Original Article

The Human Glomerular Podocyte Is a Novel Target for Insulin Action

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Microalbuminuria is significant both as the earliest stage of diabetic nephropathy and as an independent cardiovascular risk factor in nondiabetic subjects, in whom it is associated with insulin resistance. The link between disorders of cellular insulin metabolism and albuminuria has been elusive. Here, we report using novel conditionally immortalized human podocytes in vitro and human glomeruli ex vivo that the podocyte, the principal cell responsible for prevention of urinary protein loss, is insulin responsive and able to approximately double its glucose uptake within 15 min of insulin stimulation. Conditionally immortalized human glomerular endothelial cells do not respond to insulin, suggesting that insulin has a specific effect on the podocyte in the glomerular filtration barrier. The insulin response of the podocyte occurs via the facilitative glucose transporters GLUT1 and GLUT4, and this process is dependent on the filamentous actin cytoskeleton. Insulin responsiveness in this key structural component of the glomerular filtration barrier may have central relevance for understanding of diabetic nephropathy and for the association of albuminuria with states of insulin resistance. *Diabetes* 54: 3095–3102, 2005

In 1980, Morgensen et al. (1) published a study whereby they infused human subjects with insulin (while maintaining normoglycemia with glucose infusion) and observed a dose-dependent induction of albuminuria, which occurred over a period of minutes. They concluded that the glomerular filtration barrier of the kidney must be insulin sensitive in some manner, but they were not able to comment further on the link.

Passage of albumin into the urine is normally prevented by the selective sieving action of the glomerular capillary wall. In recent years, by elucidating the genetic origin of a number of single human gene defects that result in congenital or early onset nephrotic syndrome, it has become apparent that the cell type primarily responsible for this barrier to albumin is the visceral glomerular epithelial cell or podocyte (2–5). The most common cause of progressive renal damage in the developed world is diabetic nephropathy, consuming vast amounts of health resources. Diabetic nephropathy occurs as a long-term complication of reduced insulin secretion (type 1 diabetes) or of failure of insulin action (type 2 diabetes), the latter being numerically more important (6,7). The earliest clinical manifestation of diabetic nephropathy is microalbuminuria. Microalbuminuria also occurs in nondiabetic subjects, in whom it is an independent cardiovascular risk factor associated with insulin resistance (8). It has not previously been apparent why abnormalities of insulin secretion and/or action should lead to albumin leaking into the urine even before there are major structural changes in the kidney.

Using unique conditionally immortalized human podocyte cell lines (9) and human glomerular sections, we have studied the effect of insulin on these cells and the mechanisms of glucose uptake. Insulin-responsive glucose uptake has previously been described in adipocytes and cardiac and skeletal muscle in humans (10). In these cells, glucose uptake is achieved through the facilitative glucose transporters GLUT4 and to a lesser extent GLUT1 (11,12) in an actin-dependent manner. We report that podocytes take up glucose in response to insulin, share similar signaling pathways as these cells, and show that both GLUT1 and GLUT4 are involved in this mechanism.

RESEARCH DESIGN AND METHODS

All reagents were purchased from Sigma Chemical (Poole, Dorset, U.K.) unless otherwise stated.

Cell lines. Two distinct normal human podocyte cell lines were studied, as well as control cell lines. We have previously reported (9) the derivation and characterization of a normal human podocyte cell line using conditional immortalization, whereby the immortalizing transgene is inactivated by culturing the cells at 37°C and the cells express the phenotype of differentiated podocytes. A second normal human podocyte cell line was generated using the same technique from the normal pole of a kidney removed for Wilm's tumor. This cell line has been shown to express the same markers of differentiation (nephrin, podocin, and synaptopodin) as the original cell line (R.J.M.C., M.A.S., unpublished data). Passage numbers of cells used were between 5 and 20. Experiments on differentiated cells were performed after silencing of the temperature sensitive simian virus 40 transgene at 37°C for at least 14 days. Cells were cultured in RPMI-1640 and supplemented with 10% (vol/vol) FCS, with insulin, transferrin, and selenite. Before all insulin stimulation experiments, the cells were cultured in serum-free and insulin-free conditions. An immortalized proximal tubular epithelial control cell line (HK2) (13) was cultured and starved in a similar manner. We also studied conditionally immortalized human glomerular endothelial cells, which had been generated using similar methodology as human podocytes (14). Murine 3T3-L1 fibroblast clones were obtained from LGC promochem (Teddington,

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2-DOG, 2-deoxy-[^{3}H]D-glucose; BCA, bicinchoninic acid; F-actin, filamentous-actin; RIPA, radioimmunoprecipitation; siRNA, small inhibitors of RNA.

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Middlesex, U.K.). They were differentiated into adipocytes as previously described (15). Adipocytes were serum and insulin starved for 2 h before insulin stimulation experiments.

Antibodies. The monoclonal GLUT4 (1F8; R&D Systems, Minneapolis, MN) antibody was used for immunofluorescence and immunogold labeling. Polyclonal rabbit antibodies (16) against GLUT1 and GLUT4 were used in Western blotting. GLUT1 immunogold was achieved using a rabbit polyclonal antibody (17), which was a gift from Professor F. Brosius (Ann Arbor University, Ann Arbor, MI). Species-specific fluorescein isothiocyanate– and tetramethylrhodamine-labeled secondary antibodies were used in immunofluorescence (Jackson Immunoresearch, Philadelphia, PA), and horseradish peroxidase were used antibodies in Western blotting.

Protein extraction, fractionation, and Western blotting. When studying total cellular lysate, protein was extracted using a modified radioimmunoprecipitation (RIPA) lysis buffer (18). Protein was quantified using a bicinchoninic acid–based (BCA) assay (Pierce, Rockford, IL) (19), and equal quantities were loaded. Microsomal preparations of cells were initially lysed in the nondetergent HES-based buffer (225 mmol/l sucrose, 1 mmol/l EDTA, and 20 mmol/l HEPES, pH 7.2) with protease inhibitors. Cells were spun at 5,000*g* for 10 min, and then the supernatant was spun at 175,000*g* in an Optima TM ultracentrifuge for 45 min. All steps were carried out at 4°C. The resulting microsomal pellet was resuspended in 50 μl modified RIPA buffer. A similar Western blot procedure was followed as previously reported (9). Preparation of whole-cell membrane fractions was as previously described (20) with the final pellet (cell membrane) being resuspended in modified RIPA. All steps were carried out on ice.

