Comparison of the Effects of Glibenclamide on Metabolic Parameters, GLUT1 Expression, and Liver Injury in Rats With Severe and Mild Streptozotocin-Induced Diabetes Mellitus

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Summary. *Background and Objective. Glucose transport via GLUT1 protein could be one of additional mechanisms of the antidiabetic action of sulfonylureas. Here, the GLUT1 gene and the protein expression was studied in rats in the course of severe and mild streptozotocin-induced diabetes mellitus and under glibenclamide treatment.*

Material and Methods. Severe and mild diabetes mellitus was induced using different streptozotocin doses and standard or high fat chow. Rats were treated with glibenclamide (2 mg/kg daily, per os for 6 weeks). The therapeutic effect of glibenclamide was monitored by measuring several metabolic parameters. The GLUT1 mRNA and the protein expression in the kidneys, heart, and liver was studied by means of real-time RT-PCR and immunohistochemistry.

Results. The glibenclamide treatment decreased the blood glucose concentration and increased the insulin level in both models of severe and mild diabetes mellitus. Severe diabetes mellitus provoked an increase in both GLUT1 gene and protein expression in the kidneys and the heart, which was nearly normalized by glibenclamide. In the kidneys of mildly diabetic rats, an increase in the GLUT1 gene expression was neither confirmed on the protein level nor influenced by the glibenclamide treatment. In the liver of severely diabetic rats, the heart and the liver of mildly diabetic rats, the GLUT1 gene and the protein expression was changed independently of each other, which might be explained by abortive transcription, and pre- and posttranslational modifications of gene expression.

Conclusions. The GLUT1 expression was found to be affected by the glucose and insulin levels and can be modulated by glibenclamide in severely and mildly diabetic rats. Glibenclamide can prevent the liver damage caused by severe hyperglycemia.

Introduction

Diabetes mellitus and its complications cause numerous health and social problems throughout the world. The number of patients with diabetes mellitus is constantly increasing. Both the search for novel remedies with an antidiabetic activity and comprehensive studies of the mechanisms of actions of widely used antidiabetics with a goal to widen an indication spectrum are topical problems of modern medicinal chemistry and pharmacology. Glibenclamide (glyburide) is one of the most widely used antidiabetic drugs and the most popular sulfonylurea. The drug works by inhibiting ATP-sensitive potassium channels in pancreatic beta cells. This inhibition induces cell membrane depolarization, which results in opening of the voltage-dependent calcium channels, this causes an increase in intracellular calcium in beta cells, and stimulates insulin release as a consequence (1). Many studies suggest that besides stimulating insulin release, sulfonylureas have direct extra pancreatic actions (2) as well as insulin-independent blood glucose decreasing activities (3). Glibenclamide interferes with glucose transport modulation by ATP-sensitive potassium channels in peripheral tissues (4) and NO-mediated vasorelaxation induced by a high glucose level (5). Glibenclamide interferes with mitochondrial bioenergetics in nonpancreatic cells by inducing the changes in the membrane ion permeability (6). Noninsulin-dependent glucose transport via GLUT1 protein appears to be one of the possible additional mechanisms of the drug antidiabetic action. It has been reported that glibenclamide significantly increases the total content and the plasma membrane level of GLUT1 in L6 myotubes (2). The chronic application of sulfonylurea to cultured cardiomyocytes was found to produce an approximate doubling of the basal glucose uptake rates by an insulin-independent pathway most probably involving the increased protein expression of GLUT1 (7). However, the data are scarce, and the studies on GLUT1 in diabetes have shown the contradictory findings (8, 9 and references therein). To fill the existing gap and to im-

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prove the knowledge about an insulin-independent action of glibenclamide, we have performed a study of the *GLUT1* gene and the protein expression in the course of development of severe and mild diabetes mellitus induced by streptozotocin and under glibenclamide treatment. The comparison of glibenclamide effects in these 2 models of diabetes mellitus has allowed evaluating the effects of the drug under the conditions of severe or mild hyperglycemia, which is an important factor for the development of diabetic complications. The antidiabetic effects of the drug were proved by monitoring the insulin level together with several parameters of carbohydrate and lipid metabolism.

Material and Methods

Animals. Animals were obtained from the Laboratory of Experimental Animals, Riga Stradins University, Riga, Latvia. All experimental procedures were carried out in accordance with the guidelines of the Directive 86/609/EEC "European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" (1986) and were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia). Wistar male rats, each weighing 215.00±5.63 g at the beginning of the experiments, were used in all the work. The environment was maintained at a temperature of 22°C±0.5°C with a 12-hour light/dark cycle. The animals were fed ad libitum a standard laboratory diet with a free access to water before the experiments; some groups received a standard diet also in the course of experiments, and animals in several experimental groups were fed with R638 high-fat food, consisting of 18% protein, 33% fat, 8.5% fiber and 27% carbohydrates of weight (Lantmännen, Malmö, Sweden).

Chemicals. Glibenclamide and streptozotocin were purchased from Sigma-Aldrich Chemie GmbH (Germany, Taufkirchen). Glucose (anhydrous) was purchased from Chempur (Germany, Karlsruhe).

Induction of Diabetes. A severe form of diabetes mellitus, resembling human type 1 diabetes mellitus, was induced in rats by a single injection of streptozotocin at a dose of 50 mg/kg, freshly dissolved in 10-mM citrate buffer (pH 4.5) via the tail vein (injection volume, 0.2–0.25 mL) (10). The control animals received 0.2 mL of 0.9% NaCl via the tail vein. Diabetes was confirmed by measuring the fed-state blood glucose level in blood samples obtained via tail bleeding 120 hours after the induction. The rats with a blood glucose level of >13.89 mmol/L (250 mg/dL) were included in the study.

The model of mild diabetes mellitus claimed to share many features with human type 2 diabetes mellitus was induced according to Zhang et al. (11). Animals were fed with R638 high-fat food (Lantmännen, Malmö, Sweden) for 2 weeks. Then the animals received 2 intraperitoneal injections of 30-mg/kg streptozotocin in a fresh solution of 10-mM citrate buffer (pH 4.5) with a 7-day interval. The rats continued to receive R638 food to the end of the experiment. One week after the second streptozotocin injection, fasted and fed state glucose was monitored. Animals with the fed-state glucose level higher than 11.1 mmol/L and fasted state glucose level higher than 7.8 mmol/L were taken for experiments 1 week later.

Longer time needed to induce this model of experiment and other way of streptozotocin administration required a separate control group and a glibenclamide-treated group for this set of experiments. The control animals received a standard chow for 2 weeks; 2 intraperitoneal injections of 10-mM citrate buffer (pH 4.5) were made with a week interval. It should be mentioned that animals with mild diabetes mellitus were 5 weeks older than animals with severe diabetes mellitus.

Experimental Design and Glibenclamide Administration. After the proof of diabetic state, animals were randomized; in each series of experiments, animals were divided in 4 groups (10 animals in each group). In the series of experiments with a severe form of diabetes mellitus, there were control animals (Control 1), glibenclamide-treated animals (further in the text Glib 1), nontreated diabetic animals (streptozotocin group, further in the text STZ), and diabetic animals treated with glibenclamide (glibenclamide+streptozotocin group, further in the text STZ+Glib). In the second series of experiments, there were control animals (Control 2), glibenclamide-treated animals (further in the text Glib 2), nontreated diabetic animals (high fat food+streptozotocin; further in the text HS), and diabetic animals treated with glibenclamide (further in the text HS+Glib). Randomization before the beginning of the treatment was performed so that the diabetic groups to be compared (STZ versus STZ+Glib and HS versus HS+Glib) would not differ in the main metabolic parameters (Table 1).

