DIABETES TECHNOLOGY & THERAPEUTICS
Volume 13, Number 7, 2011

DOI: 10.1089/dia.2011.0041

Original Article

A Fluorescence Method for Measurement of Glucose Transport in Kidney Cells

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Abstract

Background: Diabetes may alter renal glucose reabsorption by sodium (Na^+)-dependent glucose transporters (SGLTs). Radiolabeled substrates are commonly used for in vitro measurements of SGLT activity in kidney cells. We optimized a method to measure glucose uptake using a fluorescent substrate, 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG).

Methods: Uptake buffers for 2-NBDG were the same as for ^{14}C-labeled α-methyl-D-glucopyranoside ([^{14}C]AMG). Cell lysis buffer was optimized for fluorescence of 2-NBDG and Hoechst DNA stain. Uptake was performed on cultures of primary mouse kidney cells (PMKCs), the LLC-PK_1 proximal tubule cell line, or COS-7 cells transiently overexpressing mouse SGLT1 or SGLT2 by incubating cells at 37°C in buffer containing 50–200 μM 2-NBDG. Microscopy was performed to visualize uptake in intact cells, while a fluorescence microplate reader was used to measure intracellular concentration of 2-NBDG ($[2\text{-NBDG}]_i$) in cell homogenates.

Results: Fluorescent cells were observed in cultures of PMKCs and LLC-PK_1 cells exposed to 2-NBDG in the presence or absence of Na^+. In LLC-PK_1 cells, 2-NBDG transport in the presence of Na^+ had a maximum rate of 0.05 nmol/min/μg of DNA. In these cells, Na^+-independent uptake of 2-NBDG was blocked with the GLUT inhibitor, cytochalasin B. The Na^+-dependent uptake of 2-NBDG decreased in response to co-exposure to the SGLT substrate, AMG, and it could be blocked with the SGLT inhibitor, phlorizin. Immunocytochemistry showed overexpression of SGLT1 and SGLT2 in COS-7 cells, in which, in the presence of Na^+, $[2\text{-NBDG}]_i$ was fivefold higher than in controls.

Conclusion: Glucose transport in cultured kidney cells can be measured with the fluorescence method described in this study.

Introduction

The kidney plays a major role in glucose homeostasis.[1] Glucose is filtered out of blood in the glomerulus, but it is almost completely reabsorbed in the proximal tubule (PT) by sodium (Na^+)-dependent glucose transporters (SGLT1 and SGLT2).[2] The absorbed glucose then diffuses into blood from the basolateral side via facilitative transporters (GLUT1 and GLUT2).[3] The transport maximum of glucose is 375 mg/min; therefore, in diabetes patients, when plasma glucose levels approach 400 mg/dL, unabsorbed excess glucose is excreted in the urine.[1] We and others have shown that the in vivo expression levels of SGLTs are altered in the kidneys of animal models of diabetes.[4–7] Similarly, SGLT2 expression in primary cultures of human exfoliated PT epithelial cells (HEPTECs) isolated from urine of type 2 diabetes patients was shown to be higher than in HEPTECs from healthy controls.[8] Consistently, glucose uptake was higher in HEPTECs from diabetes patients than in controls.

Radiolabeled substrate, ^{14}C-labeled α-methyl-D-glucopyranoside ([^{14}C]AMG), is commonly used to measure SGLT-mediated glucose uptake in primary cultures of human or animal PT cells and in the PT cell line LLC-PK_1.[8–10] Similarly, we have used [^{14}C]AMG in our toxicological studies to show the inhibitory effect of cadmium on SGLT activity.[11,12]

A fluorescent derivative of glucose, 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), is available.[13] Microscopy can be performed to examine 2-NBDG uptake in living cells,[14] and the intracellular concentration of transported 2-NBDG can be measured with fluorescence spectroscopy.[15] In enterocytes, 2-NBDG uptake could be blocked with phlorizin, an inhibitor of SGLTs,[14,16] whereas cytochalasin B, a GLUT inhibitor,[17] blocked 2-NBDG transport in endothelial and pancreatic

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cells,[18,19] suggesting that 2-NBDG is transported by SGLTs and GLUTs.

Measurement of glucose uptake in kidney cells by fluorescence has not previously been described. Instead of measuring radioactivity, we performed fluorescence spectroscopy to measure in vitro uptake of glucose in cultured kidney cells.

Materials and Methods

Materials

Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA). Medium 199 HEPES modification (M199), Dulbecco's modified Eagle's medium, and Ham's F12 medium were from Sigma (St. Louis, MO). 2-NBDG was from Invitrogen (Carlsbad, CA). Phlorizin, cytochalasin B, Hoechst 33258, and all other chemicals were from Sigma. Corning (Lowell, MA) 96-well black microplates with clear bottoms were used.

Cell culture

Cell lines were from the American Type Culture Collection (Manassas, VA). LLC-PK_1 cells were grown in M199 and 2.2 g/L sodium bicarbonate (pH 7.4) supplemented with 4% fetal bovine serum, 0.05 g/L penicillin, and 50 μg/mL streptomycin. COS-7 cells were grown in Dulbecco's modified Eagle's medium with 25 m*M* HEPES, 10% fetal bovine serum, and antibiotics. Primary mouse kidney cells (PMKCs) were prepared as described previously and grown in 1:1 Dulbecco's modified Eagle's medium:Ham's F12 with supplements.[20] Cells were plated in 35-mm-diameter or in six-well culture plates and were maintained at 37°C in a 5% CO_2 atmosphere.

Transient transfection

Plasmid constructs for overexpression of mouse SGLT1 (clone ID 3497611) and SGLT2 (clone ID 4235707) were from Open Biosystems (Huntsville, AL). Both constructs were subjected to restriction enzyme digestion analysis. In addition, SGLT1 and SGLT2 insert clones were fully sequenced (Functional Biosciences, Madison, WI), and their nucleotide sequences were confirmed. Each vector (1 μg) was transfected into COS-7 cells using TurboFectin 8.0 (OriGene, Rockville, MD). In addition to control untreated cells, those treated with TurboFectin alone were used as vehicle control. Experiments were performed on cells 24–48 h post-transfection.

Western blot

We custom-synthesized and peptide affinity-purified polyclonal antibodies specific against mouse SGLT1 and SGLT2 by using the services of Invitrogen. SGLT1 antibody was raised in goats against peptide AVTATDAPIPSYERIR (GenBank NP_062784); SGLT2 antibody was raised in rabbits against peptide MEQHVEAGSELGEQKV (GenBank NP_573517).

Western blotting was performed as previously described.[21] In brief, cells were lysed directly on plates, aliquots were subjected to electrophoresis, and proteins were blotted. Membranes were probed with SGLT1 or SGLT2 antibody and then incubated with horseradish peroxidase–conjugated anti-goat or anti-rabbit secondary antibody. Blots were briefly incubated with HyGLO™ chemiluminescent horseradish peroxidase antibody detection reagent (Denville Scientific, Metuchen, NJ) and documented by Kodak (Rochester, NY) Image Station 2000R.