Densitometry was performed using a Bio-Rad Gel doc 1000 mini transilluminator and processed using the Quality 1 software package (Bio-Rad, Hercules, CA).

Cellular stimulation. Podocytes were insulin and serum starved for 2–24 h before stimulation. Unless stated otherwise, 220 nmol/l insulin was used. Cells were stimulated for 15 min with insulin.

GLUT4 RT-PCR. Total RNA from differentiated podocytes was studied. The superscript (Invitrogen, Paisley, U.K.) cDNA synthesis and PCR system was used with 35 cycles of amplification. Sequencing of the PCR products was carried out using standard preparation techniques for an ABI Prism automatic sequencer (Applied Biosystems, Foster City, CA).

GLUT4-specific primers were designed against the 5′ and 3′ termini together with the intracellular and extracellular loop portions of the molecule. The sequences used were as follows: 5′-terminal forward GTCAGAGACTCC AGGATCGG, 5′-terminal reverse TTCAATCACCTTCTGAGGGG (product length 224 bp); 3′-terminal forward CTGGACGAGCAACTTCATCA, 3′-terminal reverse CGTTCTCATCTGGCCCTAAA (product length 244 bp); intracellular loop forward GCCAGAAAGAGTCTGAAGCG, intracellular loop reverse CTACCCCTGCTGTCTCGAAG (product length 239 bp); extracellular loop forward CCCCTCAGAAGGTGATTGAA, extracellular loop reverse CTTTTCC TTCCAAGCCACTG (product length 189 bp).

Immunofluorescence. Immunofluorescence of cells was performed using a modified protocol as previously described (9). Cells were washed in ice-cold PBS and put on ice to arrest glucose transport after insulin stimulation. The blocking and permeabilizing solution consisted of 0.1% Saponin, 4% FCS, 0.1% Tween 20, and PBS, which was used for dilution of both primary and secondary antibodies. Filamentous-actin (F-actin) was visualized using a Texas red–conjugated phalloidin probe (Molecular Probes, Eugene, OR).

2-deoxy glucose uptake assays. Podocytes, proximal tubular cells, and conditionally immortalized human glomerular endothelial cells were grown to confluence on 6- or 12-well plates (Iwaki, Funabashi, Japan), and each experiment was performed with equal matched samples of basal and insulin-stimulated wells. Methods used were as previously reported (15), except that cells were then stimulated for 15 min with insulin, and matched control wells received no insulin. 2-deoxy-[^{3}H]D-glucose (2-DOG) at a concentration of 50 μmol/l (1μCi/ml) was added to the basal and stimulated cells for at least 3 min. Cytochalasin B and D were added at concentrations of 10 μmol/l, 30 min before an experiment to block glucose transport binding and F-actin mediated transporter translocation, respectively.

Bis-glucose photo labeling. Bis-glucose labeling was performed as previously described (21) with the following modifications: podocytes were incubated with and without 220 nmol/l insulin for 12 min and then incubated for a further 3 min in the presence of 500 μmol/l Bio-LC-ATB-BGPA (4,4-*O*-[2-[2-[2-[2-[2-[6 (biotinylamino) hexanolyl] amino] ethoxy] exoxy] ethoxy]-4-1-azi-2,2,2-trifluoroethyl) benzoyl] amino-1,3-propanediyl]bis-D-glucose). Cells were then irradiated for 1 min in a Rayonet photochemical reactor (Southern New England Ultraviolet, Branford, CT) using 300-nm-wavelength lamps. Cell surface GLUT4 and GLUT1 were determined by Western blot as before. An unlabeled podocyte sample was processed to act as a negative control, and a 15-min insulin-stimulated 3T3 adipocyte sample was processed to act as a positive control.

Small inhibitors of RNA studies. Podocytes were differentiated and then transfected with equal amounts of either GLUT4- or GLUT1-specific small inhibitors of RNA (siRNA) (which was checked for specificity by using the National Center for Biotechnology Information, NBLAST program), or control scrambled siRNA. The GLUT4 mRNA target sequence we used was CAGAU AGGCUCCGAAGAUG (Dharmacon, Lafayette, CO). For GLUT1, a combination of two sequences were used because they resulted in the greatest amount of knockdown of GLUT1 protein (sense target sequences CCAAGAGUGUGC UAAAGAA and CAUCGUGGCUGAACUCUUC). A nonspecific control siRNA sequence (Dharmacon) containing the same GC content was used following exactly the same procedure as used for the GLUT siRNA. Cells were grown in 12-well plates, and to each well, 750 μl Optimem (Gibco BRL, Gaithersburg, MD) was added. For each well, 4.5 μl Oligofectamine (Invitrogen) was combined with 18 μl Optimem for 5 min before being combined with a solution containing 4.5 μl of 20 nmol/l siRNA and 75 μl Optimem. This was left to form complexes for 25 min, after which 48 μl Optimem was added. This solution (150 μl) was then added to the wells making a total volume of 900 μl. After 4 h, the solution was supplemented with FCS and insulin, transferrin, and selinite to standard concentrations. Western blotting for GLUT4 on microsomal preparations, GLUT1 on plasma membrane preparations, and tritiated glucose uptake were performed after 48 h.

Immunogold analysis of tissue sections. Immunoelectron microscopy, using an indirect immunogold procedure, was performed on Lowicryl ultra thin human sections with GLUT1 and GLUT4 antibodies as described previously (22).

Phosphotyrosine-probed Western blotting. Podocyte whole-cell lysates were insulin starved and then basal cell tyrosine phosphorylation was compared with insulin-stimulated phosphorylation. Cells were prepared with modified RIPA buffer supplemented with sodium orthophosphate (23). The phosphotyrosine antibody 4G10 (Upstate, Milton Keynes) was used to probe the blot using standard Western blot methodology.

Statistical analysis. For glucose uptake assays, an ANOVA was performed, and the groups were then compared with a post hoc Bonferroni multiple comparison test. The Prism 2 program was used for analysis. A two-tailed paired Student's *t* test was used for the cytochalasin D and bis-glucose experiments. P values <0.05 were deemed significant. SEM is shown for all experiments.

