Localization of the GLUT8 glucose transporter in murine kidney and regulation in vivo in non-diabetic and diabetic conditions

Mario Schiffer,1,2 Katalin Susztak,1,2 Mollie Ranalletta,3 Amanda C. Raff,1 Erwin P. Böttinger,1,2 and Maureen J. Charron3

1Division of Nephrology, Department of Medicine, and 3Department of Biochemistry, Albert Einstein College of Medicine, Bronx; and 2Department of Medicine, Mount Sinai Medical Center, New York, New York

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The kidneys play a significant role in plasma glucose homeostasis. In 24 h the kidneys filter ~180 g of glucose through the proximal tubules which accounts for ~90% of glucose reabsorption. Due to the hydrophilicity of glucose, cellular glucose uptake is accomplished via transporters. Glucose influx occurs through apical Na+/glucose cotransporters that reabsorb glucose and concentrate glucose in tubules (26). Glucose efflux follows through basolateral facilitative glucose transporters (8). The family of hexose transporters (GLUT) proteins consists of several members with different characteristic elements that define their physiological function. GLUT1, GLUT2, GLUT3, GLUT4, and GLUT5 were described as hexose transporters in the kidney (3, 8, 11, 35). GLUT8, a recently identified member of the extended GLUT family has been localized to several tissues including kidney, testis, basiocysts, brain, muscle, and adipocytes and may contribute to maintenance of normal serum glucose (6, 14, 27).

The aims of this study were to localize GLUT8 within normal and diabetic kidney and to study the regulation of its expression in a normal physiological context and in pathological states of insulin resistance. To understand the normal regulation in response to metabolic signals, fasting and feeding conditions were studied. To study pathological states two models of insulin resistance, the GLUT4−/− mouse (16) and the leptin receptor-deficient db/db mouse (30, 32), were examined. GLUT8 mRNA and protein expression levels were regulated by normal metabolic functions during fasting and feeding as well as upregulated under diabetic conditions in vivo.

Glucose transporters have been described as critical mediators for intracellular glucose utilization and ATP generation and they influence the functions of the cellular survival factor Akt (10, 12, 25). Akt, a serine/threonine kinase, participates in various signaling pathways that prevent apoptosis. We and others have shown that Akt is a critical mediator of podocyte survival (13, 29). Because GLUT8 has already been shown to be required for cell survival in other cells (23) and the loss of podocytes is a key step during the development of glomerulosclerosis and observed in diabetic patients (18, 22), the present findings might be of great relevance for the pathogenesis of diabetic nephropathy. However, the functional role of increased GLUT8 expression in cellular survival of podocytes needs to be studied in more detail.

Materials and Methods

Animals

GLUT4+/+ and GLUT4−/− mice. Female GLUT4−/− or −/+ mice on a C57BL6/CBA background (11–13 wk old) were killed in the fed state or after an overnight fast (12 h) and rapidly dissected. Tissue was frozen in liquid nitrogen and stored at −70°C until processing for mRNA or protein extraction.

Db/db and db/db mice. Male mice of C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). All mice

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were housed at the Albert Einstein College of Medicine Animal Facility with free access to food and water. Animals were killed at 20 wk of age with average blood glucose levels of ~600 mg/dl and significant proteinuria. All protocols were approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine in accordance with the Public Health Service Animal Welfare Policy.

Immunofluorescence Labeling In Situ and In Vitro

For in situ detection, antibodies specific for the following proteins were used: monoclonal anti-synaptopodin antibody (20) and affinity-purified rabbit polyclonal anti-11 COOH-terminal amino acids of GLUT8 protein amino acid sequence (LEQITAHFEGR). Antibody generation and affinity purification were described earlier (27).