Immunocytochemistry and fluorescence confocal microscopy

COS-7 cells transfected with SGLT or the vehicle control were grown on Lab-Tek II chamber slides (Thermo Scientific, Waltham, MA). Cells on slides were rinsed in Dulbecco's phosphate-buffered saline and then fixed for 30 min in 4% paraformaldehyde. After the rinses, cells were first incubated for 15 min in SuperBlock in Tris-buffered saline (Thermo Scientific) and then for 2 h with SGLT1 or SGLT2 antibody diluted in blocking buffer. Following the rinses, cells were incubated for 1 h with 5 μg/mL Alexa Fluor 647–conjugated donkey anti-goat or anti-rabbit secondary antibody (Invitrogen). Following a rinse, VECTASHIELD® mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was added.

Image analysis was performed at the Children's Research Institute Imaging Core Facility at the Medical College of Wisconsin. Fluorescent images were collected with a Carl Zeiss (Jena, Germany) LSM510 laser scanning microscope using a plan-Apochromat 20×/0.8NA lens. The image represents 3× digital zoom, 1,024×1,024 pixels, and a line average of 4. The Alexa 647 secondary antibody was excited with a HeNe laser-633 (acousto-optic tunable filter (AOTF) set at 100%), and DAPI was excited with a diode laser-405 (AOTF set at 9%). Confocal images were collected (pinhole set at 1 Airy Unit) with appropriate dichroics (MBS-405/488/561/633/KP725, DBS1-NFT635vis, and DBS3-plate) and filters (LP650 for Alexa-647 in front of channel 1 and BP420-480 for DAPI in front of channel 2) for each flurophore, and image pixel saturation was corrected with photomultiplier tube (PMT) detector gain and offset controls as per the manufacturer's recommendations.

Buffer optimization

All fluorescence measurements were made on 200-μL aliquots of samples in a BioTek (Winooski, VT) Synergy™ HT microplate reader. Fluorescence of 2-NBDG (2.5–100 μ*M*) in buffers that we routinely use for [^{14}C]AMG uptake[20] were measured. The Na^+ buffer contained 140 m*M* NaCl, 5 m*M* KCl, 2.5 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 1 m*M* KH_2PO_4, and 10 m*M* HEPES (pH 7.4); NaCl was replaced with choline chloride in Na^+-free buffer. Dilution in water was used as the control. Fluorescence was measured at excitation and emission wavelengths of 485 nm and 528 nm, respectively. An arbitrary unit (A.U.) was used to express fluorescence.

The same dilutions of 2-NBDG as above were also made in cell lysis solution containing 0.2% Triton X-100/0.01 *N* NaOH,[20] and fluorescence was measured. Because this solution abolished fluorescence (see Fig. 1B), an alternative lysis buffer composed of 1% Nonidet P-40, 1% sodium deoxycholate, 40 m*M* KCl, and 20 m*M* Tris (pH 7.4) was tested. Fluorescence of 2-NBDG in buffer without detergents and in water was also measured.

Effects of detergents on fluorescence of Hoechst were examined. Hoechst was added to the final concentration of 1 μg/mL to calf thymus DNA (0.5–15 μg/mL) in [40 m*M* KCl and 20 m*M* Tris (pH 7.4)] containing either 1% Nonidet P-40 or 1%

sodium deoxycholate. Fluorescence was measured at excitation and emission wavelengths of 360 and 460 nm, respectively.

Fluorescence microscopic analysis

PMKCs or LLC-PK_1 cultures were grown until 80% confluent. Medium was removed, and culture plates were rinsed three times in Na^+-free buffer. Cells were then incubated at 37°C with 200 μM 2-NBDG in Na^+ or Na^+-free buffer. After 1 h, buffers were removed, plates were rinsed in Na^+-free buffer, and cells were examined with an Olympus (Center Valley, PA) IX50 fluorescence microscope. As a control, the above experiments were repeated in the absence of 2-NBDG.

Fluorescence microplate assay analysis

Nine culture plates of PMKCs or three LLC-PK_1 plates were used for each uptake experiment. After medium was removed and plates were rinsed in Na^+-free buffer, cells were incubated at 37°C in Na^+ buffer containing 50–200 μM 2-NBDG. After 60 min, buffers were removed, and plates were rinsed three times in Na^+-free buffer. Cultures were then incubated at room temperature with 0.1 mL of cell lysis buffer (1% sodium deoxycholate, 40 mM KCl, and 20 mM Tris [pH 7.4]) for 10 min. Lysed cells were scraped off and homogenized by 10 passages through a 19-gauge needle. Immediately following centrifugation at 12,000 g for 5 min at 4°C, fluorescence of aliquots from supernatants were measured as described above. To quantify the intracellular concentration of 2-NBDG ($[2\text{-NBDG}]_i$), standard curve graphs were generated by measuring fluorescence of 2.5–20 μM 2-NBDG in lysis buffer.

To measure DNA, aliquots from cell homogenates were diluted in lysis buffer, Hoechst was added (1 μg/mL), and fluorescence was measured as described above. To quantify the cellular amount of DNA, standard curve graphs were generated by measuring fluorescence of Hoechst (1 μg/mL) for calf thymus DNA (0.5–15 μg/mL) in lysis buffer. The concentration of transported 2-NBDG was normalized to the amount of DNA, and $[2\text{-NBDG}]_i$ was expressed as μM 2-NBDG/μg of DNA.

2-NBDG transported in presence of Na^+ is considered as the total uptake, which is the sum of contributions from SGLT and GLUT. To determine transport by GLUT, cells were incubated for 1 h with 50–200 μM 2-NBDG in Na^+-free buffer, and $[2\text{-NBDG}]_i$ was determined. The difference between the total and the Na^+-independent uptakes was used as Na^+-dependent transport by SGLT.

Kinetic analysis of 2-NBDG transport in LLC-PK_1 cells

LLC-PK_1 cells were incubated at 37°C with 50, 75, 100, or 200 μM 2-NBDG in Na^+ buffer for 0, 5, 15, 30, 45, 60, or 75 min. The amount of background fluorescence measured in cell homogenates at time 0 were subtracted from the values after 5–75 min of incubation, and the resulting values were normalized to the amount of DNA. Measurements were fitted into hyperbolic dependence of rate on 2-NBDG concentration to determine the V_{max} value using SigmaPlot version 9.0 (Systat Software, Inc., San Jose, CA).

Competition and inhibition assays

LLC-PK_1 cells were incubated with 50 μM 2-NBDG with or without either D-glucose or AMG at 30 mM in Na^+ and Na^+-free buffers. Uptake was carried out as described above, and Na^+-dependent changes in $[2\text{-NBDG}]_i$ were determined.

To examine the effect of phlorizin, $[2\text{-NBDG}]_i$ was measured in LLC-PK_1 cells co-incubated with 2-NBDG (100 μM) and phlorizin (100 μM) in Na^+ and Na^+-free buffers. To examine the effect of cytochalasin B, LLC-PK_1 cells were incubated with 10 μM cytochalasin in Na^+-free buffer. After 5 min, plates were rinsed in Na^+-free buffer, and uptake was performed with 2-NBDG (100 μM) in Na^+ and Na^+-free buffers. Controls included uptake in the absence of an inhibitor and in the presence of ethanol (0.05%) or dimethyl sulfoxide (DMSO; 0.001%), the vehicles for phlorizin and cytochalasin, respectively.