The treatment of animals with a glibenclamide solution in 10% dimethyl sulfoxide (DMSO) in the Glib 1, STZ+Glib, Glib 2, and HS+Glib groups was performed per os at 9:00 AM (2 mg/kg daily); the control and nontreated animals received the solvent. The rats were treated for 6 weeks. The experiment was performed in spring. The treatment was begun immediately after the development of severe diabetes was proved a week later after mild diabetes was detected (week 0). Before the treatment (week 0), and at the end of weeks 2, 4, and 6 of the experiment, the following parameters were measured in the rats fasted for 12 hours: weight, blood glucose, blood triglycerides, ketone body concentration in blood.

Table 1. Descriptive Characteristics of the Groups Before the Initiation of Glibenclamide Treatment

Group	Weight, g	Fasting Glucose, mmol/L	Fed-State Glucose, mmol/L	Ketone Bodies, mmol/L	Triglycerides, mmol/L	HbA1c%
Control 1	215.00±5.63	2.88±0.14	6.41±0.43	2.56±0.16	2.50±0.11	3.12±0.03
Glib 1	203.00±3.35	3.16±0.15	6.10±0.24	2.75±0.15	2.15±0.24	3.04±0.02
STZ	208.42±3.35	16.78±1.57*	35.63±4.60*	1.26±0.27*	1.71±0.20*	4.13±0.12*
STZ+Glib	207.89±5.16	16.40±1.57*	35.10±5.72*	1.40±0.40*	1.79±0.33*	4.22±0.15*
Control 2	295.00±10.42	3.40±0.19	4.99±0.27	1.06±0.01	1.61±0.17	3.28±0.08
Glib 2	301.00±7.06	3.61±0.20	4.42±0.18	0.94±0.08	1.70±0.12	3.20±0.06
HS	273.33±12.51	13.49±2.21†	19.46±1.93†	1.85±0.46†	1.59±0.21	4.63±0.29†
HS+Glib	286.36±11.46	10.43±1.5†	18.17±1.93†	1.62±0.37†	1.54±0.17	4.22±0.29†

*$P<0.05$ versus Control 1 group; †$P<0.05$ versus Control 2 group.

The glucose level was determined also in the fed state. The level of glycated hemoglobin (HbA1c%) was measured at weeks 0 and 6. Oral glucose tolerance tests were performed at week 6. After performing the routine measurements on week 6, a cardiac puncture was performed under ether narcosis; blood for measurements of total cholesterol, free fatty acid, and insulin concentrations was collected. Later the rats were euthanized with pentobarbital at a dose of 200 mg/kg and dissected; liver, heart and kidney tissue samples were taken and frozen in liquid nitrogen for following RNA extraction, and other samples of the same organs were fixed in 10% buffered formalin for immunohistochemical examination.

Determination of Metabolic Parameters. Rat blood samples were obtained via a tail vein puncture. Blood glucose and ketone body concentrations were determined using a portative glycometer MediSense OptiumXceed (Abbott Diagnostics Ltd, Maidenhead, UK); triglyceride concentration, with a glycometer Accutrend GCT (Hoffmann-La Roche Ltd., Basel, Switzerland). HbA1c% was detected using a Nycocard reader (Axis-Shield, London, UK). The oral glucose tolerance test was performed as follows: after overnight fasting (with free access to water), the rats were weighed, and the concentration of blood glucose was determined (0 min). Afterwards, the animals received an aqueous dextrose solution (2 g/kg per os). Subsequently, the blood glucose concentration was determined at 15, 30, 60, and 120 minutes after dextrose ingestion. Insulin (ng/mL) was measured by an Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL, USA) with the BioTek Elx808 plate reader and Gen5 software. Free fatty acids (mmol/L) were determined by a Wako Nefa-HR kit (Wako Biochemicals GmbH, Neuss, Germany); total cholesterol (mmol/L) by an Instrumentation Laboratory Cholesterol determination kit (Instrumentation Laboratory, Bedford, MA, USA) on the PerkinElmer Lambda 25 spectrophotometer according to the manufacturer's instructions.

RNA Extraction and cDNA Preparation. Total RNA was isolated from the liver, kidneys, and heart using a TRI reagent (Sigma Aldrich, Taufkirchen, Germany). DNA contaminations were removed with a DNA-free kit (Ambion, Austin, TX, USA). The resulting RNA quantity and purity were determined by spectrophotometry. The integrity of RNA molecules was monitored by gel electrophoresis, and only specimens with well-pronounced rRNA bands were taken for reactions. RNA (2 μg) was reverse-transcribed using a random hexamer primer (RevertAid™ First Strand cDNA Synthesis Kit, Thermo Scientific® Fermentas, Vilnius, Lithuania) to obtain cDNA.

Real-Time RT-PCR. The mRNA expression rates of *GLUT1* in the liver, kidneys, and heart and a reference gene were determined using the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the instructions provided by the manufacturer. The amplification and detection of specific products were performed on a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following temperature-time profile: 1 cycle at 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and at 60°C for 30 seconds. To check the specificity of amplification products, the dissociation curve mode was used (1 cycle at 95°C for 15 seconds, at 60°C for 30 seconds, and 95°C for 15 seconds). The primers were designed using the Primer3 software (12). The primers were supplied by the Metabion International AG, Martinsried, Germany. The $2^{-\Delta\Delta CT}$ method was applied for analysis of the results (Applied Biosystems StepOne sofware, version 2.1). The primer sequences for GLUT1 gene were 5'-ATGATGCGGGAGAAGAAGGT-3' and 5'-GAACAGCGACACCACAGTGA-3'; the size of the expected product was 228 bp. RNA-polymerase II (5'-GCCAGAGTCTCCCATGTGTT-3' and 5'-GTCGGTGGGACTCTGTTTGT-3', 135 bp) and beta-actin (5'-AGCCATGTACGTAGCCATCC-3'and 5'-ACCCTCATAGATGGGCACAG-3', 115 bp) were chosen as reference genes.

Immunohistochemical Examination. The tissue sections were stained for the immunohistochemical visualization of GLUT1-positive cells using a rabbit polyclonal active GLUT1 antibody from Abcam Inc. (Cambridge, MA, USA) according to the previously published protocol with minor modifications (8, 9

and references therein). GLUT1-positive cells were counted in 20 high-powered fields at a magnification of ×400. In the kidneys, both GLUT1-positive cells and tubules were counted in the cortex and the medulla. In the heart tissue, GLUT1-positive cells were counted in the pericardium, endocardium, and myocardium. In liver tissue, GLUT1-positive cells were counted in 20 randomly selected high-powered fields at a magnification of ×400. The slides were evaluated by 2 pathologists in a blinded fashion (S.I. and D.S.). All cell counts were expressed as the number of cells per mm^2.

In addition, histopathological changes in the liver tissue were evaluated by the modified histological activity index (HAI) (13): infiltration of inflammatory cells (a score of 0–4); necrosis of hepatocytes around a central vein (a score of 0–6); necrosis of hepatocytes and apoptosis in the peripheral lobules (a score of 0–4); and inflammatory changes of portal tracts (a score of 0–4).

For morphological examination, at least 3 replicate measurements of GLUT1-positive cells were performed by the same observer in 10 randomly selected slides, and the intraobserver reproducibility was assessed with the coefficient of variation and the interclass correlation coefficient. The intraobserver coefficient of variation was 6%, and the intraobserver correlation coefficient was 0.92.

