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Na^+-Glucose Transporter-2 Messenger Ribonucleic Acid Expression in Kidney of Diabetic Rats Correlates with Glycemic Levels: Involvement of Hepatocyte Nuclear Factor-1α Expression and Activity

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Mutations in Na^+-glucose transporters (SGLT)-2 and hepatocyte nuclear factor (HNF)-1α genes have been related to renal glycosuria and maturity-onset diabetes of the young 3, respectively. However, the expression of these genes have not been investigated in type 1 and type 2 diabetes. Here in kidney of diabetic rats, we tested the hypotheses that SGLT2 mRNA expression is altered; HNF-1α is involved in this regulation; and glycemic homeostasis is a related mechanism. The *in vivo* binding of HNF-1α into the SGLT2 promoter region in renal cortex was confirmed by chromatin immunoprecipitation assay. SGLT2 and HNF-1α mRNA expression (by Northern and RT-PCR analysis) and HNF-1 binding activity of nuclear proteins (by EMSA) were investigated in diabetic rats and treated or not with insulin or phlorizin (an inhibitor of SGLT2). Results showed that diabetes increases SGLT2 and HNF-1α mRNA expression (~50%) and binding of nuclear proteins to a HNF-1 consensus motif (~100%). Six days of insulin or phlorizin treatment restores these parameters to nondiabetic-rat levels. Moreover, both treatments similarly reduced glycemia, despite the differences in plasma insulin and urinary glucose concentrations, highlighting the plasma glucose levels as involved in the observed modulations. This study shows that SGLT2 mRNA expression and HNF-1α expression and activity correlate positively in kidney of diabetic rats. It also shows that diabetes-induced changes are reversed by lowering glycemia, independently of insulinemia. Our demonstration that HNF-1α binds DNA that encodes SGLT2 supports the hypothesis that HNF-1α, as a modulator of SGLT2 expression, may be involved in diabetic kidney disease. (*Endocrinology* 149: 717–724, 2008)

RENAL GLUCOSE REABSORPTION is a coordinated process, which takes place in the epithelial cells of the proximal tubule, involving two classes of glucose transporters, the Na^+-glucose transporters (SGLTs) and the facilitative diffusion transporters (GLUTs) (1, 2). In the early S1 segment, in which the bulk of filtered glucose is reabsorbed, the low-affinity/high-capacity glucose transporters, SGLT2 and GLUT2, are coexpressed. SGLT2, at the brush-border membrane, is involved in the glucose uptake from the tubular fluid into the epithelial cell, for further release into the bloodstream via basolaterally expressed GLUT2-facilitative glucose transporter (2).

Uncontrolled diabetes, by increasing blood and interstitial glucose concentration, may lower the outwardly directed glucose gradient, from tubule to blood, impairing the glucose efflux from the epithelial cells and consequently the glucose reabsorption process as a whole. Thus, increases in the glucose transporters gene expression are important to renal glucose reabsorption maintenance in diabetes (3, 4). In fact, increased GLUT2 gene expression has been extensively reported in experimental models of spontaneously (5) or pharmacologically induced diabetes (3, 4, 6–9). However, concerning the SGLT2 cotransporter in experimental diabetes, there are only a few studies showing unchanged (7) or increased mRNA expression (8).

On the other hand, renal glycosuria, defined by urinary glucose excretion in the presence of a normal blood glucose concentration, has been related to defective SGLT2 gene, and 21 different mutations have already been described in families affected by this disease (10), highlighting this cotransporter on the renal glucose reabsorption physiology.

Hepatocyte nuclear factor (HNF)-1α was originally characterized as a liver-specific transcription factor that binds to the promoters of many genes (11); subsequently, it was shown to be expressed in the pancreatic islet β-cell-derived insulinoma cell line, in which it transactivates the rat insulin gene (12). Thereafter it was reported that HNF-1α-deficient mice have impaired kidney proximal tubular reabsorption of glucose, phosphate, and amino acids, thus relating this transcription factor with the renal glucose reabsorption function (13), which was further confirmed to involve decreased SGLT2 expression in tubular cells (14). Later it was shown that HNF-1α directly controls SGLT2 gene expression in mouse and man (15). Additionally, since 10 yr ago, the ma-

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Abbreviations: ChIP, Chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, facilitative diffusion transporter; HNF, hepatocyte nuclear factor; MODY3, maturity-onset diabetes of the young 3; SGLT, Na^+-glucose transporter.

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turity-onset diabetes of the young 3 (MODY3) has been related to HNF-1α mutations that impair liver and pancreatic expression of some target genes including insulin (16–18), establishing a definitive relationship between diabetes and HNF-1α but not clearly showing direct effects upon renal glucose reabsorption.

Although SGLT2 and HNF-1α genes have been extensively studied in these rare diseases, renal glycosuria and MODY3, the expression of these genes, as well as the potential role of HNF1-α as enhancer of SGLT2 transcription, has not been investigated in type 1 and type 2 diabetes. We hypothesized that HNF-1α-induced change of SGLT2 gene expression occurs in kidney of diabetic subjects, which may be related to the development and/or evolution of the nephropathy. Thus, the present study was designed to investigate the HNF-1α and SGLT2 mRNA expression in diabetic rats as well as the effects of insulin and phlorizin treatment, the latter a specific inhibitor of SGLT2 protein, which blocks the renal glucose reabsorption. This strategy allowed us to show the individual effects of insulin or plasma and urinary glucose levels on the changes of the gene expression. Additionally, HNF-1α binding activity was investigated in diabetic and insulin-treated diabetic rats to demonstrate the role of this transcriptional factor in SGLT2 mRNA expression in renal cortex. The *in vivo* binding of HNF-1α into the SGLT2 promoter in kidney was confirmed by chromatin immunoprecipitation (ChIP) assay. We observed that diabetes increased both SGLT2 and HNF-1α mRNA expression and that a 6-d treatment with insulin or phlorizin is able to reduce the increased expression of both genes. Besides, HNF-1α binding activity was seen to be modulated in parallel with its mRNA expression in diabetic and diabetic insulin-treated rats. This study shows, for the first time, a correlation between the expression of SGLT2 and HNF-1α binding activity in experimental diabetes, which may be involved in the development of diabetic renal tubular and glomerular diseases.

Materials and Methods

Experimental animals

Male Wistar rats (~260 g of body weight) were individually caged in an environment maintained at controlled temperature (23 ± 2 C) and lighting (from 0600 to 1800 h) and allowed free access to water and standard rodent chow diet (Nuvilab CR-1; Nuvital, Curitiba, Brazil). One week later, the animals were fasted overnight and rendered diabetic by a single iv injection (penis vein) of alloxan (40 mg/kg body weight). Nondiabetic control rats were injected with saline in the same volume. Animals with polyuria (>25 ml/d^{-1}), glycosuria (>2+ but without ketonuria) were chosen as diabetics. The effects of diabetes were evaluated 20 d after alloxan or saline injection.