RESULTS

Differentiated podocytes are insulin-sensitive glucose-utilizing cells. Using 2-DOG uptake assays, we found that two independent podocyte cell lines were each capable of increasing their glucose uptake by 90 and 105%, respectively, after 15 min of stimulation with 220 nmol/l insulin. Figure 1*A* shows the result for one of the two cell lines. This process was dependent on the degree of differentiation of the cells, with proliferating cells cultured at 33°C unable to significantly increase glucose uptake above baseline. A control immortalized proximal tubular epithelial cell line and an immortalized human glomerular endothelial cell line also did not significantly increase their glucose uptake in response to the same dose and duration of insulin. Cytochalasin B, a generic glucose transporter inhibitor, reduced insulin-stimulated glucose uptake by podocytes to basal levels, showing the process to be mediated by glucose transporters (data not shown). The half-maximal effective concentration for insulin in respect to glucose uptake was found to occur at 4.5 nmol/l, and full saturation of glucose uptake occurred at 75 nmol/l (Fig. 1*B*).

GLUT4 and GLUT1 are responsible for insulin-stimulated glucose uptake. GLUT4 was investigated at the messenger RNA level and found to be present in cultured podocytes, giving PCR products of the correct predicted size, and when sequenced, they showed complete homology to the 3′, 5′, intracellular, and extracellular loop portions of the National Center for Biotechnology Information published sequence for the GLUT4 molecule (Fig. 2*A*). Translation of GLUT4 mRNA to protein was demonstrated by Western blotting, which showed that it was detectable in microsomal fractions (Fig. 2*B*) correspond-

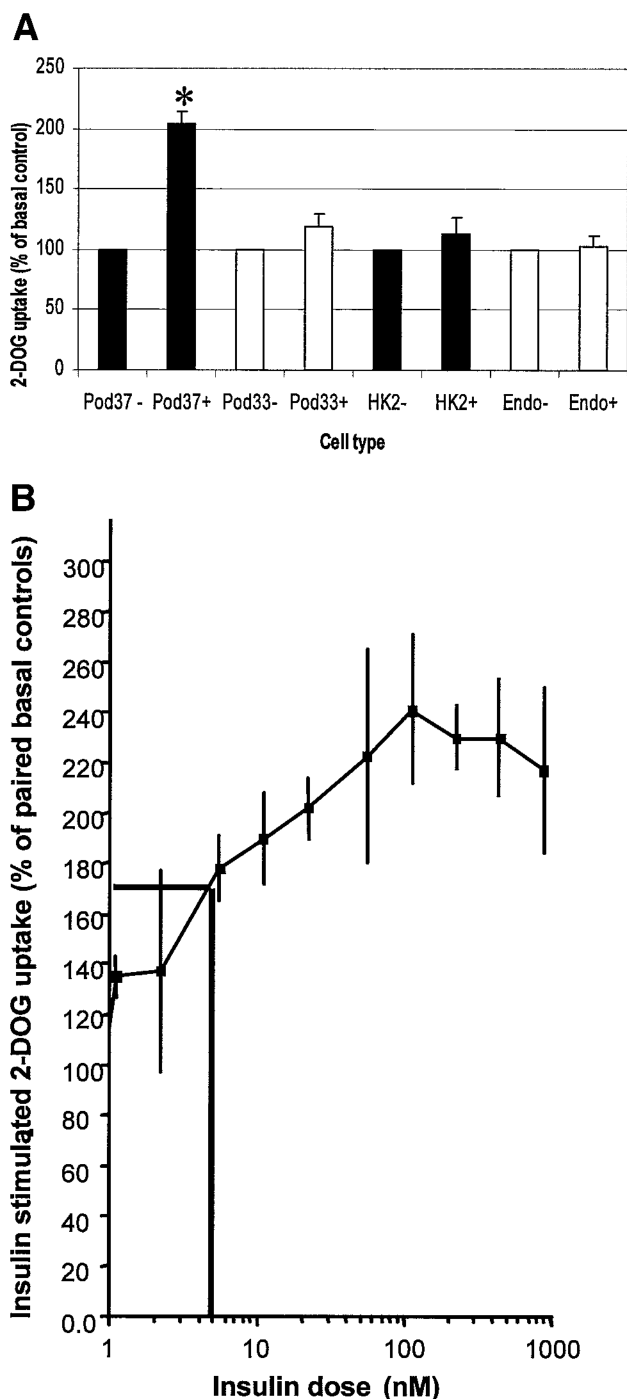


FIG. 1. Insulin-induced 2-DOG uptake in human podocytes. ***A*: Immortalized differentiated (37) and proliferating, nondifferentiated (33) podocytes (Pod), together with proximal tubular (HK2) cells and conditionally immortalized human glomerular endothelial cells (Endo) examined for glucose uptake under basal, non–insulin-stimulated conditions (−) and after 15 min of 220 nmol/l insulin stimulation (+). One-way ANOVA for all groups significant, *P < 0.0001. Post hoc analysis using Bonferroni test demonstrates significantly increased response in differentiated podocytes compared with all other cell types (P < 0.001). *B*: Fifteen-minute insulin-induced 2-DOG uptake dose-response curve for differentiated immortalized podocytes. n = 4–14 for each concentration. Insulin dose plotted on a log scale.**

ing to adipocyte-positive controls. This was the case for three different GLUT4 antibodies (two COOH-terminal and one NH_2-terminal antibody) (data is only shown for one of COOH-terminal antibodies; Fig. 2*B*). The cellular location of GLUT4 was in an intracytoplasmic, vesicular distribution in the resting cell, but upon insulin stimulation, GLUT4 translocated to the cell surface as shown by immunofluorescence. There was no GLUT4 immunostaining found in non–insulin-responsive HK2 cells (Fig. 2*C*). To confirm the presence of GLUT4 ex vivo in human podocytes, we performed immunogold electron microscopy on glomerular sections. These showed GLUT4 to be located in an intracellular vesicular distribution and at the plasma membrane of the cell. Some sections revealed GLUT4 fusing at the plasma membrane (Fig. 3).