2-NBDG uptake in cells overexpressing SGLT1 and SGLT2

Eight COS-7 culture plates per experimental condition were used. Cells were incubated at 37°C with 200 μM NBDG in Na^+ and Na^+-free buffers, and $[2\text{-NBDG}]_i$ was measured following a 30-min incubation. Cells transfected with TurboFectin alone were used as the vehicle control.

Statistical analysis

Unless otherwise stated, at least three independent cultures were used to repeat each experiment. Data analysis was performed with SigmaPlot version 11.2. The mean values of the results were calculated, and SE values of the means were determined. For statistical analysis, comparisons between multiple groups were performed using one-way analysis of variance (ANOVA); all data had passed the Shapiro–Wilk normality test.

Results

Buffer optimization

Uptake buffers. The fluorescence values of 2-NBDG diluted in buffers that we use to measure SGLT activity with [^{14}C]AMG[20] were compared with that of 2-NBDG diluted in water. Figure 1A shows that the fluorescence intensity of 2-NBDG diluted in water increased dose-dependently from 202 to 3,700 A.U. when its concentration was increased from 2.5 to 100 μM. The uptake buffers alone did not produce background fluorescence (data not shown). At each concentration, the fluorescence intensities of 2-NBDG in Na^+ and Na^+-free buffers were almost the same, and both were similar to that in water. Compared with the fluorescence of 2-NBDG diluted in water, there were small (4–11%) but statistically significant decreases in fluorescence when 2-NBDG were diluted to concentrations of 20 μM or higher in Na^+-free and Na^+ buffers. Based on the above analysis, [^{14}C]AMG uptake buffers were used in 2-NBDG uptake experiments.

Cell lysis buffer. We use 0.2% Triton X-100/0.01 N NaOH to lyse cells after [^{14}C]AMG uptake.[20] Dilution of 2-NBDG (2.5–100 μM) in this solution abolished fluorescence, and the representative effect is shown in Figure 1B. Next, the fluorescence intensity of 2-NBDG (2.5–100 μM) in an alternative lysis buffer composed of 1% Nonidet P-40, 1% sodium deoxycholate, 40 mM KCl, and 20 mM Tris (pH 7.4) was examined. Figure 1B shows that fluorescence of 2-NBDG in this lysis buffer (column B) was the same as in the same buffer without detergents (column C) or in water (H_2O).

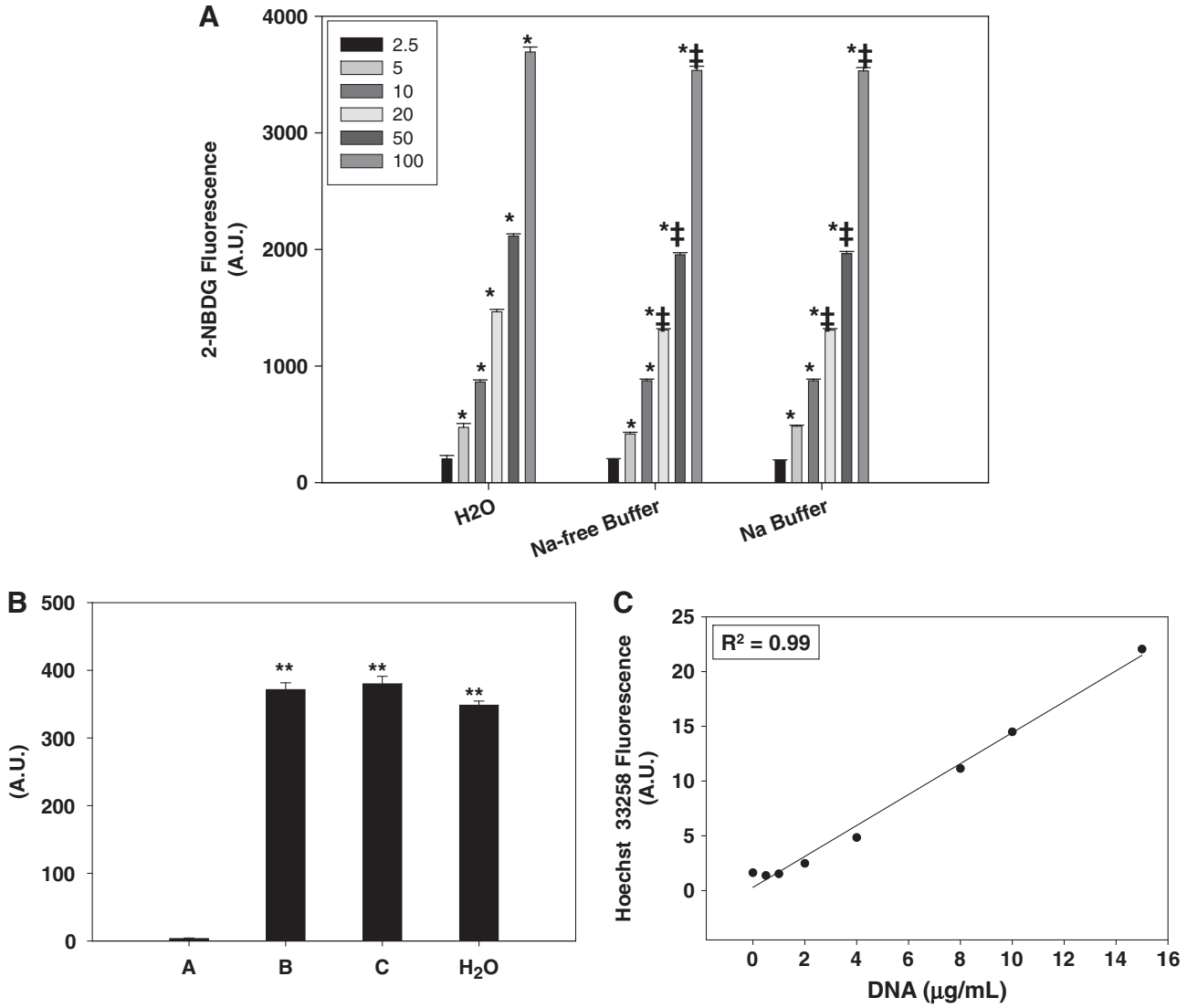

FIG. 1. Buffer optimization. A.U., arbitrary units. (**A**) Effect of uptake buffers on 2-NBDG fluorescence. 2-NBDG was diluted to final concentrations of 2.5, 5, 10, 20, 50, or 100 μM in water (H_2O) and in Na^+ and Na^+-free uptake buffers, and fluorescence was measured ($n = 3$). *$P < 0.001$, determined by comparing values at each concentration versus those at the closest lower concentration in the same solution. $^{\ddagger}P \leq 0.003$, determined by comparing values in water with those in Na^+-free or Na^+ buffer. (**B**) Representative effect of lysis solution on 2-NBDG fluorescence. Fluorescence was measured in 2-NBDG diluted to a final concentration of 10 μM in solution A (0.2% Triton X-100/0.01 *N* NaOH), B (1% sodium deoxycholate, 1% Nonidet P-40, 40 m*M* KCl, and Tris [pH 7.4]), or C (40 m*M* KCl and Tris [pH 7.4]); dilution in water was used as the control ($n = 6$). Significance was determined by comparing values obtained with each solution B, C, and water versus that of solution A. **$P = 0.004$. (**C**) Representative DNA standard curve graph. Calf thymus DNA (0.5–15 μg) was suspended in solution C with 1% sodium deoxycholate. Hoechst 33258 was added to a concentration of 1 μg/mL, and fluorescence was measured.

