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Jason I. Biederman

Edgard Vera

Rohit Rankhaniya

Clare Hassett

Giovanna Giannico

See next page for additional authors

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Effects of sulfonylureas, α-endosulfine counterparts, on glomerulosclerosis in type 1 and type 2 models of diabetes

JASON I. BIEDERMAN, EDGARD VERA, ROHIT RANKHANIYA, CLARE HASSETT, GIOVANNA GIANNICO, JERRY YEE, and PEDRO CORTES

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

Effects of sulfonylureas, α-endosulfine counterparts, on glomerulosclerosis in type 1 and type 2 models of diabetes.

Background. Previously, we showed the expression of a unique sulfonylurea receptor (SUR) and its putative endogenous ligand, α-endosulfine, in mesangial cells and isolated glomeruli. Further, this ligand was up-regulated by high glucose concentration. To investigate the possible role of α-endosulfine up-regulation in diabetes, we administered sulfonylureas, the exogenous ligands of SUR, to diabetic animals.

Methods. In streptozotocin-induced, insulin-deficient, diabetic rats, glomerulosclerosis, albuminuria, glomerular expression of fibronectin mRNA, and glomerular filtration rate (GFR) were studied for various periods up to 36 weeks. Several rat groups received either glibenclamide or tolazamide during the entire study period. Also, glomerulosclerosis and albuminuria were determined in insulin-resistant db/db mice, at 26 weeks of treatment with tolazamide.

Results. Sulfonylureas did not improve hyperglycemia or reduce glycosylated hemoglobin levels. In insulin-deficient diabetic rats, sulfonylureas significantly decreased the degree of glomerulosclerosis and completely reversed the enhanced albumin excretion. Also, glibenclamide reduced diabetes-induced glomerular overexpression of fibronectin mRNA. Because glibenclamide may improve the afferent arteriolar dilatation of diabetes, thereby reducing glomerular hyperfiltration, its effect on GFR was determined. Glibenclamide did not alter glomerular hyperfiltration or renal hypertrophy, regardless of the intensity of hyperglycemia. Finally, in insulin-resistant mice, tolazamide did not alter the extent of diabetic glomerulosclerosis or increased albuminuria.

Conclusion. Long-term treatment with sulfonylureas completely prevents glomerular injury in insulin-deficient diabetes in rats. However, this protective effect is not demonstrable in an insulin-resistant model of the disease. We postulate that mesangial α-endosulfine up-regulation in the hyperglycemic milieu of insulin-deficient diabetes may retard glomerular extracellular matrix formation and mesangial expansion.

Key words: diabetic glomerulosclerosis, sulfonylureas, endosulfine, insulin-deficiency.

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Sulfonylureas act on adenosine triphosphate (ATP)-sensitive K^+ channels (K_{ATP}). The membrane-bound pancreatic β cell K_{ATP} has been most extensively studied and represents the "classic" KATP [1–3]. Classic K_{ATPs} consist of two structurally unrelated subunits: a potassium pore and a sulfonylurea receptor (SUR). The pore belongs to the Kir6.x subfamily of weak inwardly rectifying K^+ channels while SUR is a member of the ATP-binding cassette (ABC) superfamily [4, 5]. Functional β cell KATPs are tetradimers of Kir.6.2/SUR1 [6] in which Kir6.2 confers K_{ATP} inhibition by ATP and SUR increases pore sensitivity to ATP, and regulates channel activation by magnesium adenosine diphosphate (MgADP) and sulfonylureas [3]. Thus, sulfonylurea binding to SUR inhibits the channel and closes the ion pore. The consequent cell membrane depolarization and Ca^{2+} influx through voltage-gated channels mediates insulin secretion.

Nearly 90% of the cellular K_{ATPs} are not localized to the plasmalemma, but to the endoplasmic reticulum, mitochondria, and secretory granules [7–10]. Consequently, nearly 70% of all sulfonylurea binding is cytosolic, and this intracellular locus of action of K_{ATP} appears to be an important determinant of the action of sulfonylureas [11]. Notably, the sulfonylurea membrane-binding site of the β cell K_{ATP} is cytosolic, implying the requisite membrane translocation of exogenously applied sulfonylurea to invoke its action [12] and these observations reconcile the greater potency of the more highly lipophilic sulfonylurea

compounds [13, 14]. Thus, all SUR ligands are intracellular.

The extrapancreatic SUR isoforms are the low-affinity SUR2 isoforms SUR2A and SUR2B [15–17]. SUR2B is widely expressed in the kidney, particularly in proximal tubule, ascending limb, and the collecting duct where it presumably mediates, in part, K^+ transport [18–20]. In addition, high- and low-affinity sulfonylurea-binding components are also present in isolated, metabolically active glomeruli [21]. In regard to glomerular vascular reactivity, the sulfonylurea glibenclamide reverses the hypocontractility of diazoxide-dilated afferent arterioles via K_{ATP} activation [22], and in streptozotocin-induced diabetic animals, glibenclamide produced a concentration-dependent decrease in the pathologically exaggerated afferent arteriolar diameter [23]. Therefore, sulfonylureas may potentially induce renal metabolic and functional effects that include amelioration of diabetic glomerular hypertension.

Pharmacologic studies have demonstrated the diversity of K_{ATP} among various tissues, according to their sulfonylurea affinity and sensitivity to K^+ channel openers [24]. These heterogeneous functional characteristics are based upon differing complexations of Kir6.x and SUR isoforms. Recently, we characterized a functional rat mesangial K_{ATP} containing a previously undescribed rat mesangial SUR2 splice variant [25, 26]. Expression of this novel mesangial SUR2 protein was shown in primary and cloned mesangial cell lines (16KC2) with a specific antibody that reacted with the common C-terminal epitope of SUR2A and SUR2B.

α-Endosulfine, a putative endogenous ligand of SUR, has been recently isolated [27, 28]. The gene encoding this protein (ENSA) is constitutively expressed in many tissues [29, 30], and α-endosulfine represents one of a family of neuronal cyclic adenosine monophosphate (AMP)-regulated phosphoproteins (ARPPs). These proteins are without known enzymatic activity, but possess characteristics common to intracellular proteins that alter the activities of enzymes involved in signal transduction [e.g., calmodulin and the protein kinase A inhibitor (Walsh inhibitor) protein kinase I] [31]. Of these ARPPs, two closely related isoforms have been characterized by apparent molecular mass (ARPP-16 and ARPP-19) [31], and these are capable of competitive inhibition of sulfonylurea binding to its target tissues [29, 30]. To date, only the ARPP-19/e, α-endosulfine, has been well characterized, although its actions remain largely unknown [31]. It has been proposed that α-endosulfine does not mediate its actions in an autocrine or paracrine fashion but by regulating intracellular signaling [32–34].