Acute effects of insulin or phlorizin treatment

Twenty-day diabetic rats were evaluated 4 or 12 h after a single sc 4-U NPH insulin injection (Biohulin N, Biobras, Montes Claros, MG, Brazil) plus 2 U regular insulin (Iolin R; Biobras) or sc phlorizin, 0.4 g kg body weight^{-1} (Sigma, St. Louis, MO). Phlorizin solution was 0.4 g ml^{-1} propylene glycol. Diabetic placebo-treated rats were injected with the same volume of saline, in the insulin study, or propylene glycol, in the phlorizin study. The injections were performed at 0830 h.

Short-term effects of insulin or phlorizin treatment

Twenty-day diabetic rats were evaluated after 1, 4, and 6 d of treatment with sc NPH insulin, 2 U in the morning (0830 h) and 4 U in the afternoon (1730 h) or sc phlorizin, 0.4 g kg body weight^{-1} each 12 h (0830 and 2030 h). Diabetic placebo-treated rats were injected with the same volume of saline, in the insulin study, or propylene glycol, in the phlorizin study. Twenty-four-hour urine was collected during the first, fourth, and sixth days of treatment. Animals were killed at 0830 h. For diabetes induction and final experimental procedures, animals were anesthetized with ip injection of sodium pentobarbital (40 mg/kg^{-1} body weight). Experimental procedures were approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences of the University of Sao Paulo (protocol 165/02).

RNA extraction and purification

Renal outcortices were sampled as previously described (8, 9) and immediately frozen in liquid nitrogen for further procedures. Total RNA was extracted using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA) as described by manufacturer instructions. After this, 1 mg of total RNA was processed for poly(A) RNA (mRNA) purification, using the PolyATract mRNA isolation systems (Promega, Madison, WI), and following the manufacturer instructions. In preliminary experiments of Northern blotting, total RNA did not give clear signals, which was achieved with purified poly(A) RNA.

Northern blotting analysis for SGLT2

One microgram of poly(A) RNA was fractionated by 1% agarose-formaldehyde gel electrophoresis, transferred to nylon membranes, and probed with a (α^{32}-P)dCTP-labeled rat SGLT2 cDNA (Random Primers DNA labeling system kit; Invitrogen Life Technologies). The blots were analyzed by scanner densitometry (Image Master 1D; Pharmacia Biotech, Uppsala, Sweden). β-Actin was used as an internal loading control, and the results were expressed as fold change relative to untreated diabetic animals after normalization to β-actin.

RT-PCR analysis for HNF-1α

Reverse transcription was done with 2 μg of total RNA using ImProm-II reverse transcriptase and random primers, according to manufacturer's instructions (Promega). RT-PCR assays were done in quadruplicate using recombinant GoTaq DNA polymerase (Promega) and 10 pmol of each primer in a master mix of 50 μl. The cycle numbers for each gene was defined after titration using 18 and 40 cycles and was within the logarithmic phase of amplification. The PCR primers for HNF-1α (forward, 5′-AAGATGACACGGATGACGATGG-3′; reverse, 5′-TGTTGAGGTGCTGGGACAGG-3′) amplified a 237-bp cDNA fragment with a melting temperature of 58.5 C, and those for rat β-actin (forward, 5′-ATGAAGATCCTGACCGAGCGTG-3′; reverse, 5′-CTTGCTGATCCACATCTGCTGG-3′) amplified a 510-bp fragment with a melting temperature of 55.5 C. The number of cycles was 33 for HNF-1α and 31 for β-actin. PCR products were resolved on 1.2% ethidium bromide-agarose gels, and the band intensities were determined by digital scanning followed by quantification using Scion Image analysis software (Scion Corp., Frederick, MD). Total RNA used for RT-PCR analysis was the same used for poly(A) RNA purification. β-Actin was used as an internal control, and the results were expressed as fold change relative to untreated diabetic animals after normalization to β-actin.

EMSA

Additional groups of nondiabetic, diabetic, and diabetic insulin-treated rats were prepared for renal cortex nuclear protein extraction as previously described (19). A double-stranded oligonucleotide corresponding to a sequence of rat GLUT2 promoter that contains the potential consensus binding site of HNF-1α (5′-AAAG*GTATATTGATTGAATTA*CCATC-3′) (20) was end labeled using T_4 PNK and (γ-^{32}P)ATP (Amersham Pharmacia Biotech, Amersham, UK). Binding reactions of the probes (30,000 cpm) were performed with 15 μg protein from nuclear extract, at room temperature for 20 min, in 20 μl of the binding buffer [20 mM HEPES (pH 7.6), 50 mM KCl, 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, and 2 μg polydeoxyinosinic-deoxycytidylic acid; Amersham Pharmacia Biotech]. Competitive binding assays were conducted under the same conditions, with the addition of 10-, 20-, and 30-fold molar excess of unlabeled oligonucleotide. The DNA-protein complexes

were electrophoresed on 4% nondenaturing polyacrylamide gels at 4 C in 45 mM Tris, 45 mM borate, and 1 mM EDTA buffer. The gels were dried and subjected to autoradiography. The blots were analyzed by scanner densitometry (Image Master 1D; Pharmacia Biotech), and the results of the binding activity were expressed as fold change relative to untreated diabetic animals.

ChIP assay

Renal outcortices samples were processed using buffers and reagents from EZ ChIP chromatin immunoprecipitation kit (Upstate, Lake Placid, NY; catalog no. 17-371), according to manufacturer instructions. Briefly, the fixed tissue fragments were transferred to lysis buffer after cross-linking and DNA shearing was performed by sonication. Equal amounts of protein from each sample were diluted in dilution buffer in duplicate and precleared with protein G-agarose saturated with salmon sperm. Agarose was precipitated and supernatants were transferred into a new tube. Ten microliters were picked up to be used as input, and samples were incubated with 3 μg of anti-HNF-1α antibody (sc-6547; Santa Cruz Biotechnology, Santa Cruz, CA) or 1 μg of anti-RNA polymerase II overnight at 4 C. In parallel, one precleared sample was incubated in the same conditions without antibody (no-AB control). Next, samples were incubated with protein G-agarose saturated with salmon sperm for 1 h at 4 C. Protein G-agarose was collected and the pellets were extensively washed. Cross-linked DNA was eluted from protein G-agarose and incubated for 6 h at 65 C for cross-linking reversal. Next, samples were treated with RNase A, purified by using spin filters, and recovered in elution buffer. DNA from input-, no-AB-, and HNF-1α-precipitated samples were submitted to PCR for the detection of the SGLT2 gene. Because the sequence of rat SGLT2 promoter is not available, we designed a primer set that amplifies an early fragment of the gene codifying region (U29881), between the nucleotides 10 and 172, originating a product of 163 bp. The sequences of the primers are AGGGACACGTAGAGGAAGGCTC (sense) and GCCAAGAAGTAGCCACCAACTG (antisense) with an annealing temperature of 55 C. DNA from RNA polymerase II-precipitated samples were used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter amplification, using primers provided in the kit (annealing temperature of 59 C). The amplification reactions consisted of an initial denaturation (94 C for 3 min) and 32 cycles (94 C for 20 sec, annealing temperature for 30 sec, and 72 C for 30 sec) followed by a final extension of 2 min at 72 C. The products were visualized as described in RT-PCR analysis for HNF-1α.