GLUT1 was detected in vitro by Western blotting of podocyte plasma membrane fractions. Functionally, we demonstrated GLUT1 to be rapidly insulin translocatable to the plasma membrane of the podocyte using bis-glucose photolabeling (Fig. 4*A* and *B*). The ex vivo location of GLUT1 in human podocytes was in a vesicular distribution within the cytoplasm and at the plasma membrane of the podocyte (Fig. 4*C*). The bis-glucose photolabeling technique was not sufficiently sensitive to consistently detect GLUT4 translocation in immortalized human podocytes.

GLUT4 and GLUT1 are both involved in glucose uptake. Using GLUT4- and GLUT1-specific siRNA, we specifically knocked these proteins down in differentiated podocytes as illustrated by Western blotting 48 h after application (Fig. 5*A* and *B*). There was a corresponding blunting of insulin-induced glucose uptake with both GLUT4 siRNA (15% increase) and GLUT1 siRNA (16% increase), compared with no siRNA controls (58% increase). There was no difference in the basal glucose uptake with GLUT4 siRNA, but GLUT1 siRNA resulted in a significant decrease in basal podocyte glucose uptake by 42% compared with non–siRNA-treated cells (Fig. 5*C*).

Glucose uptake and GLUT4 translocation is dependent on the actin cytoskeleton. Preincubation of podocytes with 10 μmol/l cytochalasin D disrupted the F-actin cytoskeleton in vitro. Immunofluorescence demonstrated a failure of GLUT4-rich vesicles to reach the plasma membrane of the podocyte (Fig. 6*A*) and a loss of insulin-induced tritiated glucose uptake (Fig. 6*B*). There was no decrease in the basal uptake of glucose between cytochalasin D–treated and nontreated cells, suggesting that only actin-dependent translocatable glucose transporters were affected.

Components of the insulin signaling pathway are phosphorylated in response to insulin. Stimulation of podocytes with insulin and subsequent probing of the cell lysates with anti-phosphotyrosine antibody showed increased phosphorylation of proteins at molecular weights corresponding to the level of IRS-1 and the insulin receptor (Fig. 7). This is similar to the downstream signaling events in other known cell types, such as adipocytes and muscle cells.

DISCUSSION

In this study, we have demonstrated, using two independent normal podocyte cell lines, a previously unsuspected aspect of human podocyte biology: that differentiated podocytes are insulin responsive with insulin-induced glucose uptake being mediated through GLUT4 and GLUT1. Furthermore, our data suggest that this response is specific to the podocyte in the filtration barrier of the glomerulus, because immortalized glomerular human endothelial cells did not respond to insulin.

There has been an explosion in the amount of interest and research directed toward the podocyte in recent years

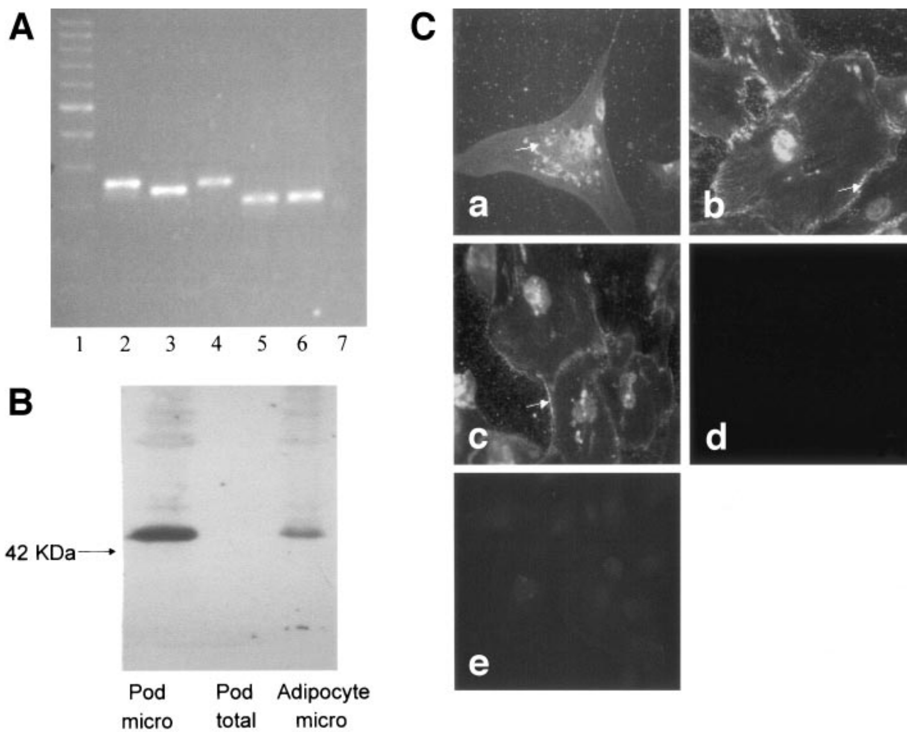


FIG. 2. GLUT4 in vitro in human podocytes. *A*: RT-PCR of differentiated podocytes and adipocytes. *Lane 1*, DNA standards. *Lanes 2–5* correspond to the 5′, 3′, intracellular, and extracellular GLUT4 domains, respectively, from podocytes. *Lane 6*, adipocyte extracellular domain–positive control. *Lane 7*, water-negative control. All bands were excised and sequenced and showed complete homology with the published National Center for Biotechnology Information database (data not shown). *B*: Western blot comparing differentiated podocyte microsomal preparation (Pod micro) with podocyte whole-cell lysate (Pod total). Adipocyte microsomal-positive control shown (Adipocyte micro). Thirty-seven micrograms of protein loaded in podocyte lanes. Representative of four blots. *C*: GLUT4 immunofluorescence of differentiated podocytes. *a*: Basal cells with vesicular cytoplasmic GLUT4 distribution. *b*: Insulin for 15-min plasma membrane location. *c*: 25 mmol/l D-glucose and insulin 15-min plasma membrane location. *d*: Mouse isotype control. *e*: HK2 cells immunostained with 1F8 GLUT4 under exactly same conditions as podocytes.

since it was proved, through positional cloning of genes responsible for a number of congenital and early onset human nephrotic syndromes, to be critical in maintaining the glomerular filtration barrier of the kidney and preventing albuminuria (2–4). However, our in vitro understanding of the biology of the podocyte has been hampered by a lack of representative human cell lines. Using a conditionally immortalized cell line, we have been able to mimic a terminally differentiated human phenotype, expressing the differentiation markers of synaptopodin, nephrin, WT1, and podocin (9).

