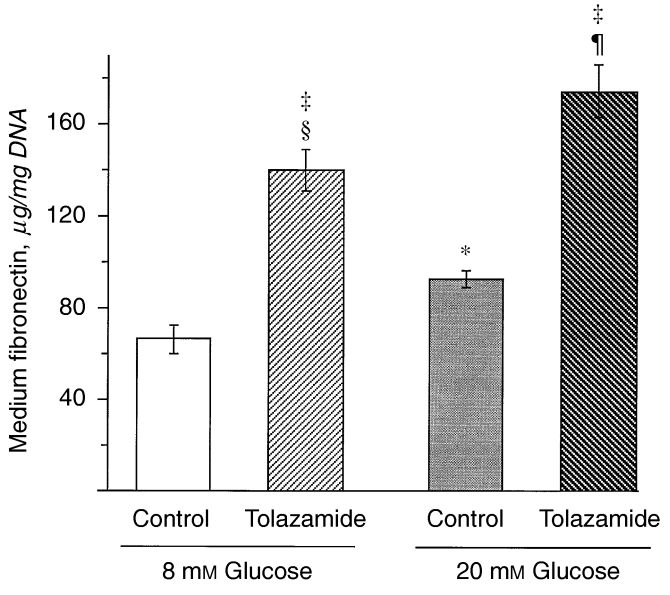

Fig. 9. Effects of medium glucose concentration and exposure to tolazamide on the formation of fibronectin by mesangial cells. The 72 hour accumulation of fibronectin was quantified in samples of media obtained from the same experiment presented in Figure 6. Each value represents the mean ± SEM of six samples. *P = 0.034 versus 8 mM control; ‡P < 0.001 versus own 8 or 20 mM untreated control; §P = 0.01 versus 20 mM control; ¶P < 0.001 versus 8 mM-tolazamide treated.

synthesis cannot be totally ruled out. In addition, tolazamide may have also enhanced the expresion and/or membrane translocation of GLUT4, as it has been described in cardiomyocytes and adipocytes exposed to the sulfonylurea, glimepiride [62, 64]. This could explain why cells incubated in 20 mM glucose and treated with 1.5 mM tolazamide synthesize more collagen than similarly treated 8 mM gucose-incubated cells, even though the latter demonstrate no additional increases in glucose transport or GLUT1 expresion.

Although most cell types demonstrate marked down-regulation of GLUT1 expression when exposed to high glucose concentrations [57, 65–67], MCs showed only a nonsignificant decrease in membrane-associated GLUT1 content, despite long-term maintenance in a 20 mM glucose medium. This result is in contrast to the findings of Heilig et al, who demonstrated that exposure of MCs to 20 mM glucose induced a 68% increase in GLUT1 protein [68]. This apparent discrepancy is likely explained by differences in GLUT1 distribution. In the present study, GLUT1 was measured in isolated membranes, however, Heilig et al measured transporter content in whole cell lysates. In any case, the MCs maintained in a high glucose concentration showed a small, but significant, increase in glucose transport. Theoretically, the lack of significant membrane-associated GLUT1 down-regulation may render MCs vulnerable to the metabolic effects of high glucose concentrations. One such adverse effect would be an exaggeration of ECM synthesis and its accumulation, as unequivocally demonstrated in our study and numerous others [3–8].

At relatively low glucose concentrations, tolazamide increased total collagen accumulation in the medium as a result of heightened synthesis, unmatched by a similar change in the catabolism of this matrix component. The magnitude of the changes in collagen metabolism attributed to tolazamide was similar to that following chronic exposure to high glucose concentrations. Notably, the addition of tolazamide to MCs maintained in high glucose concentration increased the exaggerated collagen synthesis and its accumulation even further. In addition, although the amount of collagen deposited in the cell layer was only a small fraction of the total synthesized, tolazamide also increased the quantity deposited in the cell layer. The tolazamide effect on fibronectin production was very similar to that seen for total collagen. Specifically, fibronectin accumulated in the culture medium in large quantities in MCs cultured at low and high glucose concentrations. In previous studies, we demonstrated that, in addition to