In recent work, we have demonstrated the ENSA and α-endosulfine expression in rat glomeruli and cultured rat mesangial cells [35]. Further, gene and protein expression were up-regulated in parallel during the exposure of mesangial cells to a high glucose concentration. Therefore, glomerular cells express all the components of an α-endosulfine/K_{ATP} system that is regulatable by glucose. In the present study, the potential glomerular effects of α-endosulfine in vivo have been explored by mimicking its up-regulation by the chronic administration of a sulfonylurea. Because sulfonylureas are not expected to enhance insulin secretion in diabetes induced by pancreatic β-cell injury with streptozotocin, this experimental model offered the opportunity of studying the direct glomerular effects of these drugs in the absence of the confounding metabolic effects of hyperinsulinemia. In addition, since sulfonylureas are extensively used in the treatment of type 2 diabetes, their effects in an insulin-resistant animal model of this disease, db/db mice, were also investigated.

METHODS

Materials

Streptozotocin, glibenclamide, tolazamide, fluorescein isothiocyanate (FITC)-inulin, dextran T-40, ribonucleoside vanadyl complex, and total glycated hemoglobin kits (#442) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Novolin N (NPH) (human insulin isophane suspension) and Linplant® insulin implants were purchased from Novo Nordisk Pharmaceutical Industries (Clayton, NC, USA) and Linshin Canada Inc. (Scarborough, Ontario, Canada), respectively. Methohexital (Brevital®) and isofluorane (Forane®) were obtained from Eli Lilly & Co. (Indianapolis, IN, USA) and Baxter (Deerfield, IL, USA), respectively. Albuminuria was assessed with the Nephrat® and the Creatinine Companion® kits obtained from Exocell Inc. (Philadelphia, PA, USA). Rabbit antirat albumin antibody was purchased from ICN Biomedical (Aurora, OH, USA). Ambion (Austin, TX, USA) supplied the RNase inhibitor SUPERaseIN. Qiagen Inc. (Valencia, CA, USA) provided the following kits for mRNA purification and quantification: RNeasy Mini Kit, RNase-free DNase Set, and Quantitect Probe RT-PCR Kit. Primers and probes were purchased from TIB-Molbiol LLC (Adelphia, NJ, USA).

Animals

Simonsen Laboratories (Gilroy, CA, USA) and Jackson Laboratories (Bar Harbor, ME, USA) supplied the rats and mice, respectively. Studies were carried out according to protocols approved by the Institutional Animal Care and Use Committee of Henry Ford Health Sciences Center. Insulin-deficient diabetes was induced in male Munich-Wistar rats at age 4 to 5 weeks old by a single intravenous dose of 55 mg/kg streptozotocin, dissolved in sodium citrate buffer-acidified normal saline, via the dorsal penile vein. This dose of streptozotocin results in significantly impaired ability to produce insulin and moderate to severe hyperglycemia. Long-term survival in most animals occurs without insulin treatment.

Control animals received the same volume of solution vehicle. Postprandial (morning) blood glucose and body weight measurements were obtained twice weekly for 2 weeks then once every 3 to 4 weeks during the rest of the study. In renal function and gene expression studies, a blood sample was obtained for determination of glycosylated hemoglobin at the completion of the observation period. Genetically diabetic male obese mice (C57BLKS db/db) and their nondiabetic heterozygous lean controls (db/+) entered the study at 4 weeks old, when obesity and hyperglycemia become evident in the diabetic group. Blood glucose measurements were obtained during weeks 3 to 4 and at conclusion of experiments.

Treatment protocols

Group 1 rats not regularly treated with insulin. The goal was to maintain a chronic significant hyperglycemia, ranging from 350 to 500 mg/dL. Rats in group 1 were used as diabetic controls in renal function studies, in glomerular gene expression quantification studies at 14 and 22 weeks of diabetes, respectively, and in histologic studies at 36 weeks after disease induction. Since glomerular hyperfiltration has been reported at various intervals between 1 and 44 weeks [36, 37] after streptozotocin injection, renal functional changes and hypertrophy were expected to be well established by 14 weeks of diabetes, when there is no significant glomerular injury. Similarly, a period of 22 weeks was selected for gene expression quantification because glomerular extracellular matrix mRNA expression has been reported to be increased between 16 and 24 weeks of experimental diabetes in rats and before the onset of glomerulosclerosis [38, 39]. Histologic studies were designed to evaluate the initial manifestations of morphologic glomerular disease. Although mesangial expansion is morphometrically detectable at 24 weeks after induction of the disease [40], a difference in the prevalence of glomerulosclerosis between diabetic animals and age-matched controls has only been reported by light microscopy at 38 to 56 weeks of the disease in animals partially treated with insulin [41, 42]. Histologic studies were not carried out at longer periods of diabetes because after 36 weeks there is a rapid decline in the survival of animals not regularly treated with insulin.

Although the dose of streptozotocin employed resulted in hyperglycemia levels that were within the desired range without insulin administration, 15% of the animals presented, a twice-confirmed blood glucose level of 600 mg/dL or greater during the last 4 to 8 weeks of the study. To maintain a similar degree of hyperglycemia in all animals and to avoid malnutrition and weight loss during long-term diabetes, these severely hyperglycemic animals received a single, sustained-release subcutaneous insulin implant (one third of a 7 mm long cylindric Linplant®. This implant reduced blood glucose to levels of 350 to 450 mg/dL for the remainder of the observation period. All other animals did not receive insulin at any time during the study.

Animals in group 1 were divided into the following treatment groups: (*1*) diabetic rats treated with the sulfonylurea tolazamide, (*2*) diabetic rats treated with the sulfonylurea glibenclamide, (*3*) diabetic rats given the same volume of vehicle, and (*4*) age-matched control rats given the same volume of solution vehicle. As part of this vehicle, animals in all groups received a total of 40 μL/day of dimethyl sulfoxide (DMSO). All animals received sulfonylureas or the solution vehicle by daily gavage from day 2 after injection of streptozotocin or acidified saline. Sulfonylureas were suspended in DMSO, further diluted in water and given as a 0.5 mL single daily dose. Tolazamide and glibenclamide were administered either in doses within the human pharmacologic ranges of 14 mg/kg and 0.28 mg/kg, respectively, or at ninefold greater amounts (128 mg/kg and 2.52 mg/kg, respectively).

Group 2 rats regularly treated with insulin to induce moderate hyperglycemia. The goal was to induce a moderate chronic hyperglycemia ranging from 220 to 320 mg/dL. This was achieved by daily subcutaneous administration of 3 to 7 U of Novolin N insulin. Rats in group 2 were used in renal function studies. Animals were divided into the following treatment groups: (*1*) diabetic rats treated with 0.28 mg/kg glibenclamide, and (*2*) diabetic rats given the same volume of vehicle. Glibenclamide and solution vehicle were administered by gavage as above.