Mice were perfusion fixed via the left ventricle of the heart using paraformaldehyde (PFA) as described earlier (19). Tissues were then immersion fixed overnight in 10% formaldehyde and paraffin-embedded. Tissue was cut at 4 μm, and, after standard deparaffinization, microwaved in citrate buffer (10 mM Na-citrate, pH 6.0). After being blocked with PBS/10% normal goat serum (NGS), tissue was incubated for 1 h with affinity-purified anti-GLUT8 (2 μg/ml). Detection was carried out using a Cy3-labeled anti-rabbit (1:150 in PBS/5% NGS) and a FITC-labeled anti-mouse antibody (1:150 in PBS/5% NGS; both from Jackson ImmunoResearch, West Grove, PA). Nuclear counterstain was carried out using 4', 6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO). Images were captured using a Bio-Rad MR600 confocal fluorescence microscope with a Kanton Electronic Prog/Res/3012 digital video camera and digitally processed using Adobe Photoshop 5.0.2 (Adobe Systems, San Jose, CA).

Quantitative Real-Time PCR

Total RNA was prepared using TRIzol reagent, reverse-transcribed with SuperScript II reverse transcriptase (Life Technologies, Invitrogen), and cDNA was amplified using SYBR-Green PCR Master Mix (Applied Biosystems) and specific primers for murine GLUT8 (5’-primer: ACCAAAGATGTCACGCCGT; 3’-primer: TAGGA-CACGAGACACCAGA) in an iCycler (Bio-Rad). Normalization across samples was performed using the average of the constitutive gene murine-β-actin (5’-primer: ACCGAGAAGATGACCAGA; 3’-primer: AGCCCTGATGGCTACGTACA).

Immunoblot Analysis

Kidneys were homogenized in buffer containing 250 mM sucrose, 1 mM EDTA, 20 mM HEPES, 4 mM phenylmethylsulfonyl fluoride, 20 mM leupeptin, and 20 mM aprotinin. Homogenates were centrifuged for 10 min at 12,000 rpm. The supernatant was transferred to new tubes and centrifuged at 90,000 rpm for 75 min at 4°C. Pellets were resuspended in PBS buffer containing 2% ths, 4 mM phenylmethylsulfonyl fluoride, 20 mM leupeptin, and 20 mM aprotinin. One hundred micrograms of samples were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech). Rabbit polyclonal GLUT8 antibodies (1:500) were used to detect GLUT8 in kidney preparations. Enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) detection was used with laser-scanning densitometry for quantitation.

Cell Culture and Glucose Treatments

Conditionally immortalized podocytes were cultured as previously reported (20). In brief, podocytes were grown on collagen type I (Becton Dickinson, Franklin Lakes, NJ) at 33°C with IFN-γ (10 U/ml, GIBCO BRL Life Technologies, Grand Island, NY) to drive expression of thermosensitive SV40 large T antigen (permissive conditions). To induce differentiation, podocytes were maintained at 37°C without IFN-γ for 14 days. For d-glucose treatments, cells were grown under permissive conditions in RPMI with 10 mM glucose. At 95% confluence, they were serum starved for 48 h in RPMI with 5 mM glucose, 0.2% FBS. After 48 h, the media was changed, and the glucose-treated time points had a final concentration of 30 mM d-glucose. The time course was also done at 37°C. These cells were plated in 10 mM glucose RPMI and grown for 10 days at 37°C. They were then serum starved for 48 h in RPMI with 5 mM glucose, 0.2% FBS. After 48 h, the media was changed to the glucose-treated time points, which had a final concentration of 30 mM d-glucose.