We examined whether detergents in lysis buffer affected the fluorescence of Hoechst. Standard curve graphs generated from DNA in Nonidet P-40 buffer were nonlinear (data not shown), but those made in sodium deoxycholate buffer were reproducibly linear (Fig. 1C). Therefore, we used a buffer containing 1% sodium deoxycholate, 40 m*M* KCl, and 20 m*M* Tris (pH 7.4) to lyse cells after 2-NBDG uptake.

Fluorescence microscopy

In the absence of 2-NBDG, background fluorescence was not observed in LLC-PK_1 cells incubated with Na^+ (Fig. 2A) or Na^+-free (Fig. 2E) buffer. The fluorescent cells were observed in cultures incubated with 200 μM 2-NBDG in the presence (Fig. 2B) or absence (Fig. 2F) of Na^+. Similarly, 2-NBDG was taken up by PMKCs with (Fig. 2D) or without (Fig. 2H) Na^+ in uptake buffer, and there was no background fluorescence in cells incubated with each buffer alone (Fig. 2C and G).

Transport kinetics

Kinetic analysis was performed in LLC-PK_1 cells incubated with 50–200 μM 2-NBDG in Na^+ buffer, following which

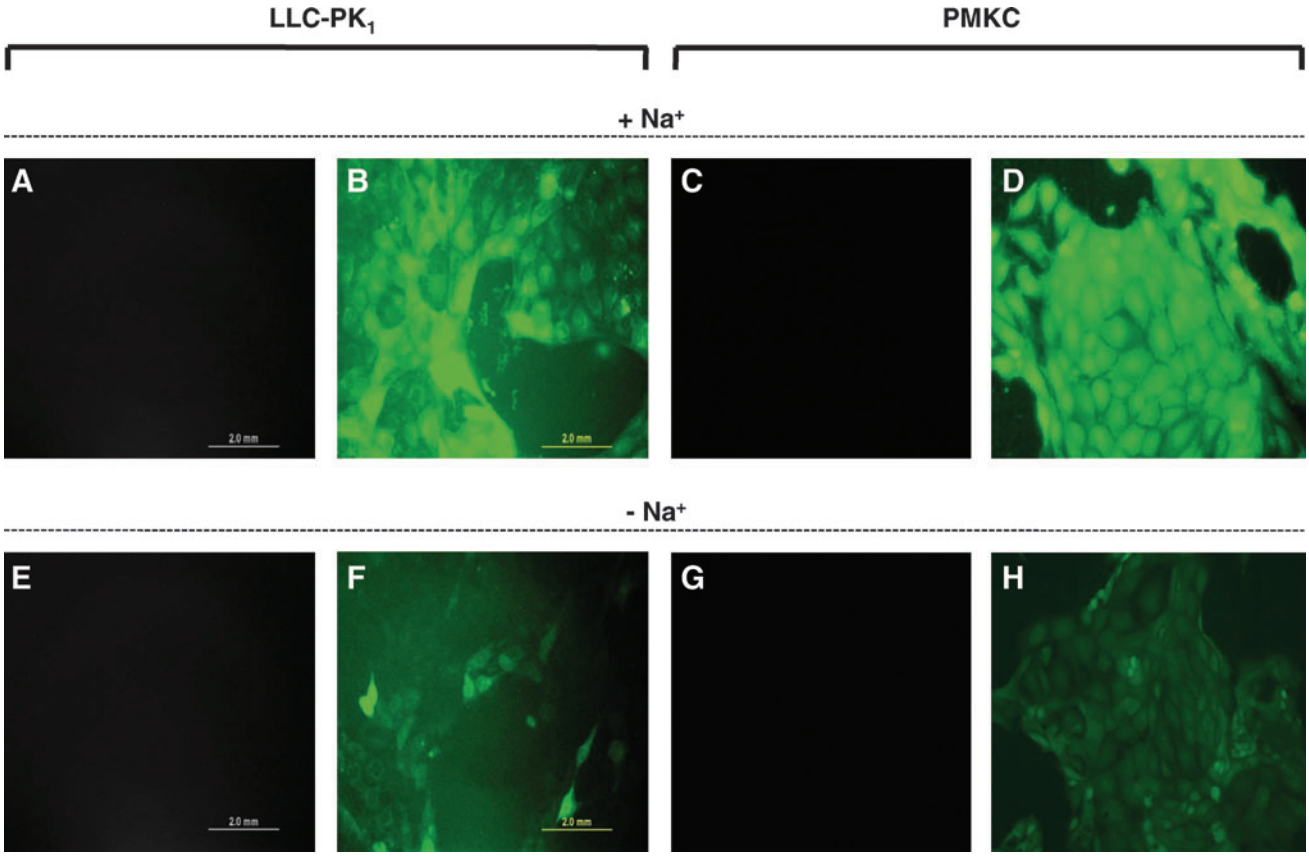

FIG. 2. Fluorescence imaging of 2-NBDG uptake. (**A**, **B**, **E**, and **F**) LLC-PK_1 cells and (**C**, **D**, **G**, and **H**) primary mouse kidney cells (PMKCs) were incubated for 1 h at 37°C in (**A**–**D**) Na^+ or (**E**–**H**) Na^+-free buffer in the presence (**B**, **D**, **F**, and **H**) or absence (**A**, **C**, **E**, and **G**) of 200 μM 2-NBDG. Images are taken at ×20 magnification.

measuring total uptake was measured after up to 75 min of incubation. The results in Figure 3A show that 2-NBDG uptake is concentration dependent; following incubation for 15 min or longer, the lowest $[2\text{-NBDG}]_i$ was observed in cells exposed to 50 μM substrate, and it dose-dependently increased as the concentration of 2-NBDG was increased to 200 μM. The data also show that uptake is time dependent and that the rate of transport remained linear for up to 60 min. The V_{max} of 2-NBDG transport was measured to be 0.05 nmol/min/μg of DNA (Fig. 3B).