Statistical Analysis. The data were expressed as mean±SEM. For analysis of weight changes and metabolic parameters (glucose, ketone bodies, triglycerides, free fatty acids, insulin, total cholesterol, HbA1c%), a one-way ANOVA followed by an independent samples *t* test was used to evaluate the significance of differences between the groups. The Mann-Whitney *U* test was used for the quantification of HAI differences and *GLUT1* gene expression rates. For GLUT1 protein expression analysis, the independent sample *t* test was used. *P* values less than 0.05 were considered significant.

Results

Therapeutic Effects of Glibenclamide in Severe Streptozotocin-Induced Diabetes Mellitus. The results are summarized in Fig 1. When administered to healthy animals, glibenclamide did not interfere much with a normal process of weight gain during the first 5 weeks; only on week 6, the animals in the Glib1 group became heavier compared to the controls. Streptozotocin-induced diabetes mellitus caused the loss of body weight in comparison to the control group, which was not corrected by glibenclamide (Fig. 1A). In animals with streptozotocin-induced diabetes mellitus, an increased blood glucose level persisted throughout the whole experiment (6 weeks). Fluctuations in both fasting and postprandial glucose concentrations were observed in the STZ and STZ+Glib groups. Significantly lower glucose levels in both fasted and fed animals were observed in the STZ+Glib group compared with the STZ group only on week 6 of the treatment (Fig. 1B and C). HbA1c% increased with the induction of diabetes and persisted during the experiment. Glibenclamide treatment did not affect the parameter (Fig. 1D). Oral glucose tolerance tests did not reveal any significant improvements in glucose tolerance in the STZ+Glib group compared with the STZ group (Fig. 3A). The effects of the drug on lipid metabolism in diabetic animals were neither pronounced (data not shown). Insulin production was increased to a small extent (0.20±0.03 ng/mL in the untreated diabetic animals versus 0.31±0.03 ng/mL in the treated rats); however, the insulin level was still much lower compared with the control group (Table 2).

Therapeutic Effects of Glibenclamide in Animals With Mild Streptozotocin-Induced Diabetes Mellitus. The animals in the control group 2 constantly gained weight throughout the experiment (Fig. 2A). Glibenclamide administration slowed down the rate of weight gain starting with week 2 of the treatment. Weight gain in the HS group was also slower than in the control animals; glibenclamide treatment did not increase the weight gain. The induction of mild diabetes mellitus provoked a stable increase in the glucose level in the fasted animals; glibenclamide administration did not decrease the glucose level significantly (Fig. 2B). The fed-state glucose level in the HS group was increased compared with the control group 2 throughout the experiment. Glibenclamide treatment significantly decreased the fed-state glucose level on weeks 2 and 4; however, the parameter was not normalized (Fig. 2C). The level of HbA1c% was increased also in the animals with mild diabetes mellitus; glibenclamide treatment did not improve the parameter (Fig. 2D). Interestingly, the glibenclamide-treated animals with mild diabetes animals gained a significantly improved, although not normal, glucose tolerance (Fig. 3B).

A triglyceride concentration in the diabetic HS animals did not differ much from that of the control 2 animals, with the exception of only on week 2 of the treatment when it appeared to be increased; glibenclamide treatment did not abolish this increase (data not shown). Ketone bodies were stably increased in the animals with mild diabetes mellitus; glibenclamide did not improve this parameter (data not shown). Insulin production (Table 2) in the rats with mild diabetes mellitus was lower than in the rats of the Control 2 group (0.76±0.07 vs. 1.01±0.19 ng/mL at the beginning of the experiment, and 0.76±0.12 vs. 1.23±0.31 ng/mL on week 6); 6-week glibenclamide treatment normalized the parameter (1.20±0.18 ng/mL).

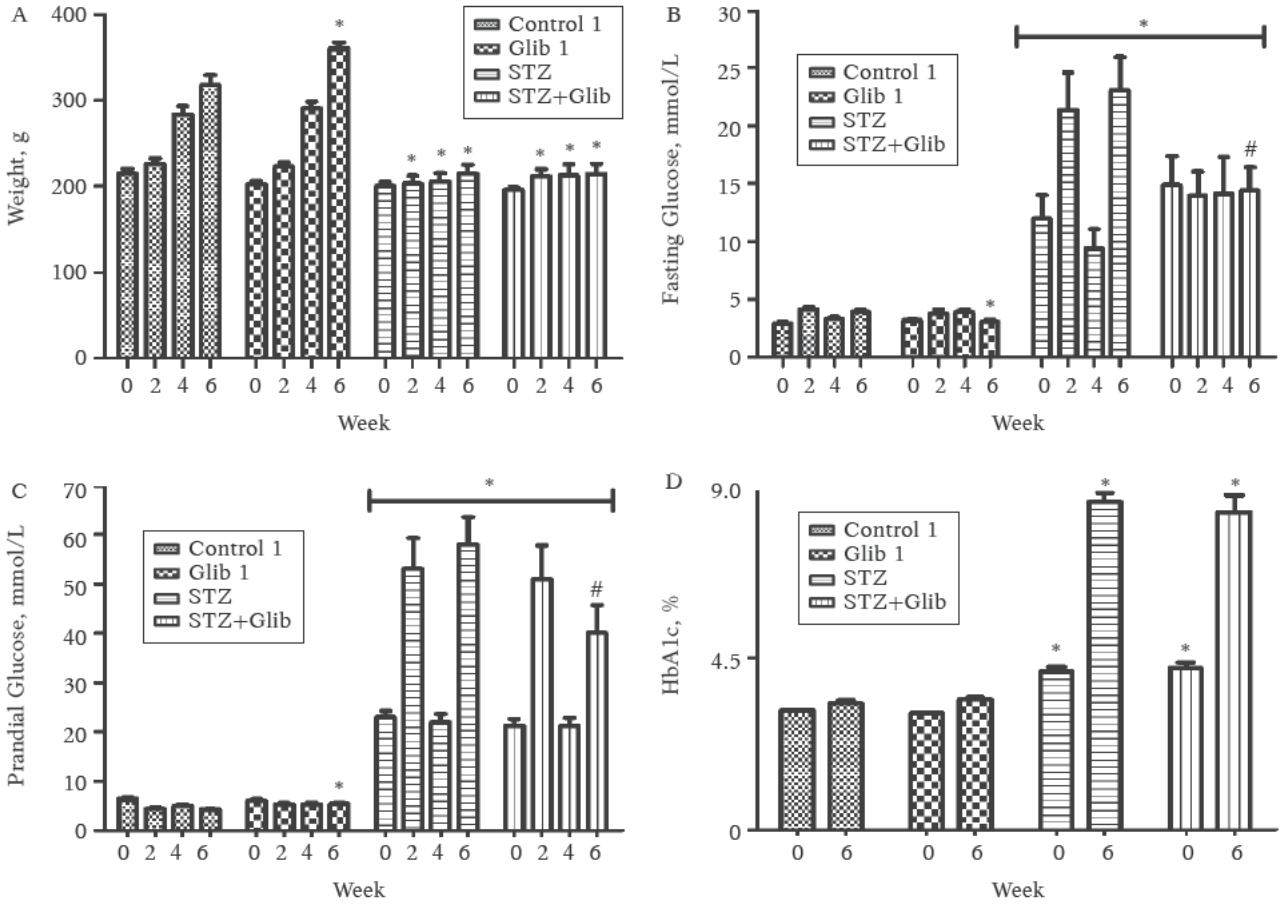

Fig. 1. Therapeutic effects of glibenclamide in rats with severe streptozotocin-induced diabetes mellitus

A, mean weight in the experimental groups during the 6-week glibenclamide treatment; B, the effect of glibenclamide on blood glucose concentration in fasted animals during the 6-week glibenclamide treatment; C, the effect of glibenclamide on blood glucose concentration in fed animals during the 6-week glibenclamide treatment; D, the level of glycated hemoglobin (HbA1c%) before and after the treatment with glibenclamide.