Group 3 insulin-resistant diabetic db/db mice not treated with insulin. The goal was to maintain significant chronic hyperglycemia. No animals required insulin administration as blood glucose concentrations remained lower than 600 mg/dL. Animals in group 3 were used for histologic analysis and albuminuria determinations at 18 and 26 weeks after study entry. Light microscopy studies were carried out at these periods of diabetes because previous observations by others only detected minimal mesangial expansion at shorter intervals (10 to 12 weeks) [43, 44]. Treatments were initiated at the second week after entry into the study and maintained for the duration of the experimental period. Mice were divided into the following groups: (*1*) diabetic tolazamide-treated by gavage with 14 mg/kg or 128 mg/kg of the drug dissolved in 0.1 mL of vehicle, (*2*) diabetic treated with the same volume of drug-solution vehicle, and (*3*) age-matched, lean heterozygous controls, gavaged with 0.1 mL vehicle.

Glomerular microdissection and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Rat glomeruli were microdissected from the left renal cortex for total RNA isolation, according to a modification of a previously described method [45]. Following

isofluorane/oxygen anesthesia, retrograde perfusion of left kidneys with 50 mL of ice-cold Hank's balanced salt solution (HBSS), containing 3.2% dextran T40, was carried out via a needle placed in the abdominal aorta below the renal arteries. Four sagittal slices from the left kidney were obtained and placed in 0.8 μm pore-filtered, ice-cold HBSS containing 5% ribonucleoside vanadyl complex. A total of 160 glomeruli were microdissected in less than 25 minutes and transferred into 50 μL of sterile, phosphate-buffered saline (PBS) containing 2 U/μL of RNase inhibitor. Following the addition of 350 μL of lysis buffer (buffer RLT) (RNeasy Mini Kit), samples were rapidly homogenized twice with 15 seconds with a rotor-strator homogenizer fitted with a 5 mm diameter probe (Omni μH Micro Homogenizer) (Omni International Inc., Warrenton, VA, USA) and stored at −70°C. Total glomerular RNA was isolated from glomerular homogenates using the Qiagen's RNeasy Mini Kit, according to the manufacturer's instructions. This isolation included an on-column DNase digestion step. RNA was stored at −70°C prior to RT-PCR.

Rapid cycle, real-time, quantitative RT-PCR was carried out using the Roche LightCycler (Roche Molecular Biochemicals, Indianapolis, IN, USA), in a multiplex, asymmetric reaction [46]. In this method, a one-step protocol was followed with the RT and PCR reactions carried out sequentially in the same reaction capillary. A commercially available reaction mix was used (QuantiTect™ Probe RT-PCR) supplemented with 2.5 U of Hot Star Taq DNA polymerase (Qiagen Inc.) [47]. In multiplex reactions, mRNA templates of interest, fibronectin and β-actin mRNA, were simultaneously amplified with specific primers following reverse transcription. Product formation was detected by fluorescence resonance energy transfer (FRET) reactions, using sequence-specific pairs of hybridization probes labeled either with fluorescein or with one of two different acceptor dyes (LC-Red 640 and LC-Red 705). All primers were specifically designed for intron-exon splice regions thereby preventing any possible genomic DNA amplification. β-actin was selected as the gene of reference because of the proven stability of its expression in diabetic glomeruli [48]. Primers and probes were for fibronectin, 5′-GAGCCTT CACACATCACCAAGTA, 5′-CATCTCCTTCCTCGC TCAGTT, 5′-CAAAGCGAGTCACTTCTTGGTGCC C, and 5′-LC-Red 640-TACTGCTGGATGCTGATGA GCTGTCCC; for β-actin, 5′-ACCCACACTGTGCCC ATCTA, 5′-GCCACAGGATTCCATACCCA, 5′-GCC ACGCTCGGTCAGGATCTTCAT, and 5′-LC Red 705-AGGTAGTCTGTCAGGTCCCGGCCA. Values were calculated as arbitrary units according to results obtained from standard curves obtained with total renal cortex RNA, and final data was expressed as the fibronectin mRNA/β-actin mRNA ratio in the same reaction. All samples were analyzed in triplicate.

Renal function

Conscious, unrestrained rats had glomerular filtration rates (GFRs) determined by a nonradioisotopic method that combined previously described techniques [49, 50]. Osmotic minipumps, containing 10 mg/mL of FITC-inulin diluted in PBS, were implanted subcutaneously in rats under 37.5 mg/kg Brevital® anesthesia. These minipumps have a nominal continuous infusion rate of 10 μL/hour over 7 days, delivering 1.67 μg/minute of FITC-inulin. Two days after minipump implantation, rats were placed in metabolic cages, acclimatized for 24 hours and then two successive 24-hour urine collections were obtained. Following washing of rat tails to remove any urinary contamination, tail vein blood samples (80 μL) were obtained before and after each urine collection. Plasma and urine samples were buffered by an equal volume of 1 mmol/L Hepes (pH 7.4). Inulin concentrations were determined in duplicate, 10 μL samples by measuring fluorescence at wavelength of 480 nm, using the real-time fluorimetry function of the LightCycler. Standard solutions of FITC-inulin in PBS yielded linear fluorescence between 1 μg/mL and 20 μg/mL. Plasma and urine blanks were included to correct for background fluorescence. GFR was calculated according to the excretion rate and mean plasma concentration of FITC-inulin. Final results were the mean value of the two urine and plasma collection periods. Kidneys were weighed at the conclusion of the study.

Renal histology

Rat and mouse kidneys were perfused as above, except that perfusion lasted for 2 minutes with HBSS perfusate at 37°C. In addition, perfusion volumes were reduced to one tenth in mice. Also, immediately following this initial period, a second perfusion with Tyrode's buffer (pH 7.4), containing 22.5% dextran T-40 and 3.7% paraformaldehyde at 37°C, was carried out for 3 minutes for in situ fixation of the tissue. The left kidney was then removed and three, 1 mm thick slices, perpendicular to the longest kidney axis, were obtained and placed in 3.7% paraformaldehyde in PBS at 4°C. Tissue slices were embedded in paraffin and 4 μm sections stained with periodic acid-Schiff (PAS). The degree of mesangial expansion and glomerulosclerosis was semiquantitatively evaluated according to a scoring system [51]. Three observers, masked as to the origin of the sample, independently scored the degree of mesangial expansion or sclerosis on a scale of 0 (no lesion), 1+ (minimal mesangial expansion), 2+ (evident mesangial expansion and/or basement membrane thickening), 3+ (marked mesangial expansion with occasional sclerosed lobules), and 4+ (sclerosis involving most of the glomerular tuft). Results were expressed as prevalence of sclerosis (number of glomeruli with ≥2+ sclerosis

Table 1. Characteristics of the rat experimental groups included in histologic studies[a]

	Control			Diabetic		
Group Treatment	None	None	Tolazimide (14 mg/kg)	Tolazamide (128 mg/kg)	Glibenclamide (0.28 mg/kg)	Glibenclamide (2.52 mg/kg)
Final number	14	57	22	21	21	24
Blood glucose (9 months mean ± SE)	94 ± 1.3[b]	422 ± 9.6	416 ± 7.7	434 ± 6.7	409 ± 6.8	391 ± 7.0[c]
Final weight	386 ± 5.3	279 ± 5.8[b]	222 ± 7.0[d]	219 ± 4.8[d]	231 ± 6.4[d]	237 ± 4.4[d]

[a]Values are mean ± SE.
[b]Blood glucose concentration and body weight in all diabetic groups vs. control ($P < 0.0001$).
[c]Blood glucose concentration vs. nontreated diabetic group ($P = 0.013$).
[d]Body weight vs. nontreated diabetic group ($P < 0.001$).

divided by the total number of glomeruli scored in a given specimen).