Other observations support effects on podocytes of

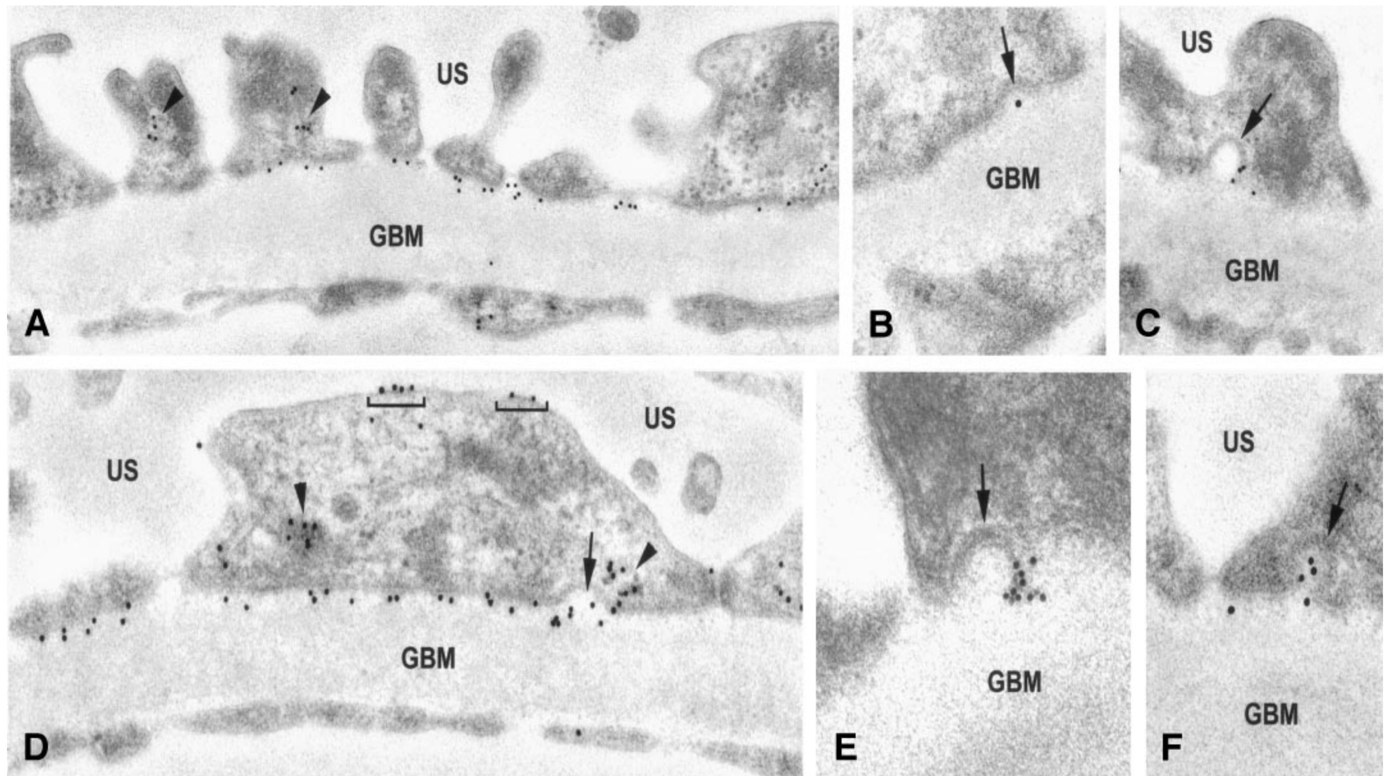


FIG. 3. GLUT4 ex vivo in human podocytes. GLUT4 immunogold electron microscopic examination of normal glomerular sections. *A* and *D*: Foot process localization of GLUT4. Vesicular localization demonstrated with arrowheads. *B–F*: Fusion of GLUT4-rich vesicles with plasma membrane (arrows). GBM, glomerular basement membrane; US, urinary space. Magnification ×20–30,000.