Control 1, the control group for a model of severe diabetes mellitus; Glib 1, intact rats treated with glibenclamide (2 mg/kg, 6 weeks); STZ, the group of animals with severe streptozotocin-induced diabetes mellitus; STZ+Glib, the rats with severe streptozotocin-induced diabetes mellitus treated with glibenclamide (2 mg/kg, 6 weeks). Bars represent the mean ± SEM (n=10).

*P<0.05 versus control group; #P<0.05 to STZ group.

Table 2. Insulin Concentration Before and After the Treatment With Glibenclamide

Group	Insulin, ng/mL	
	Week 0	Week 6
Control 1	0.95±0.03	0.87±0.02
Glib 1	0.89±0.15	1.19±0.19
STZ	0.24±0.04*	0.20±0.03*
STZ+Glib	0.21±0.08*	0.31±0.03*‡
Control 2	1.01±0.19	1.23±0.31
Glib 2	1.08±0.15	1.28±0.29
HS	0.76±0.07†	0.76±0.12†
HS+Glib	0.78±0.12†	1.20±0.18#

*P<0.05 versus Control 1 group; †P<0.05 versus Control 2 group; ‡P<0.05 versus STZ group; #P<0.02 versus HS group.

Modification of GLUT1 Gene and Protein Expression by Glibenclamide in Rats With Severe Diabetes Mellitus. The effects of the drug on the expression of *GLUT1* gene and protein differed protein expression observed both in the proximal and in the studied organs. In the kidneys of intact animals, the drug somewhat increased the gene expression; however, the difference was not statistically significant. On the contrary, the total distal canaliculi in the kidneys of healthy animals was decreased by glibenclamide (Fig. 4C and D). The induction of diabetes with streptozotocin provoked an increase in gene and protein expression; glibenclamide treatment produced a well-pronounced normalization of GLUT1 expression, and it was significantly decreased both on transcription and translation levels (Fig. 4C and D, photomicrographs in Fig. 6). The similar effects were observed in the heart tissue. Glibenclamide treatment normalized the streptozotocin-induced increase of mRNA expression, although the differences in the transporter protein expression did not reach a statistical significance (Fig. 4E and F). In the liver, the GLUT1 expression was minimal and observed predominantly in the Kupffer cells; weak cy-

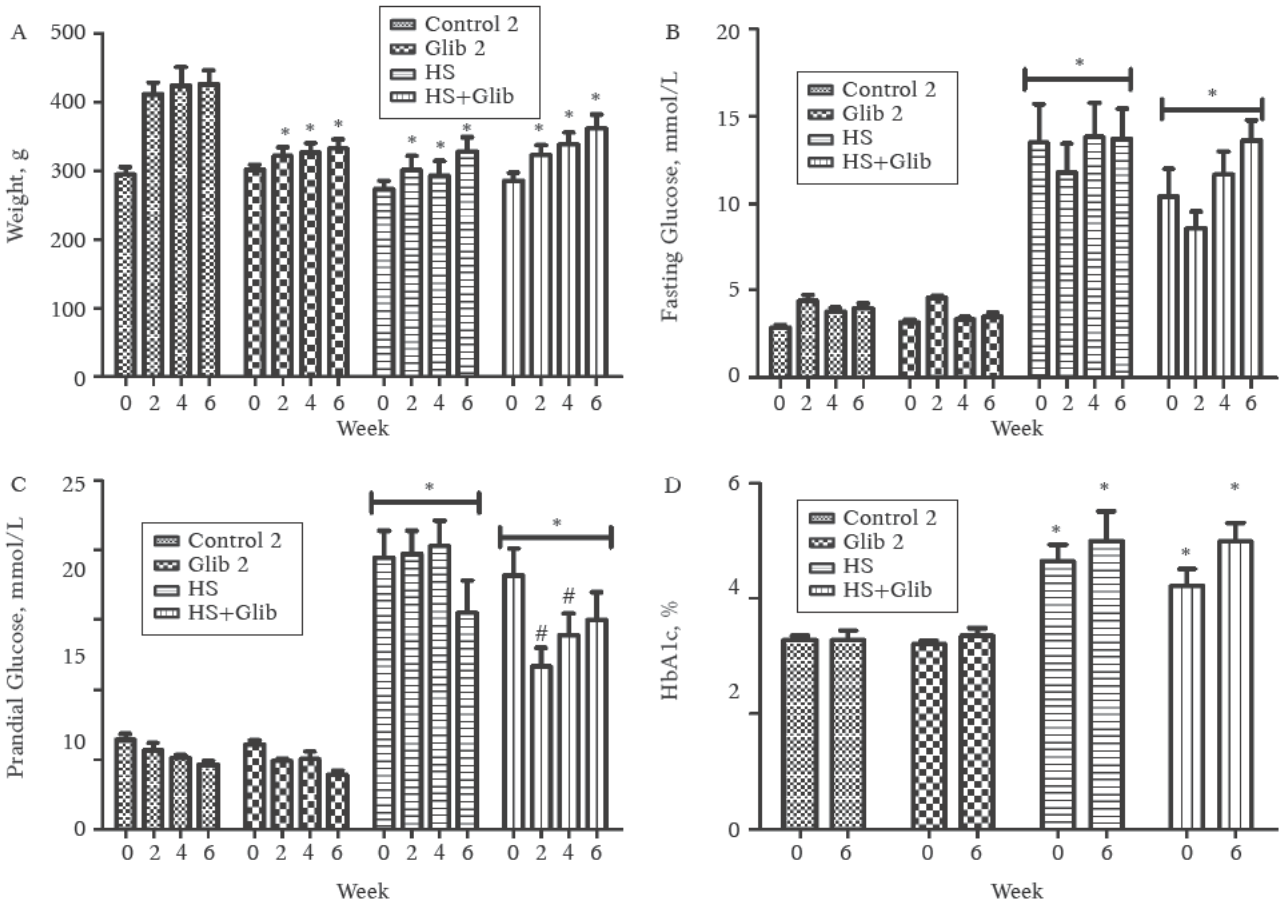

Fig. 2. Therapeutic effects of glibenclamide in rats with mild streptozotocin-induced diabetes mellitus

A, mean weight in the experimental groups during the 6-week glibenclamide treatment; B, the effect of glibenclamide on blood glucose concentration in fasted animals during the 6-week glibenclamide treatment; C, the effect of glibenclamide on blood glucose concentration in fed animals during the 6-week glibenclamide treatment; D, the level of glycated hemoglobin (HbA1c%) before and after the treatment with glibenclamide.

Control 2, the control group for a model of mild diabetes mellitus; Glib 2, intact glibenclamide-treated animals of age corresponding to that of the HS group; HS, animals with mild streptozotocin-induced diabetes mellitus; HS+Glib, the rats with mild streptozotocin-induced diabetes mellitus treated with glibenclamide (2 mg/kg, 6 weeks). Bars represent the mean±SEM (n=10). **P*<0.05 versus control group; #*P*<0.05 versus HS group.