Enzyme-linked immunosorbent assay (ELISA) and immunoblotting of urinary proteins

Rat and mouse urinary albumin was determined from aliquots of 24-hour urine collections by a colorimetric direct competitive ELISA (Nephrat®). In rats, urinary protein identification was carried out in pooled collections from four animals in each experimental group that were dialyzed against PBS and concentrated in Centriprep™-10 concentrators (Amicon, Beverly, MA, USA). Equal amounts of concentrated protein (20 μg) were dissolved in Laemmli sample buffer and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on Tris-glycine, 7.5% acrylamide gels, with proteins identified by Coumassie Blue staining. In parallel gels, resolved proteins were also transferred to a polyvinyldene fluoride (PVDF) membranes and blocked with TBS-T (Tris-buffered saline, pH 7.4, containing 0.05% Tween-20 and 5% skimmed milk powder). Membranes were incubated overnight at 4°C with antirat albumin antibody, diluted in TBS-T. Bound antibody detection was carried out using the enhanced chemiluminescence (ECL) analysis system (Amersham Pharmacia Biotech UK, Little Chalfont, Buckinghamshire, England).

Chemical analyses

Urinary creatinine was determined using the Creatinine Companion®, a colorimetric modified alkaline picrate method. The percent glycated hemoglobin content was determined in hemolyzed whole blood samples by affinity column separation. Total protein was quantified with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Statistical analysis

The main statistical analyses were carried out using the StatView® 5.0.1 software (Abacus Concepts Inc., Berkeley, CA, USA). Interrater reliability for the mean from three raters [52] was computed using a SAS macro (INTRACC) (SAS Institute, Cary, NC, USA). Except where indicated, results were expressed as mean ± SE and analyzed by analysis of variance. If significant differences were found among groups, between group comparisons were made with post-hoc testing by Fisher's protected least significant difference (PSLD) test, with significance determined at the 5% level.

RESULTS

Effects of sulfonylureas on the development of glomerulosclerosis in insulin-deficient diabetes

To investigate the effects of sulfonylureas on the development of diabetic glomerulosclerosis independently of glycemic control, streptozotocin-diabetic rats were allowed to remain substantially hyperglycemic during continued tolazamide or glibenclamide treatment after disease induction. These compounds are differentiated by their degrees of lipophilicity that presumably regulate cell membrane translocation and association with cytoplasmic binding sites [14]. All diabetic animals demonstrated a fourfold increase in their 36-week mean blood glucose concentration over control values (Table 1). All animals gained weight during the study period but, as expected, the weight gain was less in diabetic groups than in controls. Sulfonylurea treatment did not improve blood glucose levels or body weight gain in diabetic animals as compared to the nontreated diabetic control group (Table 1). Only animals treated with high-dosage glibenclamide demonstrated a small (8%) improvement in glycemia over that in nontreated diabetic rats.

In addition to glomerular mesangial expansion, basement membrane thickening and lobular sclerosis, nontreated diabetic animals demonstrated occasional glomerular vascular hyalinosis and intratubular proteinaceous casts. However, there was only minimal focal interstitial disease. The total number of glomeruli scored per animal by the three observers was 360 ± 71 (mean, SD) and similar in all experimental groups. There was a high overall reliability for the three observers averaged together (interrater reliability coefficient 0.88).

The total number of scores generated by all observers was 53,730. As compared to age-matched normal controls, 36-week diabetic, nontreated animals presented a significant increase in the degree of glomerulosclerosis (Fig. 1). The prevalence of glomerulosclerosis was significantly less in the diabetic groups treated with tolazamide or glibenclamide than in nontreated diabetic animals (Figs. 1 and 2). Further, this reduction in glomerulosclerosis appeared to be more accentuated in the groups treated with the higher dosages, particularly in the tolazamide group. Notably, diabetic animals treated with glibenclamide or high-dosage tolazamide demonstrated a significantly lower prevalence of sclerotic glomeruli than normal, age-matched controls (Fig. 1).

Urinary albumin excretion was significantly increased in diabetic animals as compared to age-matched controls (Fig. 1). In consonance with the histologic observations, excessive albumin excretion was fully normalized in all diabetic groups treated with sulfonylureas (Fig. 1). A recent study has emphasized the possible underestimation of urinary albumin in streptozotocin-diabetic rats as result of low-molecular-weight albumin fragments not detected by radioimmunoassay [53]. Thus, there was the theoretical possibility that, due to a modification of the albumin excreted by the sulfonylurea-treated animals, significant amounts of the protein were not detected by the immunoassay used. To rule this out, concentrated pooled urine samples from each of the high-dosage, sulfonylurea-treated, and control groups were analyzed by PAGE and immunoblotting. The protein excreted in the nontreated and sulfonylurea-treated diabetic groups contained a similar fraction of albumin that was immunologically detectable (Fig. 3).