Analytical procedures

Plasma, obtained from blood sampled (tail vein) at the time the animals were killed, and urine, obtained by 24-h urine sampling, were assayed for glucose by the glucose-oxidase method (Analisa Diagnostica, Sao Paulo, Brazil). Insulin was also determined in plasma samples (RIE, Coat-a-Count; Diagnostic Products, Los Angeles, CA).

Data analysis

Results from each group of saline- or propylene glycol-treated rats were pooled into placebo groups because there were no differences according to the period of treatment. All values were reported as mean ± SEM. At least three different experiments were performed, evaluating at least five different animals in each group. Detailed data of sample size were informed in the legends of figures and tables. For comparison of the mean values, one-way ANOVA, with Student-Newman-Keuls post-test, or Student *t* test was performed, using GraphPad InStat version 3.01 (GraphPad Software, San Diego, CA).

Results

Effects of diabetes

Compared with nondiabetic rats (Table 1), diabetic rats had lower body weight ($P < 0.001$), as expected. Diabetic rats showed (Table 1) very high plasma and urinary glucose levels ($P < 0.0001$) as well as very low plasma insulin ($P < 0.0001$). Figure 1 shows that diabetic rats increased both the SGLT2 (Fig. 1A) and HNF-1α (Fig. 1B) mRNAs, respectively, by 51% ($P < 0.01$) and 30% ($P < 0.05$).

TABLE 1. Effects of diabetes induction in rats

	Nondiabetic rats	Diabetic rats
Body weight (g)	324.7 ± 5.3	272.3 ± 8.6[a]
Plasma glucose (mM)	8.6 ± 0.58	22.6 ± 1.03[a]
Plasma insulin (ng/ml)	0.42 ± 0.03	0.09 ± 0.008[a]
Urinary glucose (mg per 24 h)	1.5 ± 0.5	408.1 ± 33.8[a]

Data are mean ± SEM of 10 animals in each group.
[a] $P < 0.0001$ *vs.* nondiabetic rats, Student's *t* test.

Acute effects of insulin and phlorizin treatment

Twenty-day diabetic rats were studied 4 and 12 h after a single injection of insulin, phlorizin, or placebo. Because all the results of each placebo group, saline- or propylene glycol-treated, respectively, for insulin or phlorizin showed no differences between 4 and 12 h, they were pooled. Twenty-four-hour urinary glucose before the injections was similar in all groups, showing a similar diabetic condition

Table 2 shows that the insulin injection induced a 4-h decreased ($P < 0.001$) plasma glucose, which remained low at 12 h ($P < 0.01$). Similarly, phlorizin induced a significant ($P < 0.001$) decrease in plasma glucose. Insulin-treated rats

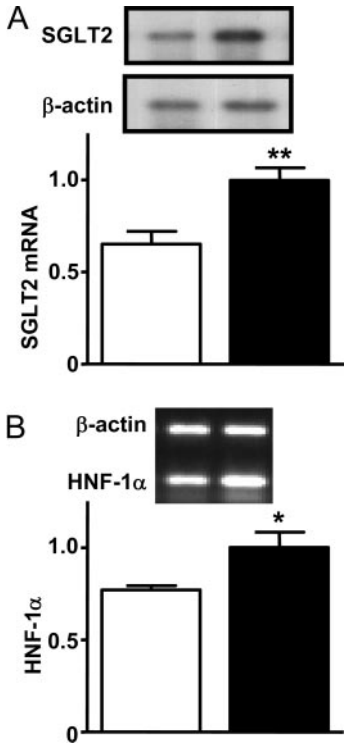

FIG. 1. SGLT2 (A) and HNF-1α (B) mRNA analysis in renal cortex of nondiabetic (*open bars*) and diabetic (*closed bars*) rats. A, SGLT2 mRNA analysis performed by Northern blotting of poly(A) RNA: at the *top*, typical images of the SGLT2 and respective β-actin mRNAs, at the *bottom*, the abundance of the SGLT2 mRNA normalized to β-actin. B, HNF-1α mRNA analysis performed by RT-PCR: at the *top*, typical images of the HNF-1α and respective β-actin amplicons, at the *bottom*, the estimated abundance of the HNF-1α mRNA normalized to β-actin value. Data are mean ± SEM of five animals in each group, evaluated in three different experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* nondiabetic rats; Student's *t* test.

TABLE 2. Acute effects of insulin and phlorizin treatment of diabetic rats

	Insulin (ng/ml)	Glucose (mM)
Insulin treatment		
Placebo (4 + 12 h)	0.16 ± 0.09	18.8 ± 0.8
4 h	18.1 ± 2.3[a]	7.4 ± 0.9[a]
12 h	7.4 ± 1.7[b,c]	13.4 ± 0.9[d,e]
Phlorizin treatment		
Placebo (4 + 12 h)	0.23 ± 0.08	20.2 ± 0.97
4 h	0.16 ± 0.02	11.7 ± 0.8[a]
12 h	0.18 ± 0.09	13.1 ± 0.6[a]

Diabetic rats were studied 4 and 12 h after a single injection of insulin or phlorizin. Placebo was saline for insulin treatment or propyleneglycol for phlorizin treatment. Results from 4- and 12-h placebo-treated rats were pooled. Data are mean ± SEM of 10 and 14 animals in saline- and propylenoglycol-placebo groups, respectively, and of six to eight animals in each insulin- or phlorizin-treated groups.

[a] $P < 0.001$ *vs.* respective placebo.
[b] $P < 0.05$ *vs.* respective placebo.
[c] $P < 0.01$ *vs.* 4 h; one-way ANOVA, Student-Newman-Keuls.
[d] $P < 0.01$ *vs.* respective placebo.
[e] $P < 0.001$ *vs.* 4 h; one-way ANOVA, Student-Newman-Keuls.

showed hyperinsulinemia; however, as expected, insulin levels of the phlorizin-treated diabetic rats remained very low.