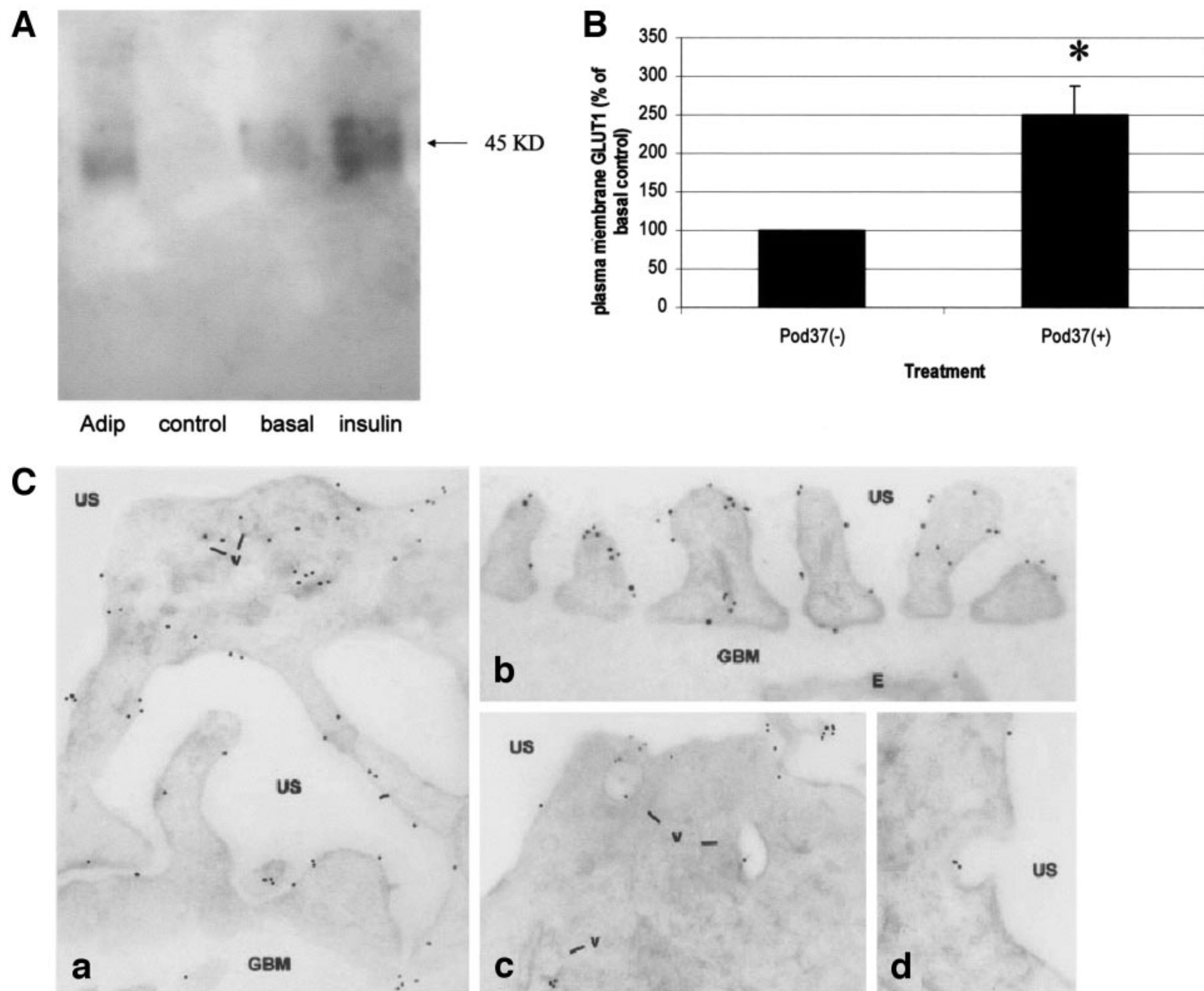


FIG. 4. GLUT1 in podocytes. *A*: GLUT1-probed bis-glucose experiment. Insulin-treated (220 nmol/l) adipocyte-positive control (Adip). Non–bis-glucose–labeled differentiated podocyte-negative control (control). Bis-glucose–labeled non–insulin-stimulated differentiated podocyte lane (basal). Bis-glucose–labeled insulin-stimulated differentiated podocyte lane (insulin). GLUT1 detected at cell surface in basal podocytes with upregulation after insulin stimulation. *B*: Densitometry of five independent bis-glucose experiments. Significant upregulation of cell surface GLUT1 between basal (−) and insulin-stimulated (220 nmol/l) differentiated podocytes (Pod37), *$P = 0.02$ using paired two-tailed Student's *t* test. *C*: GLUT1 immunogold localization in normal human glomerular sections. *a*: Section through the filtration barrier. GLUT1 is found at the podocyte cell surface and in intracytoplasmic vesicles (v). This is shown in higher power in *c*. *b*: Transverse section through the foot processes of the podocytes. *d*: GLUT1 in a plasma membrane fusion vesicle. E, endothelial cell; GBM, glomerular basement membrane; US, urinary space. Magnification ×20–30,000.

abnormalities in insulin secretion and/or its target action. These includes compelling evidence that the podocyte is directly affected in diabetic nephropathy with early podocyte loss and that alterations in its actin cytoskeleton are associated with urinary albumin loss in both type 1 (24,25) and type 2 diabetes (26–28). Microalbuminuria, which arguably results from subtle podocyte dysfunction, is known to be an important early manifestation of diabetic nephropathy and is a key feature of insulin resistance in nondiabetic subjects (it is now part of the World Health Organization criteria for diagnosis of metabolic syndrome). Many investigators have focused on the secondary effects of diabetes (e.g., hyperglycemia) in an effort to explain these cellular changes, though this fails to unify many of the clinical observations. What has been missing has been a mechanism linking podocyte damage to alterations in insulin secretion and/or action.

In this study, we analyzed podocytes from two different patients and found them both to be insulin responsive in their fully differentiated state. They both approximately doubled their glucose uptake with kinetics similar to the classic insulin-responsive muscle cell (29), using similar doses of insulin. Their saturation dose and half-maximal effective concentration are also similar to human muscle cell lines (30). We compared these cells with another immortalized human renal epithelial cell line, HK2, to ensure this effect was not a nonspecific result of in vitro insulin treatment and found that it was not. Interestingly, as the cells undergo differentiation when thermo-switched from 33°C to 37°C, they develop insulin responsiveness. Finally, we studied human glomerular endothelial cells, which had been immortalized using the same technology as used for podocytes and found them to be unresponsive to insulin. This is an important observation because endothelial cells are the only other cell type found in the glomerular filtration barrier of the kidney.

After discovering that differentiated podocytes were insulin responsive in vitro, we explored the underlying mechanism responsible for this action. The glucose transporter that is classically linked to rapid insulin stimulated glucose uptake in muscle and adipose tissue is GLUT4 (31). This resides in an intracytoplasmic vesicular distri-