RESULTS

Expression of GLUT8 in Normal Mouse Kidneys

Kidneys from 8-wk-old wild-type C57BL/6/CBA mice were harvested after perfusion fixation with PFA and sucrose for 15 min each via the left ventricle of the heart followed by regular immersion fixation and tissue processing. Coimmunostainings using affinity-purified rabbit anti-GLUT8 antibody and mouse monoclonal anti-synaptopodin antibody identified glomerular podocytes as the main glomerular cells expressing GLUT8 (Fig. 1, A–C). In general, glomerular staining intensity was much lower than the intensity in the tubular compartments. Longer exposure times led to stronger autofluorescence signals in the proximal tubules that were not quenchable (Fig. 1, D–F). In addition, we screened glomerular cells by immunocytochemistry to define cellular distribution of GLUT8. Staining of differentiated, cultured, conditionally immortalized podocytes showed a perinuclear localization of GLUT8 (Fig. 1G), which partially overlapped with the synaptopodin staining (Fig. 1, H and I). In contrast, cultured mesangial cells stained negative for GLUT8 (data not shown). Intense GLUT8 staining was also detected in tubular cells. The staining was mainly basolateral (Fig. 2, A and B) but sometimes appeared in perinuclear regions. Colocalization experiments using GLUT8 with markers for different regions of the nephron, [aquaporin-1, -2, and -3 (AQP1/2/3) and Na-K-2Cl (NKCC2)] on serial sections revealed expression of GLUT8 in the ascending loop of Henle (costaining with NKCC, Fig. 2, Ca and Ch, open arrows) and in the collecting ducts (costaining with AQP2 (Fig. 2Cc) and AQP3 (Fig. 2Cd, filled arrows)). No overlapping staining was detected using a marker for proximal tubuli (AQP1) (data not shown). There were no significant differences in the distribution pattern between distal tubular segments of the nephron in the cortical or medullary portions. The medullary collecting ducts also stained positive for GLUT8 in a basolateral distribution pattern (data not shown). However, cultured inner medullary collecting duct (IMCD) cells showed a similar perinuclear expression pattern of GLUT8 immunostaining with no plasma membrane immunostaining noted (data not shown).

GLUT8 Expression is Regulated by Fasting and Feeding in Wild-Type (GLUT4+/+) and GLUT4 Null (GLUT4−/−) Mice

It was of interest to determine whether the absence of the insulin-responsive glucose transporter GLUT4 or differences in the serum glucose concentration in vivo would influence the expression levels of GLUT8. GLUT4 has been described to be expressed in mesangial and glomerular epithelial cells of the kidney as well as in renal microvessels (17). Quantitative real-time-PCR (QRT-PCR) analysis for GLUT8 mRNA extracted from total kidney from fed and fasted GLUT4+/+ and GLUT4−/− mice was performed. Relative GLUT8 mRNA expression levels tended to be lower but did not reach statis-
GLUT8 Regulation in Kidneys of db/db Mice: A Mouse Model for Type 2 Diabetes

The expression levels for GLUT8 mRNA and protein in kidney extracts from 20-wk-old db/db mice (glucose >600 mg/dl) compared with 20-wk-old nondiabetic, heterozygous (db/m) mice were examined. Interestingly, the mRNA expression levels were more than twofold higher (P < 0.01) in hyperglycemic db/db compared with db/m mice (Fig. 4A). Expression levels of the 75-kDa protein band were significantly increased 1.5-fold (P < 0.01) in db/db compared with db/m mice (Fig. 4B). Next, kidney sections from db/db and db/m mice were examined to identify changes in the expression pattern of GLUT8 in specific cell types. A strikingly higher expression of GLUT8 was found in the glomeruli in podocytes of diabetic db/db mice, as confirmed by double staining for the podocyte-specific marker synaptopodin (Fig. 4C) compared with wild-type db/m mice (Fig. 4C). The tubular expression of GLUT8 appeared to be slightly increased, but these differences could not be captured using fluorescence microscopy (data not shown).