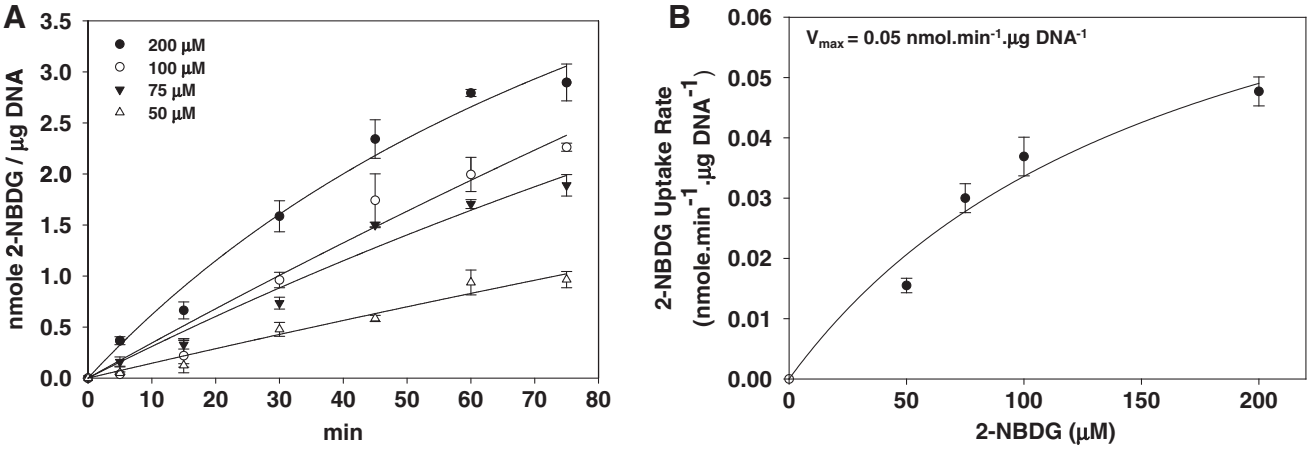

FIG. 3. Transport kinetics of 2-NBDG in LLC-PK_1 cells. (**A**) 2-NBDG transport rate. Cells were incubated with 50, 75, 100, or 200 μM 2-NBDG in Na^+ buffer at 37°C. The amount of transported 2-NBDG was measured in cells after incubation for 5, 15, 30, 45, 60, and 75 min, and values were normalized to the amount of DNA ($n = 3$). (**B**) The data shown in (**A**) were used to calculate the V_{max} value.

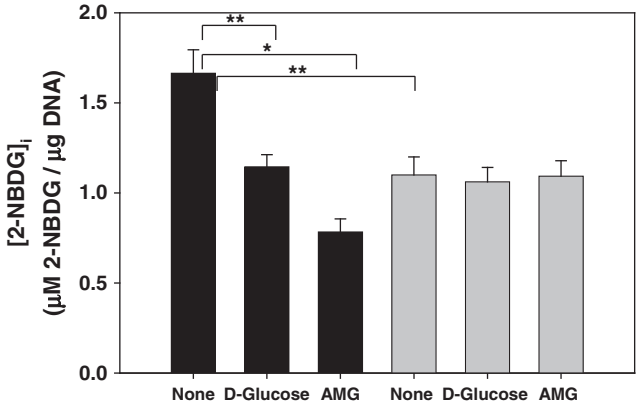

FIG. 4. Competition assay. LLC-PK_1 cells were incubated for 1 h at 37°C with 50 μM 2-NBDG in Na^+ and Na^+-free buffers in the presence or absence of 30 mM AMG or 30 mM D-glucose. Na^+-dependent (black columns) and Na^+-independent (gray columns) 2-NBDG transports were normalized to the amount of DNA, and intracellular concentration of 2-NBDG ($[2\text{-NBDG}]_i$) values were determined ($n = 2$–3). *$P < 0.001$, **$P = 0.02$.

Competition with SGLT substrate

Competition assays were performed with the SGLT substrate, AMG,[12] in LLC-PK_1 cells. The Na^+-dependent transport was measured in cells incubated with 2-NBDG (50 μM) alone or with 2-NBDG and AMG (30 mM). As the control, AMG was replaced with D-glucose (30 mM). Figure 4 shows that in the absence of a competitor, $[2\text{-NBDG}]_i$ was 1.7 $\mu M/\mu$g of DNA, and its level decreased to 1.1 $\mu M/\mu$g of DNA when D-glucose was also present. When AMG was used as the competitor, it reduced uptake by half decreasing the $[2\text{-NBDG}]_i$ to 0.8 $\mu M/\mu$g of DNA. In the absence of Na^+, AMG had no effect on 2-NBDG uptake; the $[2\text{-NBDG}]_i$ remained about 1 $\mu M/\mu$g of DNA, and 30 mM D-glucose did not block uptake of 50 μM 2-NBDG (Fig. 4, gray bars). These results support that competition by AMG was specific to the Na^+-dependent transport of 2-NBDG.

Inhibition by phlorizin

To further determine the role of SGLT in 2-NBDG uptake, the effect of the SGLT inhibitor, phlorizin, was investigated in LLC-PK_1 cells. Uptake experiments were performed in the presence and absence of Na^+ with 100 μM 2-NBDG and an equimolar concentration of phlorizin. As the control, the effect of the GLUT inhibitor, cytochalasin B, was also examined. The results in Figure 5A show that phlorizin strongly inhibited Na^+-dependent uptake, reducing $[2\text{-NBDG}]_i$ from 1.9 to 0.5 $\mu M/\mu$g of DNA, whereas exposure to ethanol, the vehicle for phlorizin, had no effect.

Control experiments showed that the Na^+-independent uptake of 2-NBDG was not affected by phlorizin (Fig. 5B). Also, while cytochalasin (10 μM) had no effect on Na^+-dependent uptake (Fig. 5A), it reduced $[2\text{-NBDG}]_i$ from 2.0 to 0.9 $\mu M/\mu$g of DNA when uptake was performed in the absence of Na^+ (Fig. 5B). Exposure to DMSO, the vehicle for cytochalasin, had no effect on the uptake.

Uptake by SGLT1 and SGLT2

Commercially available cytomegalovirus vectors for overexpression of each mouse SGLT protein (see Materials and Methods) were used to examine whether 2-NBDG is transported by SGLT1, SGLT2, or both. For verification purposes, nucleotide sequences of each SGLT clone were confirmed by DNA sequencing. BLAST analysis confirmed that these constructs encode proteins with amino acid sequences identical to mouse SGLT1 and SGLT2 with GenBank accession numbers NP_062784.3 and NP_573517.1, respectively.