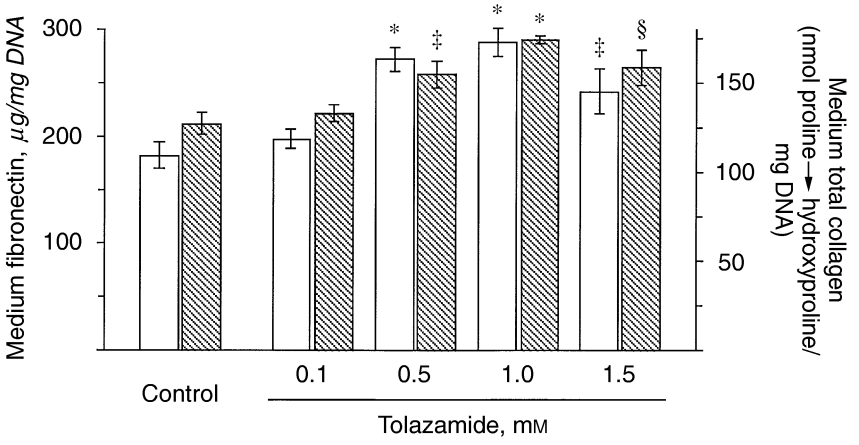

Fig. 10. Effects of increasing tolazamide concentrations on the formation of fibronectin (□) and collagen (▨) by mesangial cells. The accumulation of fibronectin and total collagen in the medium was quantified in cells cultured in 8 mM glucose as in Figures 7 and 9. Cultures were exposed to increasing amounts of tolazamide during the last five days of the experiment. Each value represents the mean ± SEM of six samples.*$P < 0.001$, ‡$P < 0.008$, and §$P < 0.003$ versus 8 mM control.

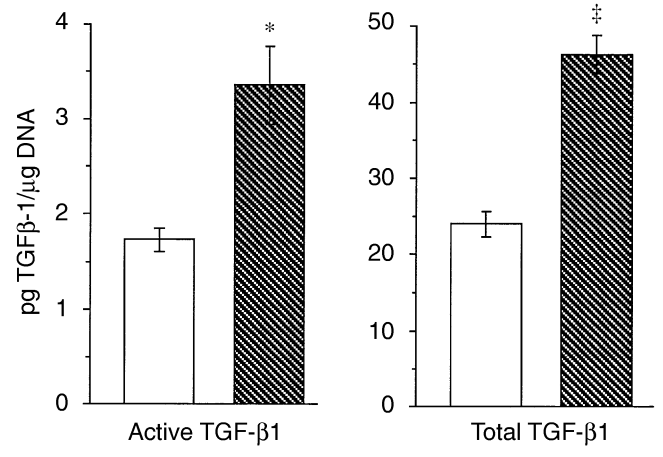

Fig. 11. Effect of tolazamide on the mesangial cell production of active and total TGF-β1. Mesangial cells were grown as in Figure 6 in a medium containing 8 mM glucose and treated with 1.5 mM tolazamide during the last five days of culture. Twenty-four hours before the termination of the experiment, the medium was replaced with a similar one, but containing 1% FCS in place of NuSerum. Active and total TGF-β1 24-hours secretion was determined in this medium using a mink lung epithelial cell line bioassay. Values are mean ± SE, $N = 6$. *$P < 0.02$ versus control; ‡$P < 0.001$ versus control. Symbols are: (□) control; (▨) tolazamide.

changes in ECM synthesis, exposure to high glucose concentrations or induction of GLUT1 overexpression in MCs results in cellular hypertrophy [25, 69]. In this study, tolazamide also resulted in MC hypertrophy, irrespective of the glucose level. Therefore, tolazamide appears to elicit a generalized increase in the accumulation of matrix components and cell hypertrophy that mimics and magnifies the metabolic effects of enhanced glucose uptake and high glucose concentration.

Concomitantly with increased glucose transport, tolazamide exposure also resulted in the stimulation of the secretion and activation of TGF-β1. Interestingly, this occurred at relatively low glucose concentrations, suggesting that the enhanced transport, and probably metabolism of the hexose, was the stimulus for increased TGF-β1 action. However, it is also known that certain growth factors, including TGF-β1, stimulate glucose uptake and the expression of GLUT1 [70, 71]. Therefore, it is also possible that the tolazamide-induced increase in glucose transport observed in MCs is a consequence of the stimulation of TGF-β1 activity by the sulfonylurea, rather than the converse. In any event, the data strongly implicates TGF-β1 action as a mediator in the enhanced collagen synthesis caused by tolazamide.