Fig. 1. GLUT8 expression in glomerular cells. A–C: indirect immunofluorescence labeling of perfusion-fixed mouse kidney incubated with affinity-purified rabbit polyclonal GLUT8 antibody reveals expression in glomerular podocytes. GLUT8 is visualized using Cy3-labeled anti-rabbit antibody (red) and colocalizes (C) with the podocyte-specific marker synaptopodin visualized using a FITC-labeled anti-mouse antibody (green; B). Arrows depict double-positive podocytes. Magnification: ×60; ×80 (inset). D–F: peptide competition experiment demonstrates autofluorescence in proximal tubules in the red channel (D), green channel (E), as well as in the overlapping picture (F) but no staining within the glomerular area (within white line). Magnification: ×40. G–I: indirect immunofluorescence labeling of GLUT8 in differentiated podocytes. GLUT8 (G) is mainly located in the cell body with perinuclear enrichment and only partially overlaps (I) podocyte differentiation marker synaptopodin (H). Magnification: ×80.
Next, studies were performed to examine whether treatment of cultured murine podocytes with high glucose would also induce GLUT8 mRNA expression. To accomplish this, differentiated podocytes were treated with 30 mM d-glucose. After 4 h of glucose exposure, a transient induction peak in GLUT8 mRNA expression levels was observed in differentiated podocytes. Replicate experiments yielded consistent induction peaks but never resulted in a greater than 0.5-fold change in GLUT8 expression (data not shown).

Similar studies were conducted to examine whether treatment with high glucose could alter the cellular expression pattern of GLUT8 in podocytes. As noted in other cell types, GLUT8 was consistently expressed throughout the cell body with a perinuclear enrichment. Treatment with glucose or insulin never led to a notable increase in protein expression or cellular redistribution of GLUT8 at the fluorescence microscopic level (data not shown). These data suggest that the expression of GLUT8 mRNA might be regulated by the glucose concentration in vivo. Because the in vitro experiments only led to a transient mRNA induction peak and no cellular redistribution, the functional significance of this induction in the kidney requires further study.

**DISCUSSION**

Expression of several glucose transporters (GLUT1, GLUT2, GLUT3, GLUT4, and GLUT5) have been previously reported in the kidney (5, 7, 11, 17). In the present study, we demonstrate that the new glucose transporter, GLUT8, is expressed in glomerular podocytes and in the distal part of the nephron (ascending limbs of Henle and collecting ducts). Additionally, we demonstrate that GLUT8 expression is regulated by physiological and pathophysiological alterations of blood glucose levels in vivo. Thus far, glucose reabsorption in the kidney was mainly attributed to GLUT1 and GLUT2. The majority of filtered glucose is reabsorbed in the convoluted proximal part of the nephron via the apical sodium glucose transporter-2 (SGLT-2) and basolateral GLUT2 (33). The remaining glucose is reabsorbed in the straight part of the proximal tubule via SGLT-1 apical and basolateral GLUT1 (33). Increased mRNA and protein levels of GLUT1 and GLUT2 were previously reported in streptozotocin-induced diabetes in rats (7). The segmental expression of GLUT8 in the ascending limbs of Henle and the collecting duct indicates that GLUT8 might not be involved in the reabsorption of filtered glucose but more so in glucose utilization for oxidative metabolism, as previously postulated for GLUT4 (4, 17).

In this report, we show that GLUT8 mRNA and protein are regulated in total kidney lysates of fasted vs. fed mice, in GLUT4+/+ and GLUT4−/− mice, and in a model of type 2 diabetes. With the use of QRT-PCR, it was shown that overnight fasting decreases the level of GLUT8 mRNA >50% in the kidneys of GLUT4+/+ mice. Detection of GLUT8 protein led to the identification of two molecular species, as previously described (9). We detected in the protein lysates a 32-kDa

Fig. 2. GLUT8 expression in tubular cells. GLUT8 expression is mainly basolateral in vivo in renal tubuloepithelial cells (arrows, A and B). Magnification: ×40. Using serial sections, tubular GLUT8 expression could be localized to the thick ascending loop of Henle [open arrows depict overlap of expression with Na-K-2Cl cotransporter (NKCC; Ca and Cb)] and in the distal tubules/collecting ducts [filled arrows depict overlap of expression with aquaporin-2 (AQP2) and aquaporin-3 (AQP3) in Ca and Cc/Cd, respectively]. Magnification: ×30.
species as described earlier (6, 14) and a higher-molecular-
mass band around 72 kDa. Fasting also significantly decreased
the expression of the 72-kDa protein band.