Transient transfection was performed in COS-7 cells. Prior to transport analysis, protein overexpression was determined using our specific antibodies to the mouse SGLT1 (Fig. 6A and B) or SGLT2 (Fig. 6C and D). Immunocytochemistry showed

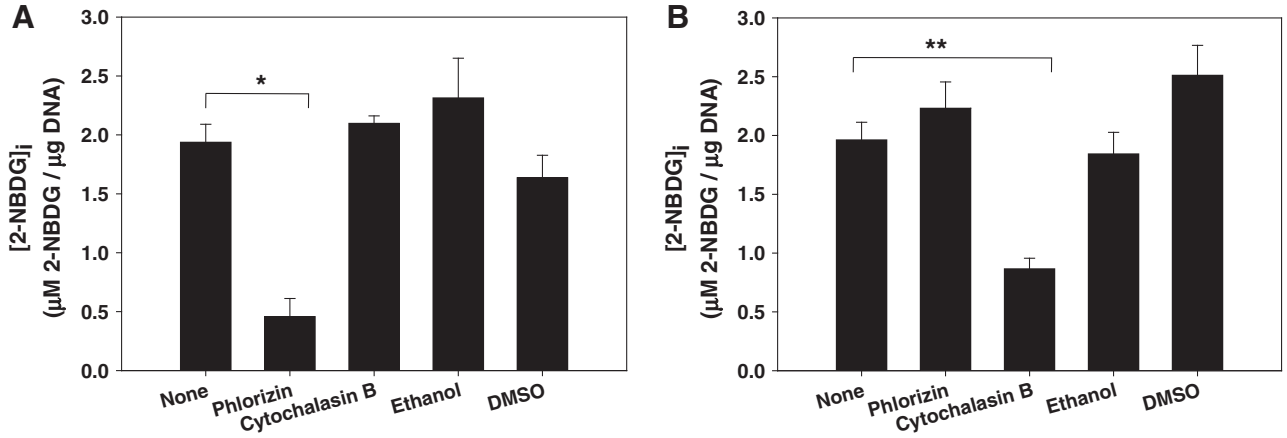

FIG. 5. Inhibition assay. Uptake was performed in LLC-PK_1 cells for 1 h at 37°C with 100 μM 2-NBDG in Na^+ and Na^+-free buffers in the presence or absence of 100 μM phlorizin. Phlorizin was replaced with 0.05% ethanol as the vehicle control. LLC-PK_1 cells were also pretreated for 5 min at 37°C with 10 μM cytochalasin B, and then uptake was performed with 100 μM 2-NBDG in Na^+ and Na^+-free buffers. Cytochalasin B was replaced with 0.001% DMSO as the vehicle control. (**A**) Na^+-dependent and (**B**) Na^+-independent 2-NBDG transports were normalized to the amount of DNA, and intracellular concentration of NBDG ($[2\text{-NBDG}]_i$) values were determined ($n = 3$–8). *$P < 0.001$, **$P = 0.006$.

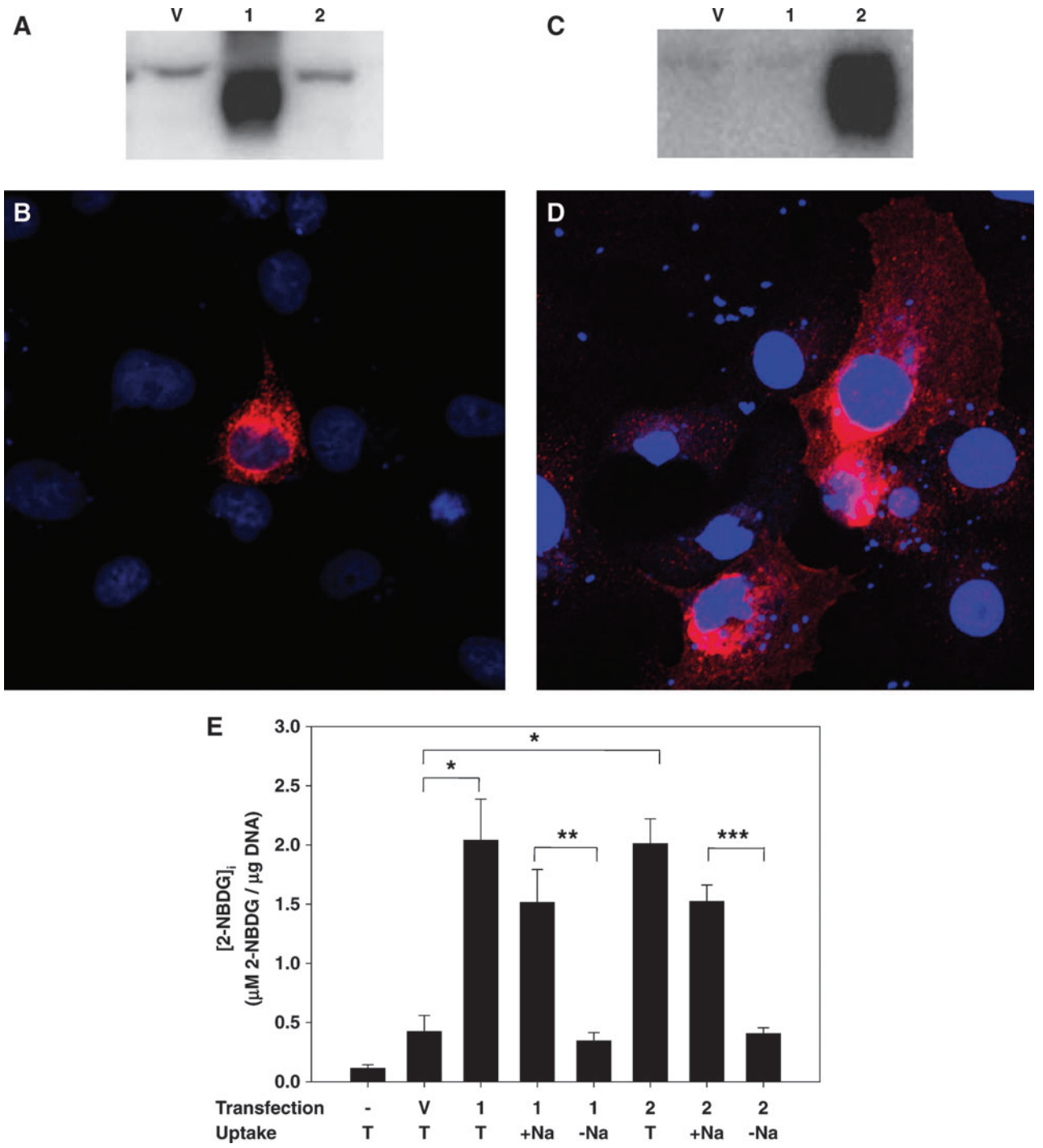

FIG. 6. 2-NBDG uptake by sodium-dependent glucose transporters SGLT1 and SGLT2. COS-7 cells were transiently transfected with vectors for overexpression of mouse SGLT1 or SGLT2. After 24–48 h, expression was analyzed using our custom antibody to (**A** and **B**) SGLT1 or (**C** and **D**) SGLT2. (**A** and **C**) Western blotting was performed on total protein from cell lysates. Immunocytochemistry and fluorescence confocal microscopy were performed on cells transfected with (**B**) SGLT1 or (**D**) SGLT2 vector. The original images without any modifications are presented. SGLT, red; DAPI, blue. (**E**) Uptake was performed 48 h post-transfection by incubating cells at 37°C with 200 μM 2-NBDG in the presence or absence of Na^+. Intracellular concentration of 2-NBDG ($[2\text{-NBDG}]_i$) values were measured after incubation for 30 min. Transfection with TurboFectin alone, SGLT1 vector, or SGLT2 vector is shown as V, 1, or 2, respectively ($n = 4$–6). Total, Na^+-dependent, and Na^+-independent uptakes are shown as T, +Na, and −Na, respectively. $^*P < 0.001$, $^{**}P = 0.002$, $^{***}P = 0.003$.

COS-7 cells expressed SGLT1 (Fig. 6B) and SGLT2 (Fig. 6D), and immunoblotting confirmed overexpression of each SGLT protein (Fig. 6A and C).