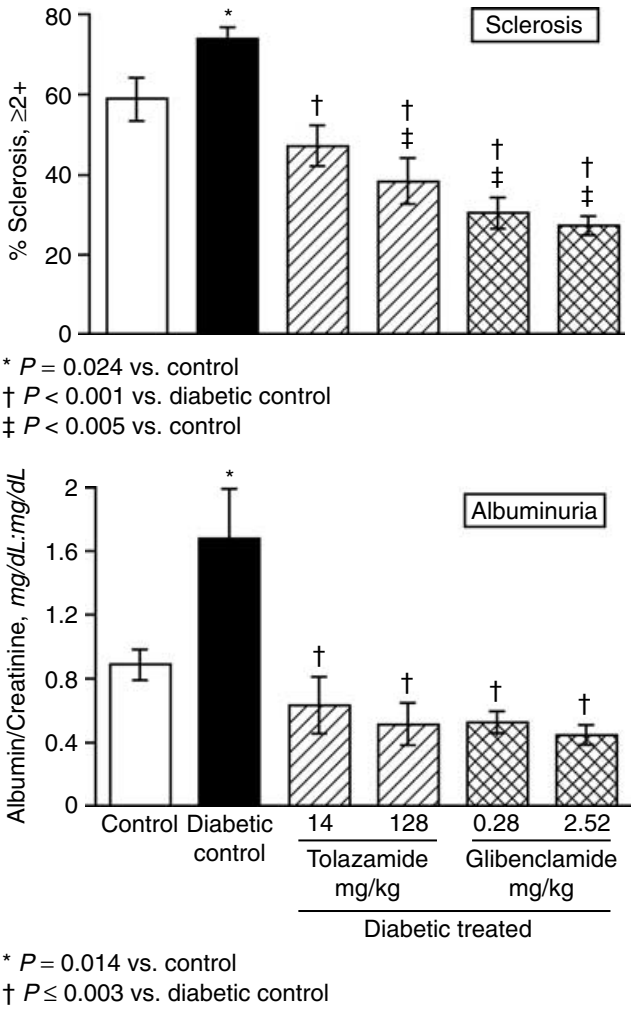


Fig. 1. Effect of treatment with sulfonylureas on the development of glomerulosclerosis and albuminuria in long-term diabetic rats. The degree of mesangial expansion, basement membrane thickening, and glomerulosclerosis were independently assessed by three observers, who were masked as to the origin of the sample, by light microscopy in periodic acid-Schiff (PAS)-stained specimens. Studies were carried out at 36 weeks after induction of diabetes. The degree of glomerular disease was defined on a scale of 0 to 4+. The mean value resulting from the individual findings by the three observers (percent of glomeruli showing conclusive disease) are presented. Albuminuria data correspond to samples obtained at the end of the observation period. Each individual albumin excretion value was the mean of two 24-hour urine collections. Control, nontreated diabetic, tolazamide-treated, and glibenclamide-treated diabetic animals were the same as those described in Table 1. Values presented are mean ± SE.

Effects of sulfonylureas on the glomerular expression of fibronectin mRNA in insulin-deficient diabetes

Additional evidence of a salutary effect of sulfonylureas in the prevention of glomerular injury was sought during an earlier period of diabetes when glomerulosclerosis is presumed to be absent. At 22 weeks of diabetes, glomerular fibronectin mRNA expression was increased over that in age-matched controls, suggesting its increased synthesis (Fig. 4). These diabetic animals presented significant hyperglycemia as compared to controls ($P < 0.0001$). As before, in these animals, treatment with glibenclamide during the entire period of diabetes did not improve the level of hyperglycemia (control 86 ± 1.4 mg/dL; diabetic 425 ± 8.9 mg/dL; and diabetic + glibenclamide 424 ± 7.6 mg/dL) (mean ± SE). In addition, the increased fraction of glycosylated hemoglobin in diabetic animals ($P < 0.0001$) was also unaltered by the administration of glibenclamide (control 5.4 ± 0.2%; diabetic 12.9 ± 0.4%; and diabetic + glibenclamide 13.2 ± 0.5%) ($N = 22$ to 40) (mean ± SE). However, treatment with glibenclamide over the entire period of diabetes resulted in a significant reduction in fibronectin mRNA expression compared to nontreated diabetic animals, although its expression was modestly greater than in normal controls (Fig. 4).

Effects of sulfonylureas on the renal functional changes and hypertrophy of insulin-deficient diabetes

Sulfonylureas have been shown to restore the impaired contractility of glomerular afferent arterioles in diabetes

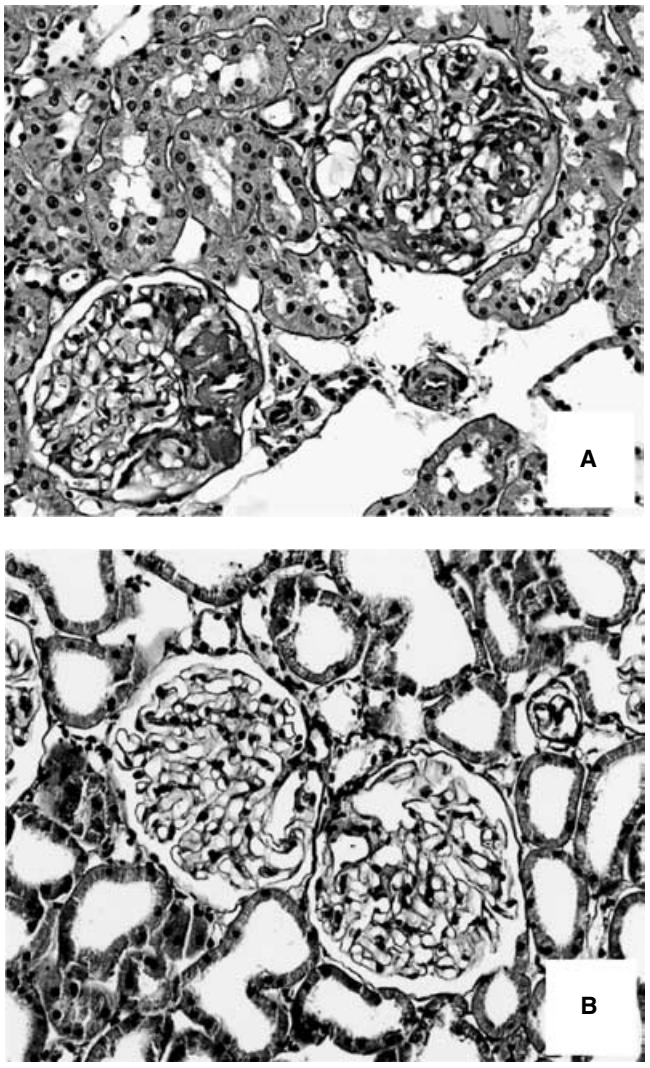


Fig. 2. Effect of treatment with sulfonylureas on diabetic glomerulosclerosis. Perfusion-fixed, periodic acid-Schiff (PAS)-stained kidney specimens are shown from a nontreated diabetic animal after 36 weeks of diabetes (*A*) demonstrating advanced mesangial expansion and sclerosis (+3) and age-matched diabetic animal that had received 0.28 mg/kg glibenclamide daily for the entire duration of diabetes (*B*) that revealed only minimal mesangial disease (1+) (original magnification ×200).