GLUT4−−/− mice, a model of peripheral insulin resistance,
are able to maintain normal glycemia despite totally lacking
insulin-responsive GLUT4 (16). Altered GLUT4 activity is
suggested to be one of the factors responsible for decreased

glucose uptake in muscle and adipose tissue in obesity and
diabetes. We observed no difference in GLUT8 mRNA
and protein levels in GLUT4+/+ vs. GLUT4−−/− mice in the fed
state; however, a significant decrease in GLUT8 mRNA and
protein expression levels was observed compared with the
fasted state. This effect was not genotype specific and was
reproducible on mRNA level in ad libitum fed vs. fasted
GLUT4+/+ and in ad libitum fed vs. fasted GLUT4−−/−
mice. We also detected this difference on the protein level in
the GLUT4−−/− mice. These data suggest that the expression
of GLUT8 is influenced by serum glucose levels in vivo
independently of the presence or absence of the glucose trans-
porter GLUT4.

In uncontrolled diabetes, glucose flux through the proximal
tubule is increased and this has been shown to affect expression
of glucose transporters in the proximal tubules (GLUT1 and
GLUT2) (7). Db/db mice have a recessive mutation in the
hypothalamic leptin receptor and develop type 2 diabetes at ~8
wk of age. Kidneys are generally enlarged in this mouse strain,
and structural glomerular changes (e.g., diffuse glomeruloscle-
rosis, glomerular basement membrane thickening) occur with-
out evidence of tubulointerstitial disease (30). Here, a signifi-
cant upregulation of GLUT8 mRNA and protein expression
in diabetic kidneys of 20-wk-old db/db mice was observed. In
contrast to overnight fasting conditions, under the high-glucose
conditions of the diabetic milieu in db/db mice, expression of
GLUT8 mRNA and 75-kDa protein was significantly (2-fold)
upregulated in db/db kidneys but a significant difference (*P < 0.01)
in protein expression levels of 75-kDa band between fasted GLUT4−−/−
kidneys and GLUT4+/+ kidneys but a significant difference
(*P < 0.01) in protein expression levels of 75-kDa band (gray bars) between fed and fasted GLUT4−−/− kidneys (GLUT4+/+, n = 5, GLUT4−−/−, n = 5).
Fig. 4. GLUT8 mRNA and protein expression in kidneys from db/m and db/db mice. A: quantitative real-time PCR shows normalized relative expression of GLUT8 in total kidney mRNA harvested from 20-wk-old db/m (filled bars, \( n = 5 \)) and 20-wk-old db/db mice (open bars, \( n = 5 \)). *\( P < 0.01 \). B: Western blots using 50 \( \mu \)g of total kidney lysate harvested from 20-wk-old db/m and db/db mice were incubated with the GLUT8 antiserum that detects 2 specific bands for GLUT8 at 75 and 32 kDa. Densitometry indicates significant difference (*\( P < 0.01 \)) in protein expression levels of 75-kDa (filled bars) between db/m and db/db kidneys. C: indirect immunofluorescence from renal cortex sections of 20-wk-old db/m (a–c) and db/db mice (d–f). Double labeling with affinity-purified antibody for GLUT8, detected with Cy3-labeled anti-rabbit antibody (red, b and e) and anti-synaptopodin antibody detected with FITC-labeled anti-mouse antibody (green, a and d), shows increased expression of GLUT8 in podocytes in diabetic db/db mice (f) compared with nondiabetic db/m mice (c).
ional studies are required to address more precisely whether an increase in GLUT8 expression in one or more region of the kidney has a beneficial or harmful effect on the development of kidney disease.