Uptake experiments were then performed in the presence and absence of Na^+ in SGLT-expressing cells as well as in cells treated with TurboFectin alone, the vehicle for transfection, and in untreated cells (Fig. 6E). In the presence of Na^+, $[2\text{-NBDG}]_i$ values in untreated and vehicle-treated COS-7 cells were 0.11 and 0.42 $\mu M/\mu g$ of DNA, respectively. Results showed that, compared with the vehicle control, total uptake of 2-NBDG increased by fivefold in cells expressing SGLT1 or SGLT2. Next, we determined the role of Na^+ in the above increased uptakes. In SGLT1-expressing cells, the Na^+-mediated transport resulted in $[2\text{-NBDG}]_i$ of 1.51 $\mu M/\mu g$ of DNA, which constituted 74% of the total uptake. Similarly, in SGLT2-expressing cells, Na^+-dependent uptake resulted in $[2\text{-NBDG}]_i$ of 1.52 $\mu M/\mu g$ of DNA, which constituted 76% of the total uptake. The Na^+-independent transport in SGLT1- and SGLT2-expressing cells resulted in $[2\text{-NBDG}]_i$ values of 0.34 and 0.41 $\mu M/\mu g$ of DNA, respectively, values that were similar to the level in vehicle control cells. Results show that 2-NBDG is transported by SGLT1 and SGLT2.

Discussion

Studies in patients and in animal models have suggested that diabetes can alter normal expression levels of SGLTs and GLUTs in the kidney.[4–8,22–24] So far, in vitro functional analyses on cultured PT cells[8] or on brush border membrane vesicles[25] from diabetic kidneys are performed with radioactive substrates. Also, the in vitro effectiveness of new antidiabetes drugs such as dapagliflozin and sergliflozin-A, which target SGLT2 activity, were investigated for their abilities to inhibit [^{14}C]AMG transport.[26,27]

We also were using [^{14}C]AMG to measure SGLT activity in LLC-PK_1 and PMKCs.[11,12] Earlier studies have shown that the in vitro Na^+-dependent and Na^+-independent glucose uptake properties of LLC-PK_1 cells were similar to that of PT cells in vivo.[28–30] Therefore, we used LLC-PK_1 as a model to investigate whether 2-NBDG can be used to measure glucose uptake in kidney cells by fluorescence. In general, advantages of 2-NBDG include use of fluorescence rather than radioactivity, measurement of a large number and a small volume of samples with a microplate reader (this study and Leira et al.[15]), and determining percentage of cells taking up glucose by flow cytometry,[31] as well as imaging (Fig. 2) or quantitative fluorescence microscopy.[19]

Because [^{14}C]AMG uptake buffers were compatible with 2-NBDG fluorescence (Fig. 1A), we used these same buffers for 2-NBDG uptake. The alkaline pH of the solution that we use to lyse cells after [^{14}C]AMG uptake interfered with fluorescence of 2-NBDG (Fig. 1B); therefore, we used an optimized buffer with pH 7.4 (Fig. 1C). Kinetic analysis showed that 2-NBDG transport was concentration and time dependent (Fig. 3).

By microscopic examinations of LLC-PK_1 cells and PMKCs, the fluorescence intensity appeared to be higher in cells incubated with 2-NBDG in the presence than in the absence of Na^+ (Fig. 2), suggesting that, in the presence of Na^+, total uptake of 2-NBDG was mediated by both SGLTs and GLUTs. Involvement of SGLTs in 2-NBDG transport was investigated in competition and inhibition studies. D-Glucose is a substrate for both SGLTs and GLUTs; however, AMG is transported only by SGLTs,[28,30] and its K_m value is 4.9 mM in LLC-PK_1 cells.[32] Competition assays in LLC-PK_1 cells showed that Na^+-dependent uptake of 2-NBDG decreased by half only when 600-fold molar excess AMG over 2-NBDG was present (Fig. 4). Consistently, 600-fold molar excess D-glucose competed with 2-NBDG and reduced its Na^+-dependent uptake by 35%. Transport inhibition studies in LLC-PK_1 cells provided further support for the role of SGLT in 2-NBDG uptake (Fig. 5). The presence of an equimolar concentration of phlorizin, a potent inhibitor of SGLTs,[16,33] strongly inhibited the Na^+-dependent uptake of 2-NBDG (100 μM) by 80%, whereas it had no effect on Na^+-independent uptake.

Expression of SGLT1 mRNA in LLC-PK_1 cells has been shown,[34] and we confirmed its expression in our cultures (data not shown). Expression of SGLT2 in these cells is not known. 2-NBDG was also transported by PMKCs (Fig. 2), which express mRNA of both SGLT1 and SGLT2.[20] To determine whether 2-NBDG is transported by one or both SGLTs, we measured 2-NBDG uptake in cells transiently overexpressing mouse SGLT1 or SGLT2 and showed that it is transported by both (Fig. 6). Similar to the results of our uptake experiments using [^{14}C]AMG, exposure of PMKCs or LLC-PK_1 cells to increasing concentrations of cadmium resulted in dose-dependent decreases in Na^+-dependent uptake of 2-NBDG (data not shown).

In LLC-PK_1 cells and PMKCs, 2-NBDG was also taken up in the absence of Na^+ (Figs. 2 and 4). The Na^+-independent transport of 2-NBDG could be blocked by the GLUT inhibitor, cytochalasin B, and was not affected by phlorizin (Fig. 5B). These data support that 2-NBDG is also transported by renal GLUTs.

In summary, we developed a microplate assay method to measure glucose transport in cultured kidney cells using fluorescent 2-NBDG and showed that 2-NBDG is transported by both SGLT1 and SGLT2. Therefore, our described method can potentially be used to measure the in vitro effectiveness of antidiabetes drugs that target SGLTs.

Acknowledgments

This study was supported in part by grants R01ES004026 and P30ES004184 from the National Institutes of Health (to D.H.P.) and a Pilot and Feasibility grant (to N.M.T.) that was funded by the parent grant P50DK079306. Also, funds from the State of Wisconsin Biomedical Technology Alliance grant LEG FY06-12368 (to N.M.T.) supported this study.

Author Disclosure Statement

The authors declare that no competing financial interests exist.

