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Glucose-Induced Regulation of NHEs Activity and SGLTs Expression Involves the PKA Signaling Pathway

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Abstract The effect of glucose on the intracellular pH (pH_i) recovery rate (dpH_i/dt) and Na^+-glucose transporter (SGLT) localization was investigated in HEK-293 cells, a cell line that expresses endogenous NHE1, NHE3, SGLT1, and SGLT2 proteins. The activity of the Na^+/H^+ exchangers (NHEs) was evaluated by using fluorescence microscopy. The total and membrane protein expression levels were analyzed by immunoblotting. In cells cultivated in 5 mM glucose, the pH_i recovery rate was 0.169 ± 0.020 ($n = 6$). This value did not change in response to the acute presence of glucose at 2 or 10 mM, but decreased with 25 mM glucose, an effect that was not observed with 25 mM mannitol. Conversely, the chronic effect of high glucose (25 mM) increased the pH_i recovery rate ($\sim 40\%$, $P < 0.05$), without changes in the total levels of NHE1, NHE3, or SGLT1 expression, but increasing the total cellular ($\sim 50\%$, $P < 0.05$) and the plasma membrane ($\sim 100\%$, $P < 0.01$) content of SGLT2. Treatment with H-89 (10^{-6} M) prevented the stimulatory effect of chronic glucose treatment on the pH_i recovery rate and SGLT2 expression in the plasma membrane. Our results indicate that the effect of chronic treatment with a high glucose concentration is associated with increased NHEs activity and plasma membrane expression of SGLT2 in a protein kinase A-dependent way. The present results reveal mechanisms of glucotoxicity and may contribute to understanding the diabetes-induced damage of this renal epithelial cell.

Keywords Glucose · Protein kinase A (PKA) · Sodium-glucose cotransporters · Na^+/H^+ exchangers

In healthy humans, more than 99% of the plasma glucose that filters through the renal glomerulus is reabsorbed in the proximal tubule (Deetjen et al. 1992; Moe et al. 2000). This reabsorption process is mediated by two classes of glucose transporters, the Na^+-glucose transporters (SGLTs) and the facilitative diffusion transporters (GLUTs) (Thorens 2001; Wright 2001). It is estimated that 90% of filtered renal glucose is reabsorbed through a high-capacity/low-affinity Na^+-glucose cotransporter, SGLT2, localized on the brush-border membrane of the epithelial cells lining in the S1 segment of the proximal renal tubule; the remaining 10% is reabsorbed through a low-capacity/high-affinity Na^+-glucose cotransporter, SGLT1, localized in the more distal S3 segment of the proximal tubule (Scheepers et al. 2004; Wright 2001). Two basolateral membrane glucose transporters facilitate the cellular glucose efflux: GLUT2, which is coexpressed with SGLT2 in the S1 segment of the renal tubule, and GLUT1, which is coexpressed with SGLT1 in the S3 segment (Rahmoune et al. 2005).

Previous in vitro and in vivo studies have demonstrated the participation of different cell signals, including protein kinase A (PKA), in the regulation of SGLT1 activity (Wright et al. 1997; Khoursandi et al. 2004; Hirsch et al. 2004; Kipp et al. 2003; Wright et al. 2003). In addition, Subramanian et al. (2009), using Chinese hamster ovary (CHO) cells stably expressing rbSGLT1, suggested an indirect (exocytosis) or direct (phosphorylation) effect of PKA on SGLT1.

On the other hand, Pontoglio et al. (2000) suggested that SGLT2 gene expression can be directly modulated by hepatocyte nuclear factor (HNF)-1α. These findings were

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confirmed by recent studies (Freitas et al. 2008), which demonstrated that diabetes increased HNF-1α mRNA and consequently SGLT2 protein expression.

It is known that there is a positive correlation between glucose levels and Na^+/H^+ exchangers (NHEs) activity (Ganz et al. 2000). The function of NHEs is fundamental to the maintenance of both intracellular pH and systemic $[Na^+]$. In mammals, multiple isoforms of NHE have been identified (NHE1–NHE10) with different tissue distributions and subcellular localizations (Orlowski and Grinstein 2004; Nakamura et al. 2005; Lee et al. 2008).