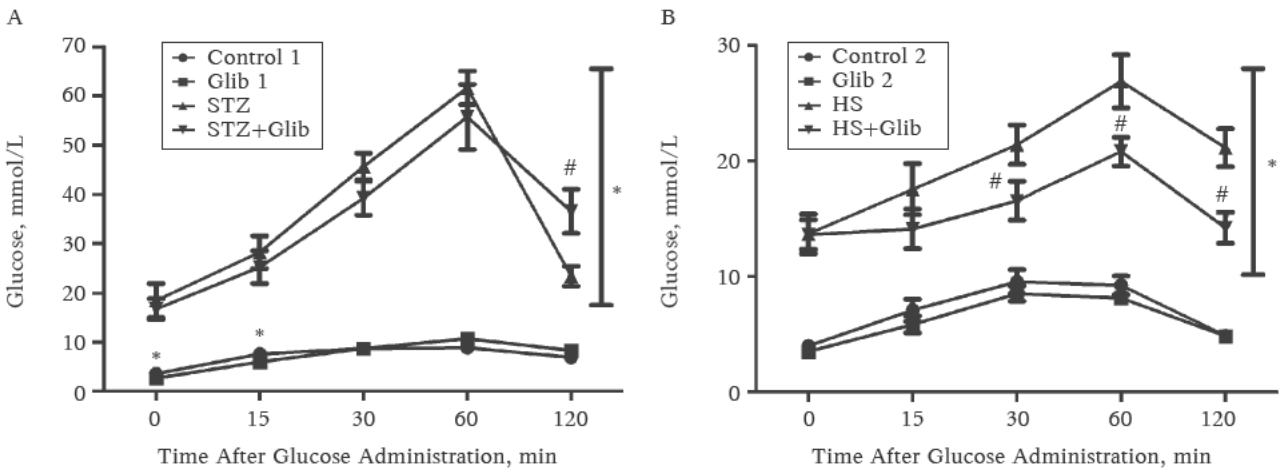

Fig. 3. Mean glucose levels obtained during the oral glucose tolerance test after the 6-week glibenclamide treatment (2 mg/kg, 6 weeks)

A, the rats with severe diabetes mellitus; B, the rats with mild diabetes mellitus. Bars represent the mean±SEM (n=10). **P*<0.05 versus Control 1 or Control 2 group; #*P*<0.05 versus STZ or HS group. All other designations are as in Fig 1.

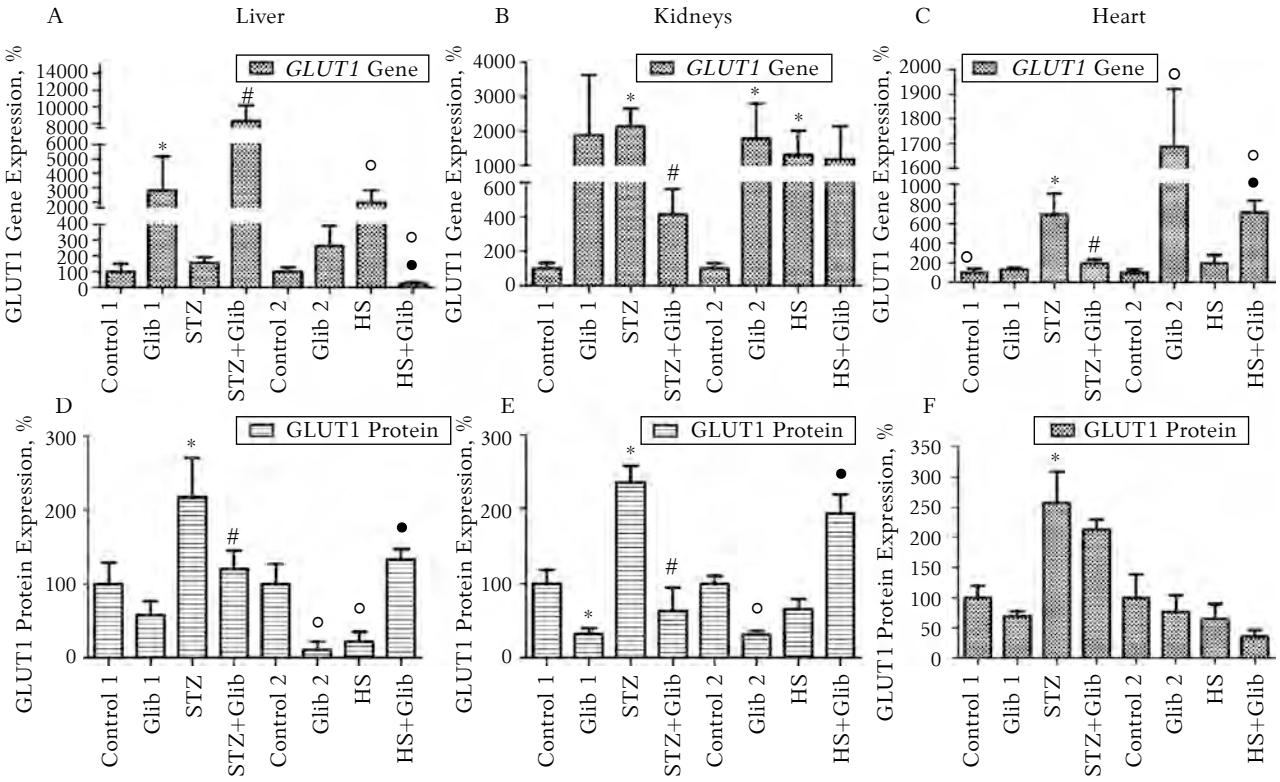

Fig. 4. Effects of glibenclamide on the *GLUT1* gene expression in the liver and the kidneys (A, C, E) and on the number of GLUT1-positive cells in the liver (B), kidneys (D), and heart (F) in rats with severe and mild streptozotocin-induced diabetes mellitus compared with control and glibenclamide-treated rats

Results are presented as percentage of the mean control values. Designation of groups as in Fig. 1.
**P*<0.05 versus Control 1 group; #*P*<0.05 versus STZ group; ○*P*<0.05 versus Control 2 group; ●*P*<0.05 versus HS group.

toplasmic staining was detected in a few hepatocytes (Fig. 8). Modifications of the GLUT1 protein expression in the liver were similar to those observed in the kidneys: the expression was increased in diabetic animals and attenuated by the glibenclamide treatment. On the contrary, the induction of diabetes mellitus with streptozotocin did not alter the level of the *GLUT1* mRNA expression level in this organ; however, the expression was upregulated when both intact and diabetic animals were treated with glibenclamide (Fig. 4A and B). In the animals with untreated streptozotocin-induced diabetes, a massive damage of liver tissue was observed: the HAI increased 4 times, and the glibenclamide treatment decreased the degree of tissue damage (Fig. 5).