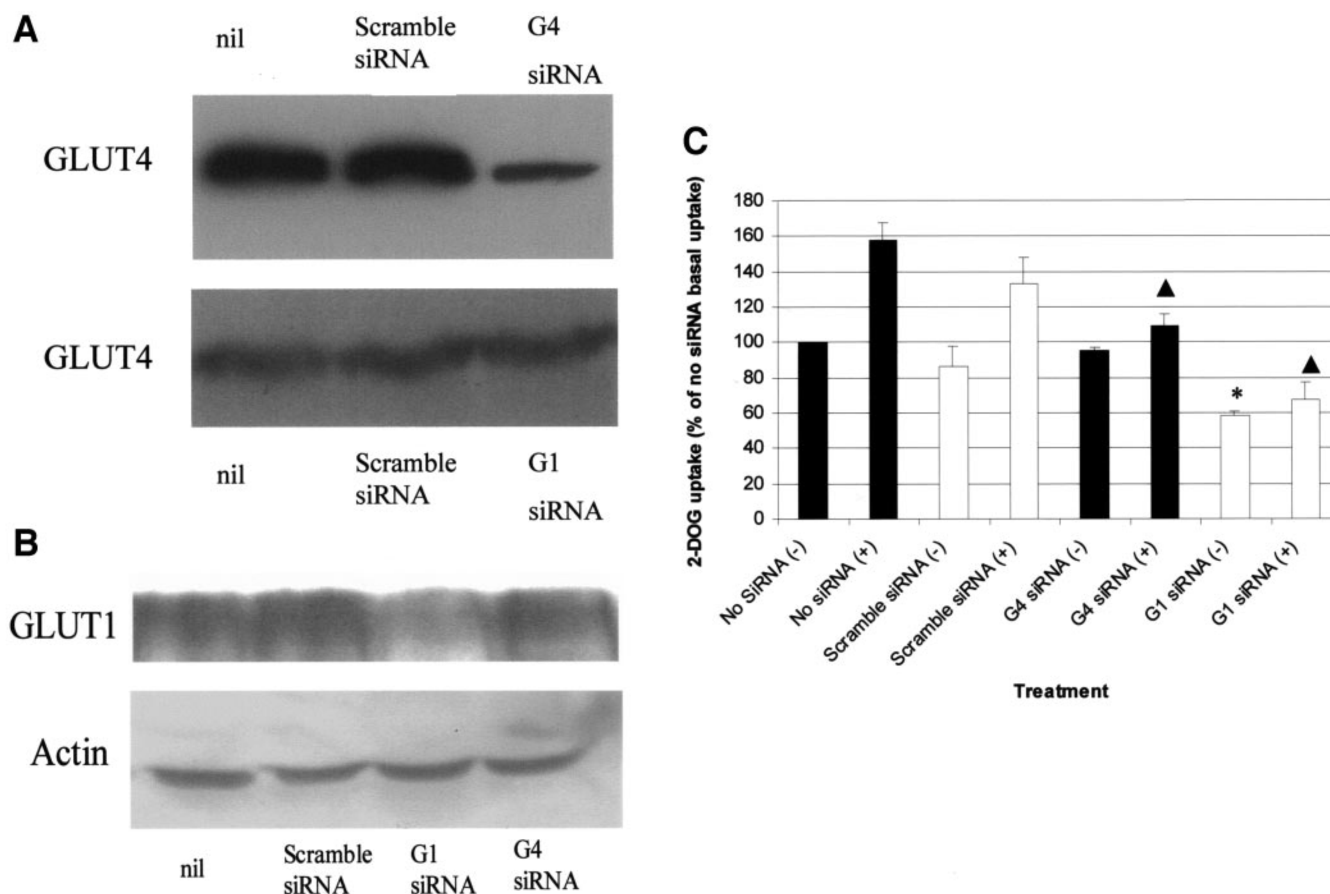


FIG. 5. GLUT4 and GLUT1 siRNA in podocytes. *A*: GLUT4 Western blot of microsomal preparations of siRNA-treated differentiated podocytes (48 h). Equal protein loading ensured by BCA assay of total lysate before high-speed centrifugation. There is no difference in GLUT4 expression compared with untreated podocytes with scramble siRNA, but appreciable reduction in signal with GLUT4 siRNA. GLUT1 siRNA did not affect GLUT4 protein expression (*bottom panel*). *B*: GLUT1 plasma membrane preparation. GLUT1 knocked down with the combined GLUT1 siRNA but unaffected by scramble and GLUT4 siRNA. Actin loading of cytoplasmic fraction shown. This was performed after BCA assay. *C*: 2-DOG uptake of basal (−) and insulin-stimulated (+) (220 nmol/l) siRNA-treated differentiated podocytes. For basal 2-DOG uptake, there was a significant difference between the groups (one-way ANOVA, $P < 0.0001$). Post hoc analysis using Bonferroni test when compared with no siRNA treatment showed only a significant difference in GLUT1 siRNA (G1siRNA)-treated podocytes; *$P < 0.001$. When comparing the fold increase in 2-DOG uptake after insulin stimulation between the different siRNA treatments one-way ANOVA showed significant difference between the groups ($P = 0.002$). Post hoc Bonferroni showed significant inhibition of insulin-induced 2-DOG uptake in both G1siRNA-treated and GLUT4siRNA (G4siRNA)-treated cells in comparison with non–siRNA-treated podocytes (vehicle only); ▲, $P < 0.02$. $n = 4$–9 independent experiments for each condition.

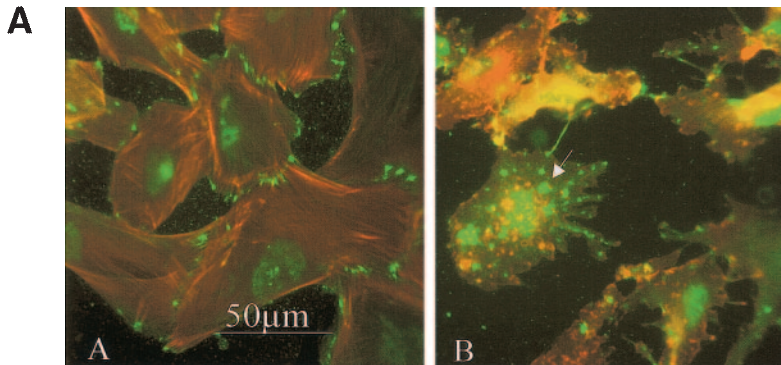
bution in the resting cell and translocates to the plasma membrane in response to insulin. We found good evidence that GLUT4 messenger RNA was present in the cells using RT-PCR and sequencing. Furthermore, a single band was found on Western blot in microsomal preparations using three different antibodies (two COOH-terminal antibodies and an NH_2-terminal antibody), all of which corresponded to a positive control of murine adipocytes prepared in a similar manner, which are categorically known to contain GLUT4 (similar to Fig. 2*B*). The typical basal cellular cytoplasmic vesicular location and insulin-induced peripheral translocation in vitro and ex vivo of GLUT4 was reproducibly demonstrated in the podocyte using immunofluorescence and immunogold with the monoclonal antibody IF8. This typical distribution is important because the 1F8 antibody has been shown to give nonspecific diffuse cellular staining in the past (32). Importantly, we found that the insulin-unresponsive HK2 cells did not stain with this GLUT4 antibody (Fig. 2*C*). Finally, we showed the functional importance of GLUT4 in insulin-induced glucose uptake using GLUT4-specific siRNA technology (Fig. 5). We feel this is strong evidence of the presence and functional importance of GLUT4 in the podocyte.