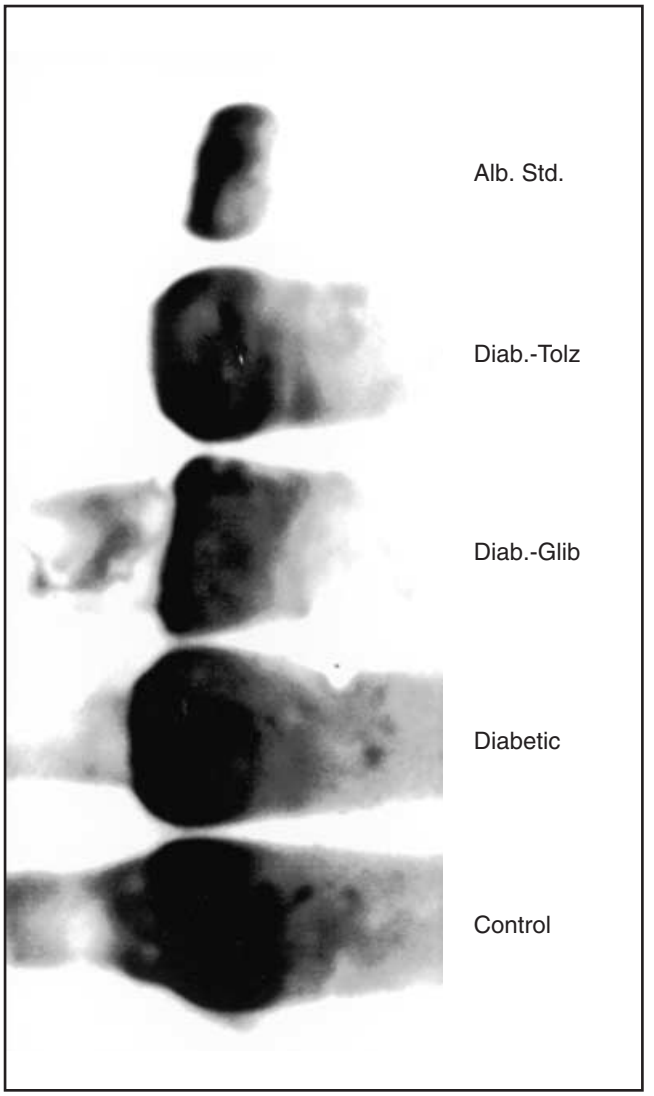
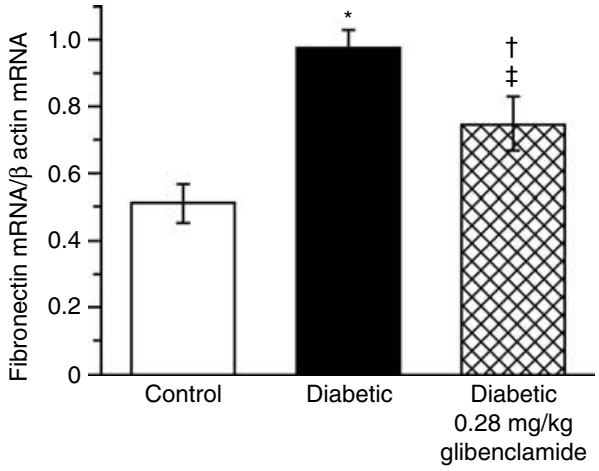


Fig. 3. Analysis of urinary proteins in long-term diabetic animals treated with sulfonylureas. Samples were obtained from the same urine collections presented in Figure 1. Urine collections from four animals in each group were pooled, dialyzed against phosphate-buffered saline (PBS) and concentrated. Twenty micrograms of each concentrated urinary protein per sample were analyzed by 7.5% sodium dodecyl sulfate-polyacryalmide gel electrophoresis (SDS-PAGE). Immunodetection was carried out with purified polyclonal antirat albumin antibody and enhanced chemiluminescence (ECL) detection.

[23]. To investigate whether the glomerular protective effects of sulfonylureas could be the result of decreased glomerular mechanical strain following restoration of glomerular pressure autoregulation and reduction of diabetic renal hyperfunction, experiments measuring GFR were carried out in insulin-deficient diabetic rats with and without treatment with glibenclamide. Studies were carried out at a time when no significant glomerular diabetic disease is apparent. Since the level of metabolic control may determine the presence of hyperfiltration, groups of diabetic animals, incompletely treated with daily insulin and demonstrating moderate hyperglycemia, were also studied. Mean blood glucose levels and the fraction of glycosylated hemoglobin were significantly higher in diabetic animals not treated with insulin than in controls (Table 2). Incomplete insulin treatment resulted in decreased severity of the metabolic derangement with blood glucose, glycosylated hemoglobin and weight gains that were intermediate those of normal age-matched controls and noninsulin treated diabetic animals (Table 2). Treatment with glibenclamide did not alter the levels of blood glucose, glycosylated hemoglobin, or final body weight in either noninsulin-treated or insulin-treated diabetic groups (Table 2).



* $P < 0.001$ vs. control
† $P = 0.017$ vs. diabetic
‡ $P = 0.018$ vs. control

Fig. 4. Effect of treatment with glibenclamide on diabetes-induced glomerular overexpression of fibronectin mRNA. Twenty-two weeks after induction of diabetes the gene expression of fibronectin was determined in samples of microdissected glomeruli by real-time, quantitative reverse transcription-polymerase chain reaction (RT-PCR). Results are fibronectin mRNA expression relative to β-actin mRNA expression, determined in the same reaction. One sample, containing 160 glomeruli, was analyzed per animal. Results are expressed as mean ± SE ($N = 21$ to 40 animals per experimental group).

Diabetic animals not receiving daily insulin demonstrated marked hyperfiltration and renal hypertrophy that were unchanged by treatment with glibenclamide (Fig. 5). Amelioration of the diabetic state by incomplete insulin therapy decreased the magnitude of hyperfiltration and renal hypertrophy. Thus, in these animals, GFR was not significantly different than in controls (Fig. 5). Again, glibenclamide administration to the insulin-treated animals did not result in any appreciable change in either GFR or renal hypertrophy.

Effects of sulfonylureas on the development of glomerulosclerosis in insulin-resistant diabetes

Sulfonylureas are extensively employed in type 2 diabetes in humans. However, there is a lack of information on their possible effects on the renal disease of insulin-resistant diabetes in animal models. To explore the effects of sulfonylureas, db/db mice were continuously treated with tolazamide, in the same dosages described for rats for 26 weeks. Tolazamide, low- or high-dosage, did not improve hyperglycemia (Fig. 6). As in the studies in rats above, renal specimens were scored for the presence of glomerulosclerosis by three independent observers who were masked as to the origin of the specimens. The mean total number of glomeruli scored per animal by the three observers was 160 ± 70 (mean, SD) without significant differences between experimental groups. As before, there was also a high overall reliability for the three observers averaged together (interrater reliability coefficient 0.92). The total number of scores generated was 13,346. The mean percent of sclerotic glomeruli determined in individual animals by the three observers was calculated and results are presented in Figure 6. Obese diabetic mice demonstrated a significantly higher prevalence of glomerulosclerosis compared to lean controls, and this observation was associated with enhanced albumin excretion (Fig. 6). In contrast to the effects in insulin-deficient rats, tolazamide did not alter the degree of glomerulosclerosis, even when it was administered in high dosage. Further, tolazamide administration did not decrease the elevated levels of albumin excretion. Additional similar studies were carried out also in a separate group of db/db mice and their controls at 18 weeks of diabetes (not shown). Although at this shorter period the degree of glomerulosclerosis and albuminuria in both diabetic and age-matched control mice were significantly lower than at 26 weeks, diabetic animals still presented significantly greater glomerulosclerosis than controls ($P < 0.001$). In addition, and as shown in the previous group, there were no differences between tolazamide-treated and nontreated diabetic animals ($P = 0.85$).