The NHE1 isoform is the housekeeping isoform of the exchanger and is ubiquitously expressed in the plasma membrane of virtually all cells. Its activity is required for a variety of physiological functions, including cell pH and volume regulation, and cell growth and proliferation (Grinstein et al. 1989; Slepkov et al. 2007). Another specialized isoform is NHE3, which is expressed almost exclusively on the apical membrane of epithelial cells. In the gut and kidney, NHE3 mediates the (re)absorption of salt, bicarbonate, and water (Lorenz et al. 1999). Previous studies have reported an inhibitory effect of adenosine 3′,5′-cyclic monophosphate (cAMP) on the Na^+/H^+ exchangers of some cell types, including the apical and basolateral exchangers in LLC-PK1 cells (Casavola et al. 1992) and the apical exchanger in OK cells (Cano et al. 1993; Casavola et al. 1992). The effect of cAMP on NHEs depends on at least two different factors: first, the primary sequence of NHE isoforms, and second, the existence of a cell-type-specific regulatory component(s) phosphorylated by a cAMP-dependent protein kinase. It is known that NHE1 (human ubiquitous isoform) is not activated by cyclic AMP/PKA (Borgese et al. 1994). However, a NHE1 mutant lacking the cAMP phosphorylation site becomes sensitive to cAMP inhibition upon its expression in the basolateral membrane of renal OK cells (Helmle-Kolb et al. 1993). In addition, Cabado et al. (1996) suggested the presence of cell-type-specific cofactors that regulate NHE isoforms, and Murtazina et al. (2007) showed that regulatory factor 1 (NHERF1) is required by PKA during inhibition of NHE3 in proximal tubule cells.

Considering the relevance of glucose in many physiological processes and its effects on the activity of SGLTs and NHEs (Ganz et al. 2000), the purpose of the current study was to investigate whether, in epithelial renal cells, the effect of high glucose on NHEs and SGLTs activity is associated with PKA signaling. To address this study, we used human embryonic kidney (HEK) cells, clone 293, expressing endogenous NHE1, NHE3, SGLT1 and SGLT2 proteins. Our results suggest that the effect of glucose on the pH_i recovery rate and SGLTs expression is associated with the same cell signaling response mediated by the PKA pathway.

Materials and Methods

Materials

The following materials were purchased: Dulbecco modified Eagle medium (DMEM) and penicillin–streptomycin (Gibco); TriZol LS Reagent kit (Life Technologies); N-methyl-D-glucamine (NMDG), mannitol, and protease inhibitor cocktail (Sigma); 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxy-methylester (BCECF-AM) and nigericin (Molecular Probes); anti-NHE1 and anti-SGLT2 antibodies (Abcam); H-89 (PKA inhibitor), anti-NHE3, and anti-SGLT1 antibodies (Chemicom); conjugated antibodies (anti-rabbit or anti-mouse) (Jackson Immuno Research Laboratories); chemiluminescence system and immunoblot reagents (Amersham Biosciences); and fetal bovine serum and trypsin solution (Cultilab). All other chemicals were obtained from Invitrogen or Sigma.

Cell Culture

HEK-293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured in DMEM (Gibco) with low or high glucose (5 and 25 mM, respectively) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L L-glutamine. Cells were grown at 37°C, pH 7.4 with 5% carbon dioxide in a CO_2 incubator. For each experiment, cells (passages 50–65) were cultured until 80–90% confluence.