Previous work on the presence of GLUT4 in the podocyte has been controversial. A number of investigators have suggested in animal studies that the podocyte expresses GLUT4 (33–35) using immunofluorescence or by detecting trace amounts of immunogold signal in glomerular tufts. However, other groups have suggested the podocyte to be devoid of this transporter (36,37). This is the first study to show GLUT4 in human podocytes.

In podocytes, as in muscle and adipocytes, GLUT4 translocation is dependent on an intact actin cytoskeleton to facilitate movement of GLUT4-rich vesicles to the periphery of the cell. By disrupting F-actin with cytochalasin-D and analyzing with GLUT4 immunofluorescence (Fig. 6*A*) and 2-DOG uptake assays (Fig. 6*B*), we showed there to be no significant increase in glucose uptake in response to insulin, although basal uptake remained unchanged. This suggests that only insulin-recruitable transporters are affected by disruption of the actin cytoskeleton.

We show that human podocytes also recruit and use GLUT1 for glucose uptake, which is similar to the other classically insulin-responsive cell types (11,12,). GLUT1 is located at the plasma membrane of most cells and is responsible for basal glucose uptake into cells (38). How-

A



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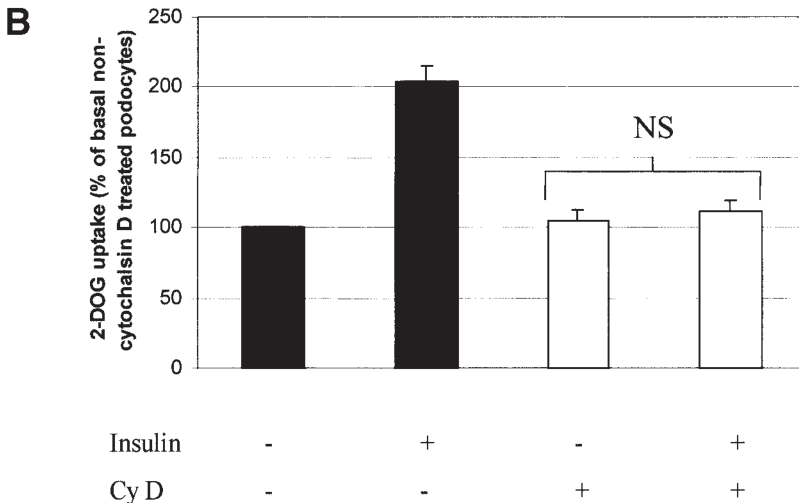

FIG. 6. GLUT4 translocation and insulin-stimulated 2-DOG uptake are dependent on the F-actin cytoskeleton. *A*: GLUT4 immunofluorescence (green) and phalloidin staining of the actin cytoskeleton (red) of insulin-treated podocytes. ***a***: Intact cytoskeleton. ***b***: Cytochalasin D disrupted cytoskeleton. Disruption of the cytoskeleton results in a failure in translocation of vesicles to the periphery of the cell in ***b*** (arrow). *B*: 2-DOG uptake in cytochalasin D (CyD)-treated basal (−) and insulin-stimulated (+) (220 nmol/l) podocytes. Compared with non–cytochalasin-treated cells. Insulin response abrogated by pretreatment with cytochalasin D ($P = 0.46$; two-tailed paired Student's t test) (NS) ($n = 5$).

ever, it is also recruited to the plasma membrane in response to insulin in muscle and adipocytes (11,12). This also occurs in podocytes in vitro as shown by our bis-glucose labeling experiment (Fig. 4*A* and *B*) and further supported by immunogold ex vivo studies demonstrating GLUT1 located in vesicles in the cytoplasm, as well as at the plasma membrane (Fig. 4*C*). The complete abrogation of insulin-stimulated (but not basal) glucose uptake after disruption of the actin cytoskeleton suggests that GLUT1 also requires the F actin cytoskeleton to move to the plasma membrane after insulin stimulation.

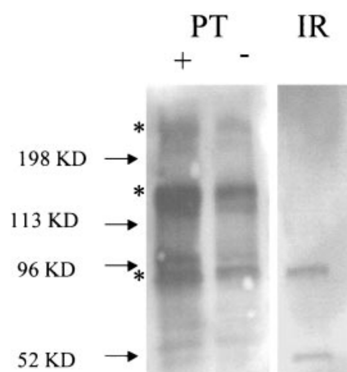

FIG. 7. Insulin-induced tyrosine phosphorylation of the podocyte. Phosphotyrosine (PT) blot of whole-cell lysate from differentiated podocytes. Basal cells (−) compared with insulin-stimulated cells (+) (220 nmol/l). Three bands detected at molecular weights of ~90, 150, and >200 kDa (*). One of these bands corresponds to the molecular weight of the insulin receptor (IR), which was run on the same gel and then probed with the insulin receptor antibody (Santa Cruz).

Functionally, GLUT1 and GLUT4 both contribute to insulin-induced glucose uptake, as we showed using GLUT1 and GLUT4-specific siRNA. GLUT4 only decreased insulin-induced glucose uptake with no reduction in basal uptake; in contrast, GLUT1 knockdown affected both basal and insulin-induced uptake. This is not surprising because GLUT1 is a constitutional glucose transporter. Intriguingly, knocking down either GLUT1 or GLUT4 resulted in an ~60% reduction in insulin-stimulated glucose uptake, compared with no siRNA vehicle controls, despite no quantitative reduction of GLUT1 protein with GLUT4 siRNA or GLUT4 protein with GLUT1 siRNA. This suggests some interdependence on each other for glucose uptake in the podocyte.

We have also demonstrated expression of other components of the downstream insulin-signaling pathway in podocytes, including the insulin receptor, insulin receptor substrate-1, insulin receptor substrate-2, and the vesicular-snare, vesicle associated membrane protein 2 (data not shown). These molecules are typical of the insulin-stimulated GLUT pathways in insulin-responsive cell types (10). Additionally, we detected phosphorylation of proteins consistent with components of this signaling pathway, in response to insulin stimulation (Fig. 7).

In conclusion, this study demonstrates that the human podocyte is uniquely insulin sensitive in the filtration barrier of the kidney. Both GLUT1 and GLUT4 are required for this process, together with F-actin reorganization. Further studies elucidating the mechanism the action of insulin on the podocyte may result in novel treatment strategies being developed for this debilitating complication of diabetes.

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