Figure 2 shows the SGLT2 and HNF-1α mRNA analysis. Because no changes in SGLT2 mRNA expression were observed in 4-h insulin- or phlorizin-treated rats, the HNF-1α mRNA was investigated only in placebo and 12-h-treated diabetic rats. Twelve hours after the insulin injection, significant decrease ($P < 0.05$) in SGLT2 mRNA was observed, compared with both placebo- or 4-h insulin-treated rats (Fig. 2A); however, no difference in HNF-1α mRNA was observed at this time (Fig. 2C). Acute phlorizin treatment did not change SGLT2 (Fig. 2B) and HNF-1α (Figs. 2D) mRNA expression.

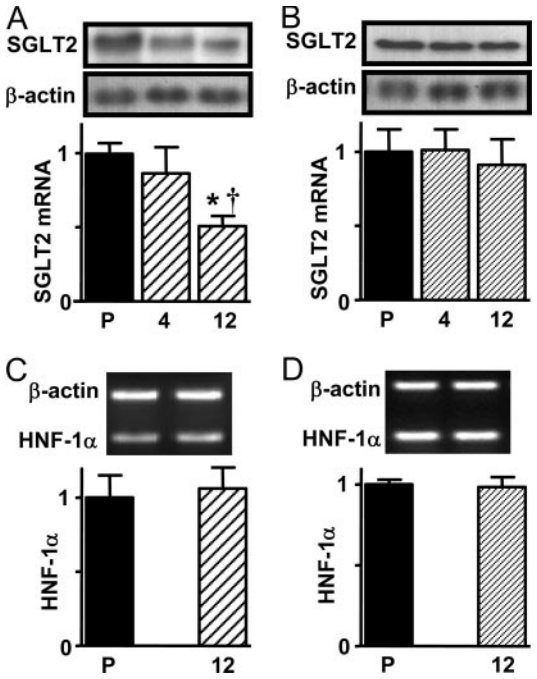

FIG. 2. SGLT2 (A and B) and HNF-1α (C and D) mRNA analysis in renal cortex of diabetic rats submitted to acute treatment with insulin (A and C) or phlorizin (B and D). Diabetic rats were studied 4 and 12 h after injection of insulin or phlorizin (*shaded bars*). Placebo treatment (P) was saline for insulin study and propylene glycol for phlorizin study. Four- and 12-h data of each placebo treatment were pooled (*closed bars*). A and B, SGLT2 mRNA analysis performed by Northern blotting of poly(A) RNA: at the *top*, typical images of the SGLT2 and respective β-actin mRNAs, at the *bottom*, the abundance of the SGLT2 mRNA normalized to β-actin value. C and D, HNF-1α mRNA analysis performed by RT-PCR: at the *top*, typical images of the HNF-1α and respective β-actin amplicons, at the *bottom*, the estimated abundance of the HNF-1α mRNA normalized to β-actin. Data are mean ± SEM of six or five animals in each group, respectively, for SGLT2 and HNF-1α analysis, evaluated in three different experiments. *, $P < 0.05$ *vs.* P (placebo); †, $P < 0.05$ *vs.* 4 h; ANOVA, Student-Newman-Keuls. No significant differences were observed in HNF-1α means tested by Student's *t* test.

Short-term effects of insulin and phlorizin treatment

Diabetic rats were submitted to insulin, phlorizin, or placebo treatment, as described in research design. Again, because the results of each placebo group (treated for 1, 4, or 6 d) showed no differences, they were pooled. On the day before the beginning of treatments, diabetic rats were randomly placed in the different groups, and 24-h glycosuria was evaluated, showing a similar diabetic state (data not shown).

Insulin treatment induced a rapid body weight gain, and 4- and 6-d insulin-treated rats were, respectively, 16 and 20% heavier than their placebo-treated rats ($P < 0.05$) (data not shown). Plasma insulin concentration of insulin-treated rats remained around 10 times higher ($P < 0.001$) than the value of the placebo-treated rats (Table 3). Phlorizin-treated rats did not change their body weight (data not shown) or their insulin concentration (Table 3). One day of insulin treatment induced a plasma glucose decrease of 24% ($P < 0.01$), and in the 6-d insulin-treated diabetic rats, the value was 60% lower ($P < 0.001$). Phlorizin treatment decreased ($P < 0.001$) the plasma glucose by 56% after 1 d (Table 3), and in the 4- and 6-d phlorizin-treated diabetic rats, the plasma glucose levels remained approximately 55% that of placebo-treated rats ($P < 0.001$). These similar glycemic profiles were accompanied by completely opposite urinary glucose excretion (Table 3).

The results of SGLT2 and HNF-1α mRNA expression are shown in Fig. 3. Because no changes in SGLT2 mRNA expression were observed in 1-d insulin- or phlorizin-treated rats, the HNF-1α mRNA was investigated only in placebo and 4- and 6-d-treated diabetic rats. The SGLT2 mRNA content (Fig. 3A) remained unchanged until 4 d of insulin treatment. However, in 6-d insulin-treated diabetic rats, the SGLT2 mRNA reduced to approximately 55% ($P < 0.001$ *vs.* placebo), a value that is similar to that of nondiabetic rats, which was accompanied by 25% reduction ($P < 0.05$ *vs.* placebo) in HNF-1α mRNA (Fig. 3C). Similarly, a 6-d phlorizin treatment reduced ($P < 0.01$) both the SGLT2 mRNA (36%, Fig. 3B) and HNF-1α mRNA (~25%, Fig. 3D). Besides, effects of phlorizin treatment could also be seen in 4-d-treated diabetic rats, when SGLT2 and HNF-1α mRNAs were already reduced by 34% ($P < 0.01$) and 11% ($P < 0.05$), respectively.

EMSA for HNF-1

EMSA analysis was performed to reinforce the correlation between HNF1-α and SGLT2 mRNA transcription in diabetes as well as in the effect of insulin treatment of diabetic rats.

TABLE 3. Short-term effects of insulin and phlorizin treatment of diabetic rats

	Insulin (ng/ml)	Glucose (mM)	Glycosuria (mg per 24 h)
Insulin treatment			
Placebo (1 + 4 + 6 d)	0.18 ± 0.05	19.9 ± 0.8	317.8 ± 37.3
1 d	9.7 ± 0.8[a]	15.1 ± 1.4[b]	156.6 ± 18.9[a]
4 d	9.3 ± 1.3[a]	9.9 ± 1.3[a,c]	21.7 ± 9.2[a,c]
6 d	10.1 ± 1.1[a]	8.3 ± 1.5[a,c]	10.7 ± 3.6[a,c]
Phlorizin treatment			
Placebo (1 + 4 + 6 d)	0.11 ± 0.05	21.1 ± 1.0	315.2 ± 20.1
1 d	0.20 ± 0.08	9.3 ± 1.1[a]	323.8 ± 20.8
4 d	0.17 ± 0.05	12.1 ± 0.7[a,c]	369.8 ± 7.4
6 d	0.15 ± 0.06	12.3 ± 0.6[a,c]	486.2 ± 17.8[d]

Diabetic rats were treated with insulin or phlorizin for 1, 4, and 6 d. Placebo was saline for insulin treatment or propyleneglycol for phlorizin treatment. Results from 1-, 4- and 6-d placebo-treated rats were pooled. Data are mean ± SEM of 10 animals in each placebo group, and six to eight animals in each insulin or phlorizin treated group.