Modification of GLUT1 Gene and Protein Expression by Glibenclamide in Rats With Mild Diabetes Mellitus. Mild diabetes mellitus induced by high-fat diet and low-dose streptozotocin injections caused the different changes in the GLUT1 expression compared with severe diabetes mellitus described in the previous section (Fig. 4). In addition, the healthy rats used in this series of experiments also exhibited some difference in the GLUT1 expression pattern, which probably should be explained by an age difference (Control 2 rats were 5 weeks

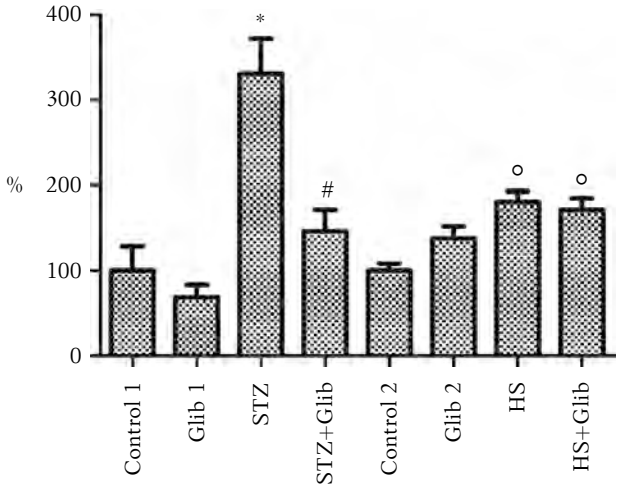

Fig. 5. The effects of glibenclamide on the histological activity index in the liver of severely and mildly diabetic rats

**P*<0.05 to Control 1 group; #*P*<0.05 versus STZ group; ○*P*<0.05 versus Control 2 group.

older than Control 1 rats). In the healthy rats of this age, the glibenclamide treatment caused a decrease in the GLUT1 protein expression and an increase in the *GLUT1* mRNA expression in the kidneys. Mild diabetes did not affect the GLUT1-positive cell count in the kidneys; however, the *GLUT1* mRNA

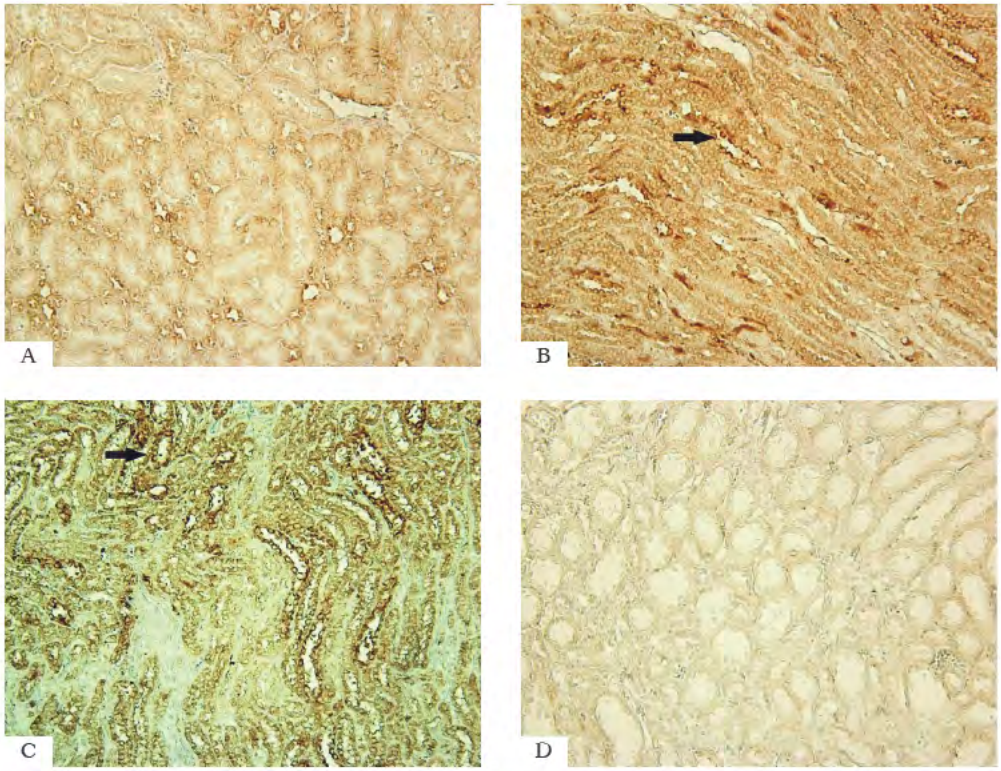

Fig. 6. Photomicrographs of rat kidney tissue stained immunohistochemically with a GLUT1 antibody in experimental series 1

A, control 1; B, the intact rats treated with glibenclamide (2 mg/kg, 6 weeks); C, the group of animals with severe streptozotocin-induced diabetes mellitus; D, the rats with severe streptozotocin-induced diabetes mellitus treated with glibenclamide (2 mg/kg, 6 weeks). Magnification, ×200. Arrows indicate positively stained cells.

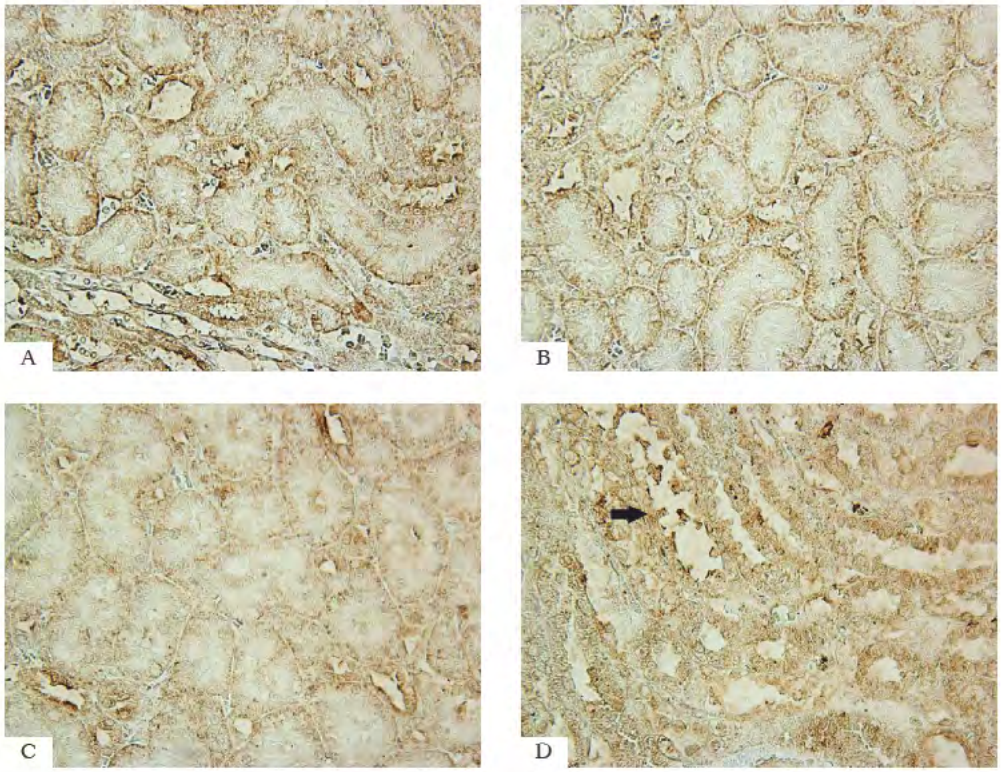

Fig. 7. Photomicrographs of rat kidney tissue stained immunohistochemically with a GLUT1 antibody in the experimental series 2

A, control 2; B, the intact glibenclamide-treated animals (2 mg/kg, 6 weeks) of age corresponding to that of the HS group; C, the group of animals with mild streptozotocin-induced diabetes mellitus (HS); D, the rats with mild streptozotocin-induced diabetes mellitus treated with glibenclamide (2 mg/kg, 6 weeks). Magnification, ×200. Arrows indicate positively stained cells.