Metformin, a biguanide antihyperglycemic agent, may also increase insulin-stimulated glucose transport, cell membrane translocation of GLUT1 and total cellular GLUT1. These effects have been documented in human skeletal muscle and L6 cells, adipocytes and fibroblasts [31–33, 35, 36, 72]. The response to this agent is evident beyond 16 hours of incubation at concentrations of 8 to 200 μM and is characteristically enhanced by high glucose concentrations [31–33, 36, 72]. In addition, the metformin-induced changes are maintained, being still present after at least 16 days of treatment. In this study, MCs exposed to 200 μM metformin for a five-day period did not manifest changes in glucose transport, membrane-associated GLUT1 content, total collagen metabolism, or cell growth, regardless of glucose concentration. Only at 500 μM, a metformin concentration five- to tenfold higher than therapeutic plasma levels [73], was there a small increment in MC glucose uptake and fibronectin accumulation. These results imply that the metabolic effects induced by metformin may not be universal for all cell types.

The data presented here do not cast light on the mechanism(s) through which sulfonylureas alter MC metabolism. However, substantial progress has recently been made regarding the elucidation of the mode of action of these drugs in other cell types. Sulfonylureas are readily capable of penetrating cell membranes according to their lipid-solubility characteristics (tolazamide ≫ glibenclamide) [74]. Therefore, they may exert their actions at either membrane-bound or cytosolic loci. At the cell membrane sulfonylureas act by their interaction with ATP-sensitive K^+ channels (K_{ATP}). These channels, present in pancreatic

β, brain, skeletal muscle, and vascular and non-vascular smooth muscle cells, play an important role in the control of cell behavior by linking the cellular metabolic status to the membrane potential [75, 76]. K_{ATP} channels are formed by the intimate association of an ion channel subunit (an inwardly rectifying K^+ channel, K_{ir}) and a sulfonylurea receptor (SUR), a member of the transporter superfamily of ATP-binding cassette proteins [77–80]. Two SURs, have been identified to date, SUR1 and SUR2. The first is a high affinity receptor present in pancreatic β cells and likely the SUR also found in cerebral cortex and smooth muscle cells [81, 82]. The second, SUR2, a low affinity receptor, was initially described in heart and skeletal muscle [78], and exists in two isoforms, SUR2A and SUR2B [80]. SUR2A is expressed exclusively in mouse heart while SUR2B is ubiquitously expressed in all tissues, including the kidney. The presence of a specific isoform in a given tissue may determine its response to sulfonylureas, since it has been shown that SUR confers, in part, the ATP sensitivity and pharmacological characteristics of K_{ATP} channels [78]. Within the cell, sulfonylureas may also act at sites independent from those in relationship to the K_{ATP} channels described above. This could be related to the presence of an as yet uncharacterized pool of specific receptors to which internalized sulfonylureas might bind [83, 84]. Our recent demonstration of a functional, membrane-associated SUR2 in MCs suggests its involvement in mediating the metabolic effects of tolazamide [85].

Many aspects of MC function that had been initially demonstrated in tissue culture have been duplicated *in situ*, including their responses to specific vasoactive agents and growth factors. From the studies reported herein, it follows that sulfonylureas may enhance matrix deposition in the mesangium, thereby triggering and/or accelerating the development of glomerulosclerosis in diabetes. In addition, this adverse effect may occur despite acceptable levels of glycemia. Surprisingly, there are no studies available on the effects of sulfonylureas on the progression of diabetic glomerulosclerosis in animal models. By the same token, there is a lack of data regarding the influence of these drugs on the progression of human diabetic renal disease. In the largest prospective, randomized study available on vascular disease in type II diabetes, the degree of histological glomerular disease among individuals in groups receiving different treatment regimens was not analyzed separately [84]. Therefore, we conclude that performance of studies regarding the renal effects of antihyperglycemic agents in human and animal models of type II diabetes is an imperative.

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