DISCUSSION

To envision the potential effects of glomerular α-endosulfine up-regulation in diabetes, this study has investigated sulfonylureas as exogenous mimics of this sulfonylurea-like intracellular protein. A model of insulin-deficient diabetes offered the opportunity of testing the glomerular effects of these drugs independently of their action on insulin secretion and in a setting of severe hyperglycemia. As expected, in streptozotocin-diabetic rats, sulfonylureas did not alter ambient glycemia levels or the decreased weight gain at any of the periods of diabetes studied. In this work, two extensively administered forms of sulfonylureas were used in dosages equivalent to those within the therapeutic range in humans. In this regard, simply factoring the dosage administered by body weight is misleading. It has been extensively reported that humans metabolize drugs much slower than rats with a median rat/human plasma clearance ratio per unit body weight of 7.18 [54]. Thus, the actual human equivalent dosage for the 0.28 mg/kg glibenclamide, used in this study in rats, is only 0.04 mg/kg, approximating a daily dose of 2.5 mg for the average adult.

Sulfonylureas are notable for their low incidence of side effects. Tolazamide was developed in 1966 and shown to have extremely low toxicity in animals with an LD50 of 5 g/kg [55]. Further, in doses of 100 mg/kg/day for 2 years, it did not cause obvious adverse effects in normal Wistar rats, except for islet β cell hyperplasia. Therefore,

Table 2. Characteristics of the rat experimental groups included in renal function studies[a]

Group	Control		Diabetic		
Treatment	None	None	Insulin	Glibenclamide (0.28 mg/kg)	Glibenclamide (0.28 mg/kg) plus insulin
Number	10	14	13	14	17
Blood glucose *mg/dL*[b]	93 ± 3.8[c]	463 ± 12.8	243 ± 22.3[d]	464 ± 7.6	316 ± 11.0[d]
Glycosylated hemoglobin%	5.0 ± 0.14[c]	14.6 ± 0.37	8.4 ± 0.61[d,e]	14.9 ± 0.46	9.1 ± 0.35[d,e]
Final weight *g*	313 ± 5.9[c]	246 ± 4.6	295 ± 6.2[d,f]	240 ± 5.8	289 ± 4.6[d,f]

[a] Values are mean ± SE.
[b] Value for each individual animal is the mean of all measurements over the 14-week period of observation.
[c] Blood glucose concentration, glycosylated hemoglobin and body weight in all diabetic groups vs. control ($P < 0.001$).
[d] Blood glucose concentration, glycosylated hemoglobin and body weight in all diabetic insulin-treated groups vs. noninsulin-treated ($P < 0.001$).
[e] Blood glucose concentration and glycosylated hemoglobin in all insulin-treated groups vs. control ($P < 0.001$).
[f] Body weight in all insulin-treated groups vs. control ($P < 0.032$).

it is highly unlikely that the results obtained in this study are the consequence of confounding toxic side-effects.

At 36 weeks, the degree of mesangial expansion and glomerulosclerosis was increased in diabetic animals over age-matched controls. Previous studies documented significantly increased glomerulosclerosis in diabetic rats only after 12 months of disease [56, 57]. However, in these studies animals received partial insulin treatment to maintain moderate hyperglycemia and high survival rates. Regarding the effect of sulfonylureas on glomerular injury, diabetic animals treated with these drugs had a significant reduction in the degree of sclerosis, especially in the glibenclamide- and high tolazamide-treated groups. In these groups, the extent of sclerosis was reduced to levels below control, suggesting that the beneficial effect of sulfonylureas may extend beyond the diabetic state. In previous studies, we reported a progressive increase in the glomerular collagen content of normal rats aging from 5 weeks to 2 years [58]. Conceivably, because of pathogenetic similarities between diabetes and aging [59], sulfonylureas may also act as a inhibitors of age-related glomerulosclerosis occurring predominantly in male rats [60, 61].

The mechanism by which sulfonylureas may limit mesangial matrix deposition remains speculative, however, the reduction of glomerular fibronectin gene expression suggests that inhibition of extracellular matrix synthesis may play a role. Finally, the beneficial effect of sulfonylureas on the histologic glomerular disease was associated with complete normalization of diabetic-induced increased albumin excretion in all sulfonylurea-treated groups. In addition, suboptimal immunologic reactivity for albumin was eliminated as the reason for this observation. However, albumin excretion in sulfonylurea-treated animals was not reduced to the levels established by age-matched controls.

Glomerular hypertension and the associated mechanical mesangial strain are considered important pathogenetic factors in the accumulation of mesangial extracellular matrix, and these are aggravated by an environment of high glucose concentration [62, 63]. Since

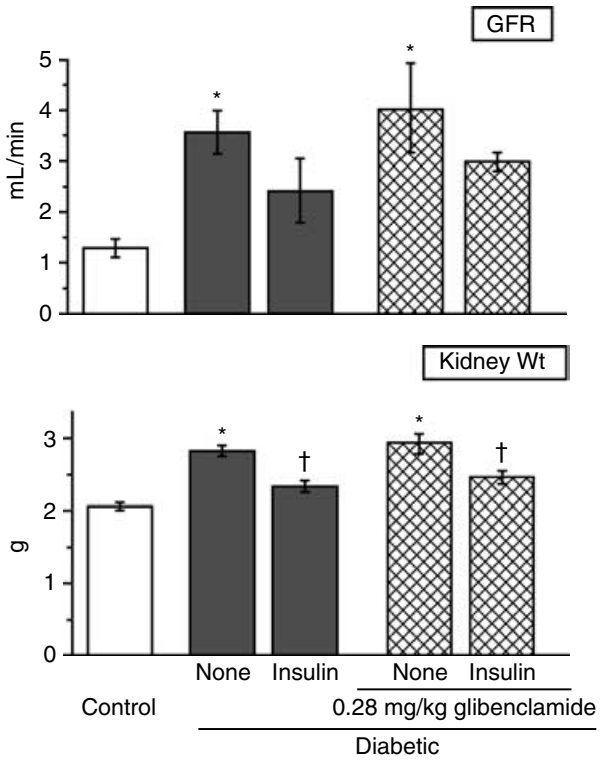


Fig. 5. Glomerular filtration rate (GFR) and renal hypertrophy in rats treated with glibenclamide and suboptimal amounts of insulin. Animals were the same as those described in Table 2. Streptozotocin-injected rats were given daily doses of NPH-insulin to maintain partial metabolic control or left untreated for 14 weeks. Groups of untreated and insulin-treated animals were also given a daily glibenclamide dose. GFR was determined in unanesthetized, unrestrained rats according to the clearance of fluorescein isothiocyanate (FITC)-inulin. Calculations were based on FITC-inulin excretion in two consecutive 24-hour urine collections and plasma concentration in tail blood samples at the start, and end of the urine collection periods. The combined weight of both kidneys was obtained at the conclusion of the experimental period. Values are mean ± SE.

glibenclamide could reduce glomerular hypertension and hyperfiltration by normalizing diabetic-induced afferent arteriolar hypocontractility [23], a functional effect was a plausible mechanism for the glomerulo-protective