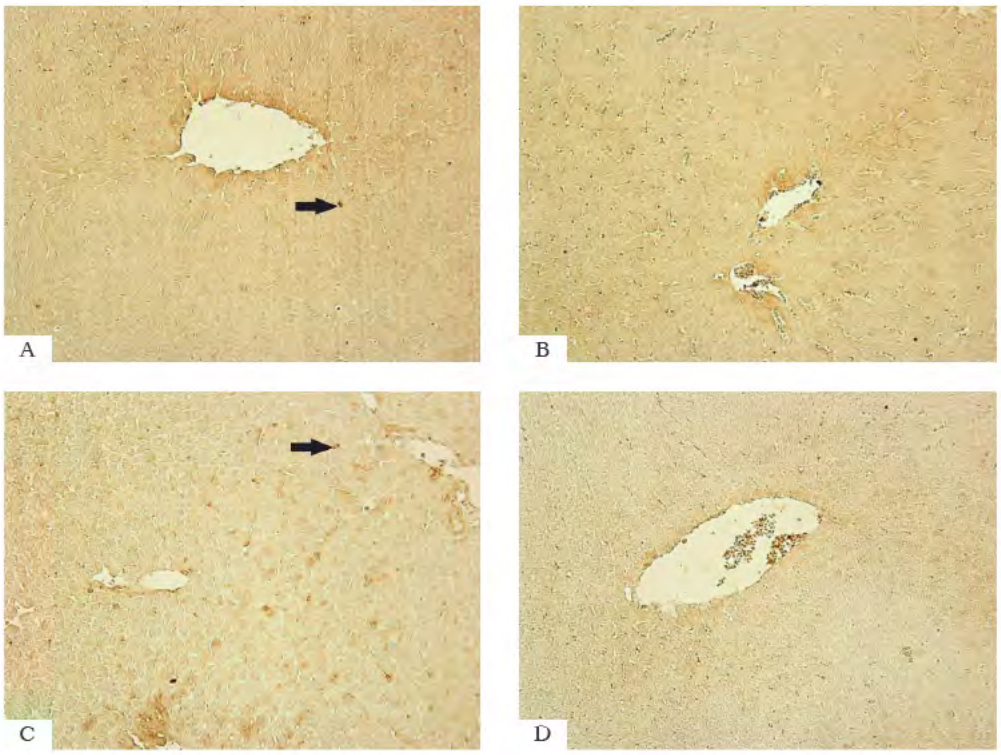

Fig. 8. Photomicrographs of rat liver tissue stained immunohistochemically with a GLUT1 antibody in the experimental series 1
A, control 1; B, the intact rats treated with glibenclamide (2 mg/kg, 6 weeks); C, the group of animals with severe streptozotocin-induced diabetes mellitus; D, the rats with severe streptozotocin-induced diabetes mellitus treated with glibenclamide (2 mg/kg, 6 weeks). Magnification, ×200. Arrows indicate positively stained Kupffer cells.

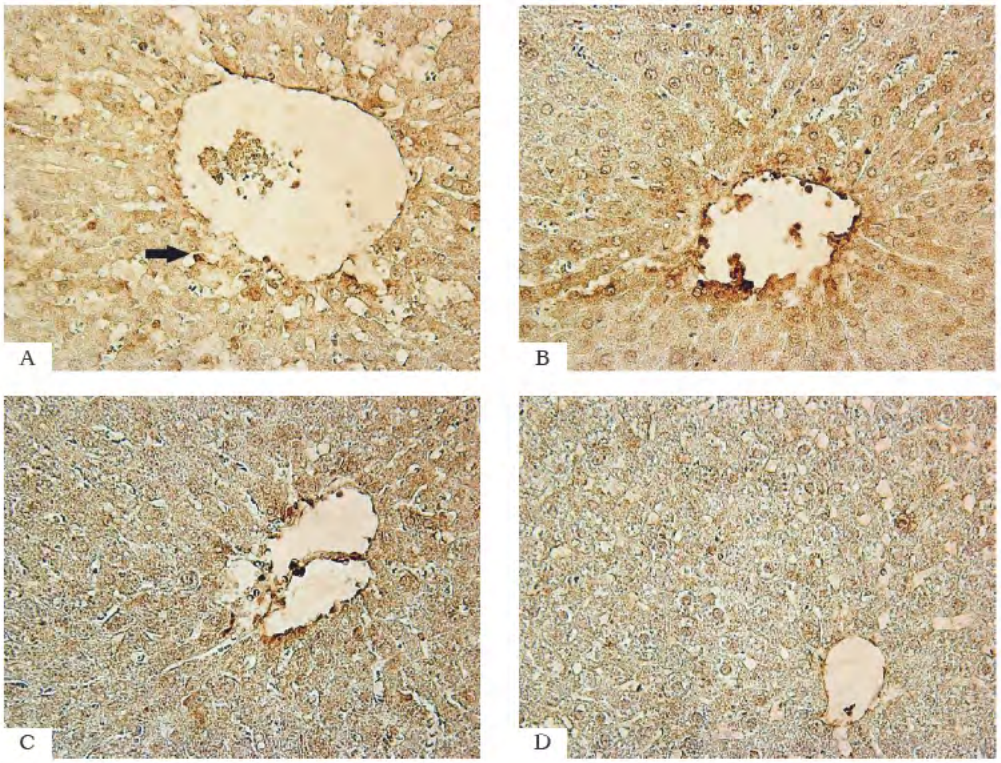

Fig. 9. Photomicrographs of rat liver tissue stained immunohistochemically with a GLUT1 antibody in the experimental series 2
A, control 2; B, the intact glibenclamide-treated animals (2 mg/kg, 6 weeks) of age corresponding to that of the HS group; C, the group of animals with mild streptozotocin-induced diabetes mellitus (HS); D, the rats with mild streptozotocin-induced diabetes mellitus treated with glibenclamide (2 mg/kg, 6 weeks). Magnification, ×200. Arrows indicate positively stained Kupffer cells.

level was increased. The glibenclamide treatment in mildly diabetic rats increased the GLUT1 protein expression, but did not affect the *GLUT1* gene expression in the kidneys (Fig. 4C and D; Fig. 7). It appeared that an increase in the GLUT1 expression in the kidneys observed in severely diabetic rats was not documented in the model of mild diabetes. Further, a glibenclamide effect in this model of diabetes was opposite to that observed in severely diabetic rats.

In the heart, the GLUT1 protein expression was not changed in any group. The *GLUT1* gene expression was increased by glibenclamide in healthy and mildly diabetic rats (Fig. 4E and F).

In the liver, the GLUT1 protein expression in the control 2 group was decreased by the glibenclamide treatment (Fig. 4A and B; Fig. 9). Mild diabetes mellitus did not alter the GLUT1 protein expression, but increased the *GLUT1* gene expression in the liver (Fig. 4A and B). The glibenclamide treatment in mildly diabetic rats decreased the upregulated gene expression; however, it caused an increase in the GLUT1 protein expression (Fig. 4A and B). Thus, the effects of mild diabetes mellitus and glibenclamide on the GLUT1 protein and the mRNA expression were opposite in the liver and the kidneys; this could be explained by the abortive gene transcription and posttranscriptional regulation of the GLUT1 expression. The induction of mild diabetes mellitus provoked a certain hepatotoxic effect registered as an increase in the HAI. The glibenclamide treatment did not cure the affected liver (Fig. 5).