References

1. Bakris GL, Fonseca VA, Sharma K, Wright EM: Renal sodium-glucose transport: role in diabetes mellitus and potential clinical implications. Kidney Int 2009;75:1272–1277.
2. Wright EM: Renal Na^+-glucose cotransporters. Am J Physiol Renal Physiol 2001;280:F10–F18.
3. Wright EM, Loo DD, Hirayama BA, Turk E: Surprising versatility of Na^+-glucose cotransporters: SLC5. Physiology 2004;19:370–376.
4. Tabatabai NM, Sharma M, Blumenthal SS, Petering DH: Enhanced expressions of sodium-glucose cotransporters in

the kidneys of diabetic Zucker rats. Diabetes Res Clin Pract 2009;83:e27–e30.
5. Vestri S, Okamoto MM, de Freitas HS, Aparecida Dos SR, Nunes MT, Morimatsu M, Heimann JC, Machado UF: Changes in sodium or glucose filtration rate modulate expression of glucose transporters in renal proximal tubular cells of rat. J Membr Biol 2001;182:105–112.
6. Osorio H, Bautista R, Rios A, Franco M, Santamaria J, Escalante B: Effect of treatment with losartan on salt sensitivity and SGLT2 expression in hypertensive diabetic rats. Diabetes Res Clin Pract 2009;86:e46–e49.
7. Freitas HS, Anhe GF, Melo KF, Okamoto MM, Oliveira-Souza M, Bordin S, Machado UF: Na^+-glucose transporter-2 messenger ribonucleic acid expression in kidney of diabetic rats correlates with glycemic levels: involvement of hepatocyte nuclear factor-1alpha expression and activity. Endocrinology 2008;149:717–724.
8. Rahmoune H, Thompson PW, Ward JM, Smith CD, Hong G, Brown J: Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulin-dependent diabetes. Diabetes 2005;54:3427–3434.
9. Fine LG, Sakhrani LM: Proximal tubular cells in primary culture. Miner Electrolyte Metab 1986;12:51–57.
10. Schlatter P, Gutmann H, Drewe J: Primary porcine proximal tubular cells as a model for transepithelial drug transport in human kidney. Eur J Pharm Sci 2006;28:141–154.
11. Tabatabai NM, Blumenthal SS, Lewand DL, Petering DH: Mouse kidney expresses mRNA of four highly related sodium-glucose cotransporters: regulation by cadmium. Kidney Int 2003;64:1320–1330.
12. Blumenthal S, Lewand D, Krezoski SK, Petering DH: Comparative effects of Cd^{2+} and Cd-metallothionein on cultured kidney tubule cells. Toxicol Appl Pharmacol 1996;136:220–228.
13. Yoshioka K, Takahashi H, Homma T, Saito M, Oh KB, Nemoto Y, Matsuoka H: A novel fluorescent derivative of glucose applicable to the assessment of glucose uptake activity of *Escherichia coli*. Biochim Biophys Acta 1996;1289:5–9.
14. Roman Y, Alfonso A, Louzao MC, Vieytes MR, Botana LM: Confocal microscopy study of the different patterns of 2-NBDG uptake in rabbit enterocytes in the apical and basal zone. Pflugers Arch 2001;443:234–239.
15. Leira F, Louzao MC, Vieites JM, Botana LM, Vieytes MR: Fluorescent microplate cell assay to measure uptake and metabolism of glucose in normal human lung fibroblasts. Toxicol In Vitro 2002;16:267–273.
16. Ehrenkranz JR, Lewis NG, Kahn CR, Roth J: Phlorizin: a review. Diabetes Metab Res Rev 2005;21:31–38.
17. Estensen RD, Plagemann PG: Cytochalasin B: inhibition of glucose and glucosamine transport. Proc Natl Acad Sci U S A 1972;69:1430–1434.
18. Gaudreault N, Scriven DR, Laher I, Moore ED: Subcellular characterization of glucose uptake in coronary endothelial cells. Microvasc Res 2008;75:73–82.
19. Yamada K, Saito M, Matsuoka H, Inagaki N: A real-time method of imaging glucose uptake in single, living mammalian cells. Nat Protoc 2007;2:753–762.
20. Tabatabai NM, Blumenthal SS, Lewand DL, Petering DH: Differential regulation of mouse kidney sodium-dependent transporters mRNA by cadmium. Toxicol Appl Pharmacol 2001;177:163–173.
21. Tabatabai NM, Blumenthal SS, Petering DH: Adverse effect of cadmium on binding of transcription factor Sp1 to the GC-rich regions of the mouse sodium-glucose cotransporter 1, SGLT1, promoter. Toxicology 2005;207:369–382.
22. Brosius FC, Heilig CW: Glucose transporters in diabetic nephropathy. Pediatr Nephrol 2005;20:447–451.
23. Kamran M, Peterson RG, Dominguez JH: Overexpression of GLUT2 gene in renal proximal tubules of diabetic Zucker rats. J Am Soc Nephrol 1997;8:943–948.
24. Freitas HS, Schaan BD, David-Silva A, Sabino-Silva R, Okamoto MM, Alves-Wagner AB, Mori RC, Machado UF: SLC2A2 gene expression in kidney of diabetic rats is regulated by HNF-1α and HNF-3β. Mol Cell Endocrinol 2009;305:63–70.
25. Marks J, Carvou NJ, Debnam ES, Srai SK, Unwin RJ: Diabetes increases facilitative glucose uptake and GLUT2 expression at the rat proximal tubule brush border membrane. J Physiol 2003;553:137–145.
26. Meng W, Ellsworth BA, Nirschl AA, McCann PJ, Patel M, Girotra RN, Wu G, Sher PM, Morrison EP, Biller SA, Zahler R, Deshpande PP, Pullockaran A, Hagan DL, Morgan N, Taylor JR, Obermeier MT, Humphreys WG, Khanna A, Discenza L, Robertson JG, Wang A, Han S, Wetterau JR, Janovitz EB, Flint OP, Whaley JM, Washburn WN: Discovery of dapagliflozin: a potent, selective renal sodium-dependent glucose cotransporter 2 (SGLT2) inhibitor for the treatment of type 2 diabetes. J Med Chem 2008;51:1145–1149.
27. Pajor AM, Randolph KM, Kerner SA, Smith CD: Inhibitor binding in the human renal low- and high-affinity Na^+/glucose cotransporters. J Pharmacol Exp Ther 2008;324:985–991.
28. Mullin JM, Fluk L, Kleinzeller A: Basal-lateral transport and transcellular flux of methyl α-D-glucoside across LLC-PK_1 renal epithelial cells. Biochim Biophys Acta 1986;885:233–239.
29. Mullin JM, McGinn MT, Snock KV, Kofeldt LM: Na^+-independent sugar transport by cultured renal (LLC-PK_1) epithelial cells. Am J Physiol 1989;257:F11–F17.
30. Weiss ER, Amsler K, Dawson WD, Cook JS: Development of Na^+-dependent hexose transport in cultured renal epithelial cells (LLC-PK_1). Ann N Y Acad Sci 1985;456:420–435.
31. Dimitriadis G, Maratou E, Boutati E, Psarra K, Papasteriades C, Raptis SA: Evaluation of glucose transport and its regulation by insulin in human monocytes using flow cytometry. Cytometry A 2005;64:27–33.
32. Del Valle PL, Trifillis A, Ruegg CE, Kane AS: Characterization of glucose transport by cultured rabbit kidney proximal convoluted and proximal straight tubule cells. In Vitro Cell Dev Biol Anim 2002;38:218–227.
33. Diez-Sampedro A, Lostao MP, Wright EM, Hirayama BA: Glycoside binding and translocation in Na^+-dependent glucose cotransporters: comparison of SGLT1 and SGLT3. J Membr Biol 2000;176:111–117.
34. Clancey CJ, Lever JE: Differential regulation of three glucose transporter genes in a renal epithelial cell line. J Cell Physiol 2000;185:244–252.

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