[a] $P < 0.001$ *vs.* respective placebo.

[b] $P < 0.01$ *vs.* respective placebo.

[c] $P < 0.01$ *vs.* 1 d.

[d] $P < 0.001$ *vs.* all groups; one-way ANOVA, *vs.* 4 h; one-way ANOVA, Student-Newman-Keuls.

Specificity of the binding was confirmed by adding 50- or 100-fold molar excess of unlabeled oligonucleotide (Fig. 4A). Diabetes induced an approximately 2-fold increase (Fig. 4B) in the HNF1 binding activity ($P < 0.01$). Analyzing the 12-h insulin-treated diabetic rats and the HNF-1α mRNA, its binding activity was shown to be unchanged (Fig. 4C). Finally 6 d of insulin treatment (Fig. 4B) reduced the binding activity to a value similar to that of nondiabetic rats ($P < 0.01$ *vs.* diabetic and 1- and 4-d insulin-treated diabetic rats; and $P = 0.2347$ *vs.* nondiabetic rats).

HNF-1α ChIP assay

To confirm the relationship between the HNF1-α transcriptional factor and SGLT2 gene expression in renal cortex, ChIP assay was performed in samples from nondiabetic and 7-d saline-treated diabetic rats (Fig. 5). ChIP assay results showed an increase of 77% in HNF1-α binding into the promoter region of SGLT2 gene in kidneys from diabetic rats (Fig. 5, B and C). No differences were found when GAPDH promoter amplification was performed from RNA polymerase II-precipitated duplicates (Fig. 5A), indicating the presence of equal amounts of DNA among samples before the immunoprecipitation procedure. This result is in accordance with EMSA results demonstrated herein.

Discussion

In the present study, we provide evidences that HNF-1α may be involved in the SGLT2 mRNA expression in renal cortex of diabetic rats. The SGLT2 mRNA increases in diabetic rats and decreases after insulin or phlorizin treatments, correlating with the plasma glucose levels, despite the strong differences in plasma insulin and urinary glucose excretion between both treatments. Additionally, the nuclear proteins binding to a HNF-1α consensus motif increased in diabetic rats and reduced in 6-d insulin-treated diabetic rat, suggesting that this transcription factor modulates SGLT2 expression in kidney of diabetic rats. *In vivo* binding of HNF-1α into

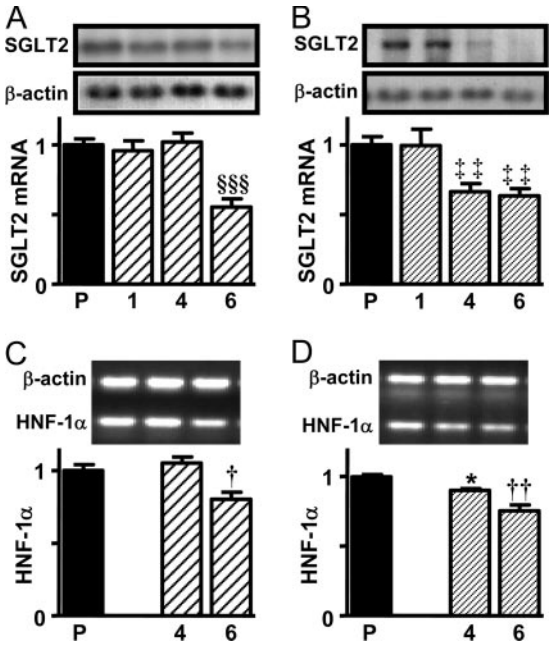

FIG. 3. SGLT2 (A and B) and HNF-1α (C and D) mRNA analysis in renal cortex of diabetic rats submitted to short-term treatment with insulin (A and C) or phlorizin (B and D). Diabetic rats were studied after 1, 4, and 6 d of treatment with insulin or phlorizin. Placebo treatment (P) was saline for insulin study and propylene glycol for phlorizin treatment. One-, 4- and 6-d data of each placebo treatment were pooled. A and B, SGLT2 mRNA analysis performed by Northern blotting of poly(A) RNA: at the *top*, typical images of the SGLT2 and respective β-actin mRNAs, at the *bottom*, the abundance of the SGLT2 mRNA normalized to β-actin. C and D, HNF-1α mRNA analysis performed by RT-PCR: at the *top*, typical images of the HNF-1α and respective β-actin amplicons, at the *bottom*, the estimated abundance of the HNF-1α mRNA normalized to β-actin. Data are mean ± SEM of six and five animals in each group, respectively, for SGLT2 and HNF-1α analysis, evaluated in three different experiments. *, $P < 0.05$ *vs.* P (placebo); †, $P < 0.05$; ††, $P < 0.01$ *vs.* P (placebo) and 4 d; ‡‡, $P < 0.01$ *vs.* P (placebo) and 1 d; §§§, $P < 0.001$ *vs.* all groups; ANOVA, Student-Newman-Keuls.

renal SGLT2 gene promoter region, observed in ChIP assay, reinforced this correlation.

Increased glucose reabsorption in the renal proximal tubule of diabetic rats is an adaptive condition in uncontrolled diabetes (3, 4, 21), which, in addition to hyperglycemia, contributes to a further elevation in the interstitial renal glucose concentration. High interstitial glucose concentration increases GLUT1 expression and glucose metabolism in mesangial cells, which is involved in nephropathy development (22–26). In this process, up-regulation of GLUT2 gene expression is considered fundamental and has also been related to the development of nephropathy (3, 4, 8, 9, 23, 24, 27).