Immunohistochemistry stainings for GLUT8 on renal cortex sections were performed to determine how fasting and feeding or hyperglycemia altered cellular expression patterns. No convincing difference could be demonstrated in the GLUT8 expression pattern in glomerular or tubular cells in response to fasting and feeding of GLUT4+/+ and GLUT4−/− mice (data not shown). Thus a conclusion about the cell types involved in GLUT8 regulation under physiological conditions will require more sensitive techniques (e.g., immunoelectron microscopy). However, on immunofluorescence examination in a direct comparison of diabetic kidneys to nondiabetic kidneys, a strong increase in GLUT8 protein signal in glomerular podocytes was detected (Fig. 4C). Differences in tubular expression of GLUT8 could not be reliably demonstrated using confocal fluorescence microscopy (data not shown). To clarify this point, experiments in isolated glomerular and tubular compartments are necessary and will be a part of further studies on GLU8 regulation in the kidney.

Experiments examining glucose transporters in vitro are often a difficult task because it is likely that glucose effects on other glucose transporters are masked by counterregulatory effects of GLUT1, the major glucose transporter. The examination of GLUT8 expression in conditionally immortalized murine podocytes (Fig. 1, G–I) demonstrated a prominent perinuclear distribution. However, high-glucose treatment led only to a slight and transient induction of GLUT8 mRNA in cultured differentiated podocytes. Challenging cultured podocytes with high glucose, insulin, or glucocorticoids did not lead to a cellular redistribution of GLUT8 at the level of fluorescence microscopy (data not shown). More detailed studies in vitro with isoform-specific knockdown and overexpression experiments are necessary to study possible effects on glucose uptake and intracellular membrane redistribution of GLUT8 in kidney cells after high-glucose treatments. In vivo studies have examined the redistribution of GLUT8 in neurons of the rat hippocampus using an in vivo glucose challenge, and enrichment of GLUT8 in the endoplasmic reticulum was noted (24).

Podocytes constitute an important component of the glomerular filtration barrier and contribute to the stabilization of capillary tuft, basement membrane turnover, and have immunological functions (31). Because podocytes are considered incapable of replication postnatally, the ability of the glomerulus to compensate for podocyte loss may be limited (2, 21). We and others previously demonstrated that podocyte loss is an early mechanism in glomerulosclerosis development in mice (28, 29) and serves as a positive predictor for the progression of diabetic glomerulosclerosis in patients with type 2 diabetes (18). The expression and upregulation of GLUT8 in podocytes could be an indicator of adaptation to cellular stress, but it remains unclear and requires further study to clarify whether these effects have a protective or harmful effect on podocytes. There is evidence for a potential protective effect against apoptosis and involvement in important cellular survival signaling pathways in murine blastocysts (23). Whether the upregulation of GLUT8 in podocytes in diabetic conditions is an indicator of higher cellular stress remains to be tested. More detailed studies of GLUT8 involvement in cellular survival signaling pathways and as an intracellular regulator of glucose concentration to maintain the intracellular milieu in different cellular compartments are necessary for a better understanding of GLUT8 regulation and its potential involvement in the pathogenesis of diabetic nephropathy.

In summary, we present the first studies on the localization and regulation of GLUT8 in the murine kidney under normal, insulin-resistant, and diabetic conditions. GLUT8 expression was found in podocytes and distal tubular epithelial cells in the kidney. GLUT8 mRNA and protein expression levels in the kidney are dependent on the plasma glucose levels in vivo under normal physiological (metabolic) as well as pathological (diabetic) conditions. GLUT8 expression is strongly upregulated in glomerular podocytes in diabetic db/db mice. GLUT8 is expressed in podocytes in culture and shows a perinuclear expression pattern; however, high glucose has no obvious influence on cellular redistribution of GLUT8. Our data suggest an important role for GLUT8 in glucose metabolism in podocytes in normal and pathological conditions. Further functional studies are necessary to elucidate the precise role of GLUT8 as a regulator of intracellular glucose milieu or its involvement in podocyte survival/apoptosis, and both functions have important implications for the treatment of diabetic nephropathy.

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