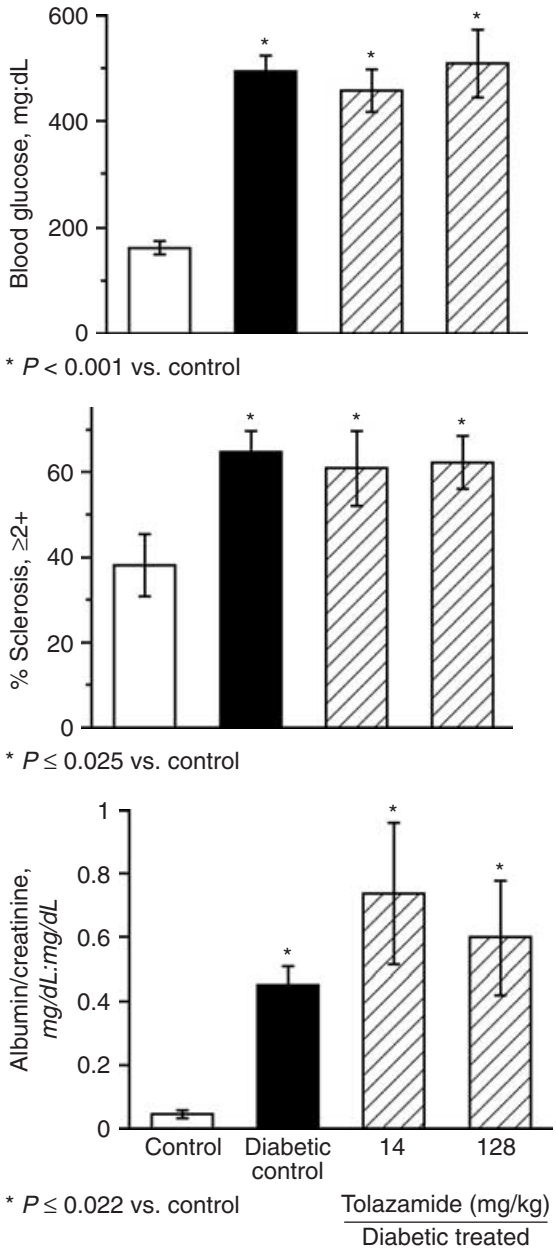


Fig. 6. Effect of treatment with sulfonylureas on the development of glomerulosclerosis in long-term diabetic db/db mice. Obese diabetic mice and lean normal controls entered the study at 4 to 6 weeks old and studies performed 26 weeks later. Diabetic animals received by gavage a daily dose of tolazamide, in the dosages indicated, during the last 24 weeks of the experimental period. As in studies in rats (Figure 1), the degree of mesangial expansion, basement membrane thickening and glomerulosclerosis was independently assessed by three observers, who were masked as to the origin of the sample, in periodic acid-Schiff (PAS)-stained specimens. The degree of glomerular disease was defined on a scale of 0 to 4+. The individual findings by the three observers were combined and presented as the percent of glomeruli showing conclusive disease. Albuminuria data correspond to samples obtained at the end of the observation period. Each individual albumin excretion value was the mean of two 24-hour urine collections. Values presented are mean ± SE ($N = 11$ to 17 animals per experimental group).

properties of sulfonylureas in diabetes. This possibility was explored in unanesthetized, unrestrained diabetic animals by measuring GFR under conditions of severe or moderate hyperglycemia. Contrary to historical studies in anesthetized rats, but in agreement with more recent observations in conscious animals with blood glucose of 360 to 530 mg/dL [64, 65], it was shown that noninsulin treated diabetic rats with marked hyperglycemia developed hyperfiltration. Nevertheless, this alteration, and the concomitant renal hypertrophy was not modified by glibenclamide administration, regardless of whether animals were receiving suboptimal insulin treatment and demonstrating milder glomerular hyperfunction and renal hypertrophy. Thus, the preventive effects of sulfonylureas on the development of glomerulosclerosis are unlikely to be related to improved afferent arteriolar contractility.

Since sulfonylureas did not diminish hyperglycemia, diabetic hyperfiltration, or the characteristic renal hypertrophy, the glomerular effects elicited by these drugs occurred in a setting wherein the major pathogenetic mechanisms of diabetic glomerular injury were not corrected (i.e., high glucose concentration, glomerular hyperfiltration, and renal hypertrophy [42, 66–71]).

The results of this study suggest that the glomerular effects of sulfonylureas in diabetes are due to a direct metabolic effect, possibly by interrupting or modulating signaling pathways through which glucose exerts its stimulating effects on extracellular matrix synthesis. This would be in consonance with the postulated mechanism of action of α-endosulfine. Since activation of the protein kinase C/mitogen-activated protein kinase cascade plays a major role in the mediation of the glomerular injurious effects of high glucose [72, 73], this pathway appears as a likely target for sulfonylurea action. This postulate is supported by preliminary results from our laboratory in mesangial cells in tissue culture treated with low concentrations of glibenclamide [abstract; Cortes P, et al, *J Am Soc Nephrol* 14:129A, 2003].

The glomerular effects of long-term sulfonylurea administration suggest that up-regulation of α-endosulfine expression in mesangial cells may attenuate the stimulating effects of high glucose concentrations on the formation and deposition of extracellular matrix. However, the interplay between α-endosulfine and its exogenous counterparts remains speculative. An attractive possibility is that the effects of sulfonylureas are, at least in part, mediated by the up-regulation of mesangial cell ENSA and α-endosulfine expression (unpublished observations).

The long-term administration of tolazamide did not alter the degree of glomerular disease or albuminuria in an animal model of type 2 diabetes. However, in this model, sulfonylureas failed to improve hyperglycemia. These findings suggest that the glomerular effects of sulfonylureas differ in insulin-deficient and insulin-resistant

diabetes, unless these drugs effectively improve glucose levels. In agreement with this postulate are results from controlled clinical studies. The United Kingdom Prospective Diabetes Study (UKPDS) determined the incidence of microvascular complications in type 2 diabetic patients that were treated by either conventional means (dietary modification), intensive therapy with one of three sulfonylureas (chlorpropamide, glipizide, and glibenclamide), a biguanide (metformin), or insulin [74, 75] This study demonstrated a reduction in the progression of albuminuria and renal insufficiency in all the intensively treated groups. However, no significant differences among these groups were shown, suggesting that sulfonylureas do not specifically alter the course of nephropathy other than by correcting the metabolic state.

CONCLUSION

This study demonstrates that the long-term administration of low-dosage sulfonylureas, particularly glibenclamide, prevents the development of glomerulosclerosis and albuminuria in experimental, insulin-deficient diabetes. This effect takes place independently of hyperglycemia, glomerular hyperfunction, or renal hypertrophy. Results suggest that up-regulation of the mesangial SUR endogenous ligand, α-endosulfine, may be a suppressor of the glucose-enhanced accumulation of mesangial matrix. In regard to the potential therapeutic use of sulfonylureas, clinical testing will be required to demonstrate that these drugs also prevent nephropathy in human type 1 diabetes.

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Reprint requests to Pedro Cortes, M. D., Division of Nephrology and Hypertension, CFP-5, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202
E-mail: cortes.pedro@usa.net.

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