On the other hand, the glucose cotransporter SGLT2, which coexpresses with GLUT2 in proximal tubular cells, has not been studied in kidney of diabetic subjects, which may be related to methodological difficulties (2). However, in the human nondiabetic renal glycosuria, different mutations in SGLT2 gene were already reported, highlighting the important role of this cotransporter in renal glucose reabsorption (10, 28). In diabetic rats, SGLT2 mRNA was first reported as unchanged (7) in a study with different methodological approach. Later we suggested increased SGLT2 mRNA in di-

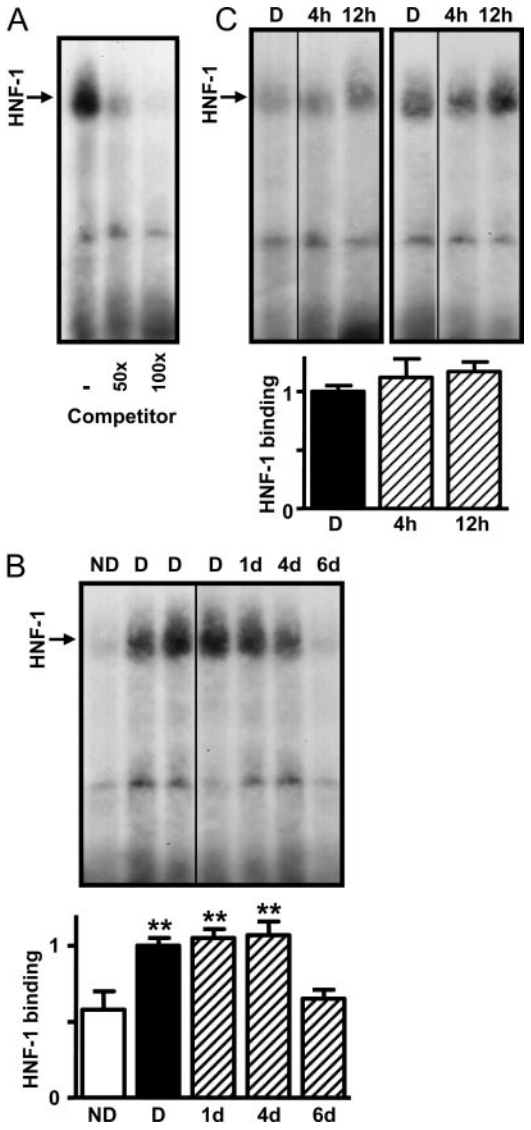

FIG. 4. EMSA of renal cortex nuclear proteins of nondiabetic (ND), placebo-treated diabetic (D), and acutely (4 and 12 h) or short-term (4, 1, and 6 d) insulin-treated diabetic rats. Acute effect of insulin was studied 4 (4 h) and 12 (12 h) h after a single injection, and short-term effect of insulin was studied in 1- (1d), 4- (4d), and 6-d (6d)-treated rats. EMSA was performed using a consensus motif for HNF-1. For details see *Materials and Methods*. A, Effect of 50- or 100-fold molar excess of unlabeled oligonigluclotide. B, Effect of diabetes state and 1, 4, and 6 d of insulin treatment: at the *top*, typical image, at the *bottom*, quantification of the data. C, Effect of 4 and 12 h of insulin treatment: at the *top*, typical images from two experiments, at the *bottom*, quantification of the data. Each *square* of autoradiogram shows images from the same gel, and *vertical line inside the square* indicates that the lanes were not contiguous. Values in graphs were expressed as fold change relative to untreated diabetic rats. Data are mean ± SEM of six animals (D group) or four animals (other groups), evaluated at least in three different experiments. **, $P < 0.01$ *vs.* ND and 6d, ANOVA, Student-Newman-Keuls.

abetic rats by using SGLT1 probe and low stringency conditions (8). Now we are definitively showing that diabetes induces an increase in the SGLT2 mRNA expression using a full-length SGLT2 probe and poly(A) mRNA from renal cortex. In addition, a recent and elegant study reported that the

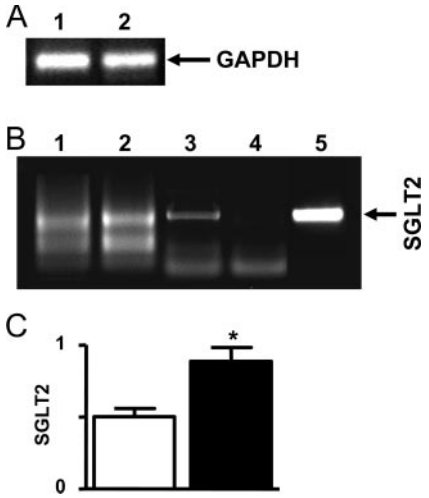

FIG. 5. *In vivo* binding of HNF-1α to SGLT2 gene in kidney. Chromatin was immunoprecipitated from renal cortex of nondiabetic (lane 1) and diabetic (lane 2) rats using anti-RNA polymerase II (A) or anti-HNF-1α (B) antibodies. Anti-RNA polymerase II-precipitated chromatin was used to analyze GAPDH promoter as a control (A). Anti-HNF-1α-precipitated DNA was amplified by PCR with primers flanking nucleotides 10–172 of the rat SGLT2 codifying region. The SGLT2 fragment was also amplified (B) from the total sheared DNA from renal cortex of nondiabetic rats (lane 3), the no-antibody sample (lane 4), and a rat kidney cDNA (lane 5). After the amplification, the reactions were visualized in a 2% agarose gel containing ethidium bromide exposed to UV light. The graph (C) represents HNF-1α-bound SGLT2 from kidney of nondiabetic and diabetic rats (mean ± SEM of three different experiments). *, $P < 0.05$ *vs.* nondiabetic; Student's *t* test.

SGLT2 mRNA was increased in human renal proximal tubular cells isolated from the urine of patients with type 2 diabetes (29).

The HNF-1α transcription factor was described as an activator of the SGLT2 gene (15), but its expression or activation in kidney of diabetic animals or human type 1 or type 2 diabetes has never been studied. However, mutations in HNF-1α gene are a feature of MODY3 patients (16–18, 30), in whom precocious nephropathy (31, 32) and renal malformations (33) were described.

In the present study, parallel increase of SGLT2 and HNF-1α mRNA expression in diabetic rats was observed. Furthermore, 6-d treatment of diabetic rats with both insulin and phlorizin induced a suppression of both mRNAs, which returned to similar values of nondiabetic rats, suggesting that HNF-1α transcription factor has an important role in the diabetes-induced modulations of its target gene SGLT2. Additionally, EMSA analysis showed that HNF-1 binding activity increased in diabetic rats and decreased in 6-d insulin-treated diabetic rats, suggesting an important role for this transcriptional factor on SGLT2 gene expression in diabetes. ChIP assay data reinforced these correlations.

Considering that insulin or phlorizin treatment induced a similar reduction in plasma glucose 6 d later but accompanied by completely different plasma insulin and urinary glucose levels, the expression of the genes must be correlated with the plasma glucose levels only. Thus, it seems that plasma glucose concentration is an important modulator of the HNF-1α transcription factor expression and activity and consequently of the SGLT2 expression.

The present results also show two interesting responses: 1) in insulin-treated rats, an acute (12 h) reduction of SGLT2 mRNA, with no changes of HNF-1α mRNA or its nuclear protein binding activity; and 2) in phlorizin-treated rats, an earlier reduction of SGLT2 and HNF-1α mRNAs (4 d), as compared with insulin treatment.