Discussion

The aim of this study was not to show the effectiveness of glibenclamide that has been successfully used in the treatment of type 2 diabetes mellitus for decades, but to link the effects of glibenclamide on some metabolic features (pronounced or mild hyperglycemia, hyperlipidemia, etc.) with its effects on the GLUT1 expression in some organs and liver damage. In the present study, we have shown that the glibenclamide treatment produced some improvement in metabolic parameters and insulin production in both the models of severe and mild diabetes mellitus when compared with the corresponding diabetic control group; thus, the anticipated therapeutic effect was achieved. No effect on HbA1c% was observed, but this can be explained by the fact that in the model of severe diabetes mellitus, the small increase in insulin secretion was significant but not sufficient in the STZ+Glib group due to the loss of the major amount of beta cells after streptozotocin administration. In the model of mild diabetes mellitus, we consider that the duration of experiment was not enough for the changes in HbA1c% to become obvious; still, improved glucose tolerance and normalized insulin secretion in the HS+Glib group are the proof of the effectiveness of the drug.

There are few studies that have investigated the effects of sulfonylureas on GLUT1 expression. It was reported that glibenclamide significantly increased the total content and the plasma membrane level of GLUT1 in L6 myotubes (2). Sulfonylurea gliclazide increased GLUT 1 protein synthesis and mRNA expression through protein kinase A-mediated pathway in cultured rat L6 myoblasts (14). The chronic application of sulfonylurea to cultured cardiomyocytes was found to produce an approximate doubling of the basal glucose uptake rates by an insulin-independent pathway most probably involving an increased protein expression of GLUT1 (7). In cultured cardiomyocytes, sulfonylurea glimepiride caused an increase in the GLUT1 protein expression in crude membrane fractions (15). It was reported that sulfonylurea tolazamide increased the GLUT1 protein expression, but did not affect the GLUT1 mRNA level in cultured mesangial cells (16). Thus, sulfonylureas appear to be GLUT1-upregulating drugs.

Our data partly support the above statement. A trend to upregulate the mRNA expression of GLUT1 was observed in the kidneys of animals in the intact control 1 group; the effect was better pronounced and reached a statistical significance in the older animals of the Control 2 group. In this animal group, glibenclamide upregulated the GLUT1 mRNA expression in the myocardium as well. It was not the case in the younger animals of the Control 1 group; however, in this group, glibenclamide upregulated the mRNA expression in the liver tissue. We have not observed the upregulation of the GLUT1 protein in any organ in intact animals; on the contrary, the protein was decreased in the kidneys of both Glib 1 and Glib 2 groups and in the liver of Glib 1 group. Thus, the effect of glibenclamide on the GLUT1 expression in healthy rats is organ-specific and depends on the age of the animal. Moreover, the drug appears to trigger an abortive transcription of the *GLUT1* gene. The opposite effects of glucose on the GLUT1 expression in animal models and cultured cells have already been observed (8, 9 and references therein).

Literature data on the modifications of the GLUT1 expression in diabetic animals are scarce and contradictory despite the utmost importance of the transporter for glucose transport. An increase in the GLUT1 expression in the kidneys is most studied, as the overexpression of GLUT1 favors the development of diabetic nephrosclerosis (17). Many publications indicate an upregulation of the gene in diabetes; however, the down-regulation or the lack of any effect was also observed. Moreover, diabetes can cause the changes in the GLUT1 expression that differ from tissue to tissue, and also due to different

experimental protocols (14–16). Our results confirm the upregulation of the transporter in diabetic animals (Fig. 4). Only severe diabetes provoked an increase in the transporter protein expression; the effect of mild diabetes was limited to the upregulation of the mRNA transcription. Probably this was due to more pronounced hyperglycemia in the former form of the disease.

Discrepancies in data on mRNA and protein expression can be explained by the existence of several mechanisms related to the regulation of gene expression. For example, the induction of increased stability of mRNA is known to be one of the ways for the regulation of the *GLUT1* expression (18). Alternatively, an increase in the mRNA copies without an increase in the GLUT1-positive cell number could be detected as a result of abortive transcription. Regulation of the protein expression on translation level could also explain the phenomenon (19).

In animals with severe diabetes mellitus, the glibenclamide treatment produced a GLUT1 downregulating effect. It was well-pronounced in the kidneys, where both gene and protein expression were reduced almost to normal levels. Surprisingly, the effect of the drug in animals with mild diabetes appears to be sooner upregulating. According to our opinion, the different effects of the drug in 2 models of the disease can be explained by the different insulin levels in the 2 animal groups. The glibenclamide treatment normalized the insulin level in animals with mild diabetes; the rats with severe disease still suffered from hypoinsulinemia. It should be kept in mind that GLUT1 is partly dependent on insulin; for example, transporter redistribution is mediated by insulin in insulin-sensitive tissues (19). Upregulation might be due to the insulin action.

To explain the downregulating effects of glibenclamide in the kidneys of animals with severe diabetes, several mechanisms can be suggested. It has been shown that KATP channels modulate the basal and insulin- or high glucose level-stimulated glucose transport (20); however, the mechanism of this effect has not been clarified. We can suggest that the opening of KATP channels stimulates the expression of GLUT1 with a subsequent increase of sodium and glucose influx into the cells. Blockade of these channels with glibenclamide could decrease the expression of GLUT1 by a feedback mechanism. The GLUT1-decreasing effect of glibenclamide could be achieved also by the increasing effectiveness of glucose utilization for energy generation. It has been shown that glibenclamide induces the permeabilization of the inner mitochondrial membrane to Cl^- by opening the inner mitochondrial anion channel. Cl^- influx induced by glibenclamide facilitates K^+ entry into mitochondria, thus promoting a net Cl^-/K^+ cotransport, Deltapsi dissipation, and stimulation of state 4 respiration rate (6). Improved bioenergetics could also favor the downregulation of GLUT1 expression by feedback mechanisms (21).

In this study, we have examined the effect of glibenclamide treatment on histopathological changes in the liver tissue probably due to nonalcoholic steatohepatitis, which were evaluated by the HAI (Fig. 5). In the rats with severe diabetes mellitus, this parameter increased and was normalized by the glibenclamide treatment. However, in mildly diabetic animals, glibenclamide could not reduce the elevated HAI-counts. Thus, we can conclude that the glibenclamide treatment reduces the pathophysiological changes in the liver in case of chronic severe hyperglycemia, but cannot protect the liver in case of increased fat consumption with food. In the literature, there are data on both negative (22) and positive (23, 24) effects of glibenclamide on liver lipid metabolism in humans.

Thus, glibenclamide normalizes the GLUT1 expression in the kidneys of severely diabetic rats; it is prospective for the treatment of diabetic nephropathy. Our results are additive to results of other groups studying the effects of glibenclamide on the development of diabetic nephrosclerosis. Clinically relevant concentrations of glibenclamide in vivo and in vitro suppress the high glucose-enhanced accumulation of collagen I, collagen IV, and fibronectin in mesangial cells (25).

Glibenclamide can also prevent the liver damage caused by severe hyperglycemia, but not hyperlipidemia. GLUT1 expression is affected by glucose and insulin concentrations and can be modulated by glibenclamide in rats with severe and mild diabetes mellitus.

Conclusions

The GLUT1 expression is affected by the glucose and insulin levels and can be modulated by glibenclamide in severely and mildly diabetic rats. Glibenclamide can prevent the liver damage caused by severe hyperglycemia.

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Statement of Conflict of Interest

The authors state no conflict of interest.

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