Concerning the 12-h effect of insulin, although we did not observe any change in HNF-1 binding activity in EMSA, we cannot discard a HNF-1-induced reduction of SGLT2 mRNA. Interestingly, the intestinal SGLT1 gene transcription, which is also enhanced by HNF-1α, was shown to be suppressed by heterodimerization of HNF-1α and HNF-1β, with a higher band shift in EMSA (34). In the present study, in some experiments, a band shift was noted in samples from 12-h insulin-treated diabetic rats, and thus, we could hypothesize a transitory heterodimerization of HNF-1, thus reducing SGLT2 mRNA. Using the same protocol of treatment, we already reported a rapid (from 4 to 12 h), transitory, and converse modulation of GLUT2 gene expression in kidney of insulin-treated diabetic rats (9). These rapid effects of insulin on SGLT2 and GLUT2 expression needs further investigations to clarify whether they have some functional role as well as the involved mechanisms.

Concerning the reduction of SGLT2 and HNF-1α mRNAs observed in 4-d phlorizin-treated diabetic rats, it seems not to be related to the plasma glucose lowering, as it is later (6 d) because the plasma glucose levels were similar in insulin- and phlorizin-treated diabetic rats. However, this may be related to some intracellular glucose effect. It is known that diabetes induces increased renal glucose production (35), which is achieved by high gluconeogenic activity in renal proximal tubule (36), and is suppressed by insulin as in liver (35, 37). In the present study, the tubular gluconeogenic activity of the diabetic rats should be reduced with the insulin treatment, but it should remain increased with the phlorizin treatment, which could induce different intracellular concentration of frutose-2,6-biphosphate. In this regard, HNF-1α expression has already been reported as modulated by the intracellular frutose-2,6-biphosphate content, independently of insulin concentration (38). Thus, in 4-d phlorizin-treated diabetic rats, it is possible that an intracellular signal on HNF-1α expression and activation is preceding the effect of plasma glucose lowering observed after 6 d of treatment.

Finally, we cannot exclude the participation of other transcriptional factors in the observed modulations of SGLT2 mRNA. In fact, the EMSA analysis also revealed the HNF-3 [α, β, and γ, also called forkhead box A (Foxa) 1, 2, and 3, respectively] nuclear protein binding (lower band in EMSA images), which was expected because Foxa consensus-responsive element has been shown inside the sequence of the HNF-1 binding site (20). However, although the Foxa transcription factors have been described as important modulators of GLUT2 (20, 39), they have never been related to SGLT2 expression.

In summary, this study provides evidence that experimental diabetes induces increased mRNA expression of SGLT2 and HNF-1α in renal cortex. Moreover, 6-d treatment with insulin or phlorizin improves the glycemic control, and that is accompanied by reduction of SGLT2 and HNF-1α mRNAs to levels similar to those observed in nondiabetic rats. Because a similar regulation was observed in insulin- or phlorizin-treated diabetic rats, despite their controversial effects on plasma insulin and renal tubular glucose rate, reduction in plasma glucose levels seems to be involved in the observed modulations. Additionally, HNF-1α mRNA modulations observed in diabetic and insulin-treated diabetic rats were accompanied by parallel changes in HNF-1 binding activity to nuclear proteins, and *in vivo* HNF-1α binding into SGLT2 promoter was shown, pointing out the HNF-1α regulation of SGLT2 gene. These results unravel mechanisms involved in SGLT2 expression and suggest that its enhanced expression may contribute to the development of the kidney tubule-glomerular disease in diabetes.

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References

1. **Thorens B** 1996 Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes. Am J Physiol 270:G541–G553
2. **Wright EM** 2001 Renal Na(+)-glucose cotransporters. Am J Physiol 280:F10–F18
3. **Dominguez JH, Camp K, Maianu L, Feister H, Garvey WT** 1994 Molecular adaptations of GLUT1 and GLUT2 in renal proximal tubules of diabetic rats. Am J Physiol 266:F283–F290
4. **Dominguez JH, Song B, Maianu L, Garvey WT, Qulali M** 1994 Gene expression of epithelial glucose transporters: the role of diabetes mellitus. J Am Soc Nephrol 5(Suppl 1):S29–S36
5. **Kamran M, Peterson RG, Dominguez JH** 1997 Overexpression of GLUT2 gene in renal proximal tubules of diabetic Zucker rats. J Am Soc Nephrol 8:943–948
6. **Chin E, Zamah AM, Landau D, Gronbcek H, Flyvbjerg A, LeRoith D, Bondy CA** 1997 Changes in facilitative glucose transporter messenger ribonucleic acid levels in the diabetic rat kidney. Endocrinology 138:1267–1275
7. **Adachi T, Yasuda K, Okamoto Y, Shihara N, Oku A, Ueta K, Kitamura K, Saito A, Iwakura I, Yamada Y, Yano H, Seino Y, Tsuda K** 2000 T-1095, a renal Na+-glucose transporter inhibitor, improves hyperglycemia in streptozotocin-induced diabetic rats. Metabolism 49:990–995
8. **Vestri S, Okamoto MM, de Freitas HS, Aparecida Dos Santos R, Nunes MT, Morimatsu M, Heimann JC, Machado UF** 2001 Changes in sodium or glucose filtration rate modulate expression of glucose transporters in renal proximal tubular cells of rat. J Membr Biol 182:105–112
9. **Freitas HS, Schaan BD, Seraphim PM, Nunes MT, Machado UF** 2005 Acute and short-term insulin-induced molecular adaptations of GLUT2 gene expression in the renal cortex of diabetic rats. Mol Cell Endocrinol 237:49–57
10. **Santer R, Kinner M, Lassen CL, Schneppenheim R, Eggert P, Bald M, Brodehl J, Daschner M, Ehrich JH, Kemper M, Li Volti S, Neuhaus T, Skovby F, Swift PG, Schaub J, Klaerke D** 2003 Molecular analysis of the SGLT2 gene in patients with renal glucosuria. J Am Soc Nephrol 14:2873–2882
11. **Courtois G, Morgan JG, Campbell LA, Fourel G, Crabtree GR** 1987 Interaction of a liver-specific nuclear factor with the fibrinogen and α1-antitrypsin promoters. Science 238:688–692
12. **Emens LA, Landers DW, Moss LG** 1992 Hepatocyte nuclear factor 1α is expressed in a hamster insulinoma line and transactivates the rat insulin I gene. Proc Natl Acad Sci USA 89:7300–7304
13. **Pontoglio M, Barra J, Hadchouel M, Doyen A, Kress C, Bach JP, Babinet C, Yaniv M** 1996 Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. Cell 84:575–585
14. **Friedlander G, Runembert I, Vrtovsnik F, Terzi F** 1999 Renal tubular cells cultured from genetically modified animals. Exp Nephrol 7:407–412
15. **Pontoglio M, Prie D, Cheret C, Doyen A, Leroy C, Froguel P, Velho G, Yaniv M, Friedlander G** 2000 HNF1α controls renal glucose reabsorption in mouse and man. EMBO Rep 1:359–365

16. **Yamagata K, Oda N, Kaisaki PJ, Menzel S, Furuta H, Vaxillaire M, Southam L, Cox RD, Lathtrop GM, Boriraj VV, Chen X, Cox NJ, Oda Y, Yano H, Le Beau MM, Yamada S, Nishigori H, Takeda J, Fajans SS, Hattersley AT, Iwasaki N, Hansen T, Pedersen O, Polonsky KS, Bell GI** 1996 Mutations in the hepatocyte nuclear factor-1α gene in maturity-onset diabetes of the young (MODY3). Nature 384:455–458
17. **Elbein SC, Teng K, Yount P, Scroggin E** 1998 Linkage and molecular scanning analyses of MODY3/hepatocyte nuclear factor-1α gene in typical familial type 2 diabetes: evidence for novel mutations in exons 8 and 10. J Clin Endocrinol Metab 83:2059–2065
18. **Hattersley AT** 1998 Maturity-onset diabetes of the young: clinical heterogeneity explained by genetic heterogeneity. Diabet Med 15:15–24
19. **Andrews NC, Faller DV** 1991 A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499
20. **Cha JY, Kim H, Kim KS, Hur MW, Ahn Y** 2000 Identification of transacting factors responsible for the tissue-specific expression of human glucose transporter type 2 isoform gene. Cooperative role of hepatocyte nuclear factors 1α and 3β. J Biol Chem 275:18358–18365
21. **Noonan WT, Shapiro VM, Banks RO** 2001 Renal glucose reabsorption during hypertonic glucose infusion in female streptozotocin-induced diabetic rats. Life Sci 68:2967–2977
22. **Heilig CW, Concepcion LA, Riser BL, Freytag SO, Zhu M, Cortes P** 1995 Overexpression of glucose transporters in rat mesangial cells cultured in a normal glucose milieu mimics the diabetic phenotype. J Clin Invest 96:1802–1814
23. **D'Agord Schaan B, Lacchini S, Bertoluci MC, Irigoyen MC, Machado UF, Schmid H** 2001 Increased renal GLUT1 abundance and urinary TGF-β 1 in streptozotocin-induced diabetic rats: implications for the development of nephropathy complicating diabetes. Horm Metab Res 33:664–669
24. **D'Agord Schaan B, Lacchini S, Bertoluci MC, Irigoyen MC, Machado UF, Schmid H** 2003 Impact of renal denervation on renal content of GLUT1, albuminuria and urinary TGF-β1 in streptozotocin-induced diabetic rats. Auton Neurosci 104:88–94
25. **Nose A, Mori Y, Uchiyama-Tanaka Y, Kishimoto N, Maruyama K, Matsubara H, Iwasaka T** 2003 Regulation of glucose transporter (GLUT1) gene expression by angiotensin II in mesangial cells: involvement of HB-EGF and EGF receptor transactivation. Hypertens Res 26:67–73
26. **Gnudi L, Viberti G, Raij L Rodriguez V, Burt D, Cortes P, Hartley B, Thomas S, Maestrini S, Gruden G** 2003 GLUT-1 overexpression: link between hemodynamic and metabolic factors in glomerular injury? Hypertension 42:19–24
27. **Schaan BD, Irigoyen MC, Bertoluci MC, Lima NG, Passaglia J, Hermes E, Oliveira FR, Okamoto M, Machado UF** 2005 Increased urinary TGF-β1 and cortical renal GLUT1 and GLUT2 levels: additive effects of hypertension and diabetes. Nephron Physiol 100:43–50
28. **Calado J, Loeffler J, Sakallioglu O, Gok F, Lhotta K, Barata J, Rueff J** 2006 Familial renal glucosuria: SLC5A2 mutation analysis and evidence of salt-wasting. Kidney Int 69:852–855
29. **Rahmoune H, Thompson PW, Ward JM, Smith CD, Hong G, Brown J** 2005 Glucose transporters in human renal proximal tubular cells isolated from theurine of patients with non-insulin-dependent diabetes. Diabetes 54:3427–3434
30. **Fehmann HC, Gross U, Epe M** 2004 A new mutation in the hepatocyte nuclear factor-1α gene (P224S) in a newly discovered German family with maturity-onset diabetes of the young 3 (MODY 3). Family members carry additionally the homozygous I27L amino acid polymorphism in the HNF1α gene. Exp Clin Endocrinol Diabetes 112:84–87
31. **Bowden DW, Sale M, Howard TD, Qadri A, Spray BJ, Rothschild CB, Akots G, Rich SS, Freedman BI** 1997 Linkage of genetic markers on human chromosomes 20 and 12 to NIDDM in Caucasian sib pairs with a history of diabetic nephropathy. Diabetes 46:882–886
32. **Iwasaki N, Ogata M, Tomonaga O, Kuroki H, Kasahara T, Yano N, Iwamoto Y** 1998 Liver and kidney function in Japanese patients with maturity-onset diabetes of the young. Diabetes Care 21:2144–2148
33. **Malecki MT, Skupien J, Gorczynska-Kosiorz S, Klupa T, Nazim J, Moczulski DK, Sieradzki J** 2005 Renal malformations may be linked to mutations in the hepatocyte nuclear factor-1α (MODY3) gene. Diabetes Care 28:2774–2776
34. **Rhoads DB, Rosenbaum DH, Unsal H, Isselbacher KJ, Levitsky LL** 1998 Circadian periodicity of intestinal Na+/glucose cotransporter 1 mRNA levels is transcriptionally regulated. J Biol Chem 273:9510–9516
35. **Stumvoll M, Meyer C, Mitrakou A, Gerich JE** 1999 Important role of the kidney in human carbohydrate metabolism. Med Hypotheses 52:363–366
36. **Conjard A, Martin M, Guitton J, Baverel G, Ferrier B** 2001 Gluconeogenesis from glutamine and lactate in the isolated human renal proximal tubule: longitudinal heterogeneity and lack of response to adrenaline. Biochem J 360:371–377
37. **Gerich JE, Meyer C, Woerle HJ, Stumvoll M** 2001 Renal gluconeogenesis: its importance in human glucose homeostasis. Diabetes Care 24:382–391
38. **Wu C, Okar DA, Stoeckman AK, Peng LJ, Herrera AH, Herrera JE, Towle HC, Lange AJ** 2004 A potential role for fructose-2,6-bisphosphate in the stimulation of hepatic glucokinase gene expression. Endocrinology 145:650–658
39. **Shen W, Scearce LM, Brestelli JE, Sund NJ, Kaestner KH** 2001 Foxa3 (hepatocyte nuclear factor 3γ) is required for the regulation of hepatic GLUT2 expression and the maintenance of glucose homeostasis during a prolonged fast. J Biol Chem 276:42812–42817

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