Cells were treated for 20 days in culture media containing glucose 5 mM and/or 25 mM (chronic effect of treatment). For the fluorescent measurement of pH_i, confluent cells on glass coverslips were dyed by exposure to 15 μM of BCECF-AM for 20 min in low or high glucose solutions, as follows: low (in mM): NaCl 138, KCl 5, $MgSO_4$ 0.81, NaH_2PO_4 0.9, $CaCl_2$ 1.8, Hepes 10, NaOH 8, glucose 5, pH 7.4); or high (in mM): NaCl 136, KCl 5, $MgSO_4$ 0.81, NaH_2PO_4 0.9, $CaCl_2$ 1.8, Hepes 10, NaOH 5, glucose 25, pH 7.4. Then the glass coverslips were rinsed with low or high glucose solutions to remove the BCECF-AM-containing solution and were placed into a thermoregulated chamber mounted on an inverted epifluorescence microscope (Nikon, TMD). The measured area under the microscope had a diameter of 260 μm. The coverslips remained in a fixed position, and bathing solutions were rapidly exchanged without disturbing its position so that the same cells were studied throughout the experiment. All experiments were performed at 37°C. The cells were alternately excited at 440 or 495 nm with a 150 W xenon lamp and the fluorescence emission was monitored at 530 nm by a photomulitiplier-based fluorescence system (Georgia Instruments, PMT-400) at time intervals of 5 s. The 495/440 excitation ratio corresponds to a specific pH_i.

At the end of each experiment, the calibration of the BCECF signal was achieved using the high K^+-nigericin method (Thomas et al. 1979), exposing cells for 15 min to a high K^+ solution ([in mM] NaCl 20, KCl 130, $MgCl_2$ 1, $CaCl_2$ 1, Hepes 5) containing 10 μM nigericin adjusted to various pH values.

The cell pH recovery rate was examined after acid loading (NH_4Cl pulse technique; Boron and Weer 1976) by exposing cells for 2 min to 20 mM NH_4Cl solution containing low or high glucose (low glucose [in mM]: NaCl 140, KCl 5, $MgSO_4$ 0.81, NaH_2PO_4 0.9, $CaCl_2$ 1.8, Hepes 10, Glicose 5, NaOH 5, NH_4Cl 20, pH 8.0; high glucose [in mM]: NaCl 115, KCl 5, $MgSO_4$ 0.81, NaH_2PO_4 0.9, $CaCl_2$ 1.8, Hepes 10, Glicose 25, NaOH 5, NH_4Cl 20, pH 8.0). In acute treatment, the NH_4Cl solution was replaced by the following external situations: (1) low glucose solution alone, (2) Na^+-free solution (NaCl from low glucose solution was replaced by 134 mM NMDG; pH 7.4), (3) different glucose concentrations (2, 10, and 25 mM) and (4) glucose (5 or 25 mM) plus mannitol at the corresponding concentrations. However, in chronic treatment, the NH_4Cl solution was replaced by the following: (1) high glucose solution alone, (2) high glucose solution at different periods (24 or 48 h, or 5 or 20 days), and (3) high glucose solution plus H-89 (PKA inhibitor, 10^{-6} M) for 30 min at 37°C. In all experiments, the initial pH_i recovery rate (dpH_i/dt; pH units/min) was calculated during the first 2 min after acid loading by linear regression analysis.

Immunoblotting

Control or treated cells in the same conditions of the pH_i experiments were rinsed with ice-cold phosphate-buffered saline 1% and scraped from the plate with a rubber scraper. The cellular suspension was pelleted by centrifugation at $3000\times g$. The pellet was resuspended in 50 μl of sodium phosphate buffer (5 mM pH 8.0) plus protease inhibitor cocktail. All protein concentrations were determined by Micro BCA assay (Pierce). Samples containing total proteins were diluted 1:1 in buffer A (Tris–HCl 62.5 mM pH 6.8, sodium dodecyl sulfate [SDS] 2%, glycerol 20%, β-mercaptoethanol 1.96%, bromophenol blue 0.05%) plus protease inhibitor cocktail, resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membrane. After blocking with 5% nonfat milk plus Tween-20, 0.1%, for 1 h, blots were probed in the same buffer overnight at 4°C with anti-NHE1 (1:1000), anti-NHE3 (1:1000), anti-SGLT1 (1:3000), or anti-SGLT2 (1:1000) antibodies. Blots were washed in 0.05% Tween-20 five times for 10 min and then incubated with secondary antibodies (anti-rabbit for anti-SGLT1 [1:5000], and anti-mouse for anti-SGLT2 [1:2000], anti-NHE3 [1:2000] and anti-NHE1 [1:2000] for 1 h at room temperature. Blots were washed as described above and then visualized with an enhanced chemiluminescence kit. To evaluate the specificity of anti-SGLT2 antibody, we used as a negative control Caco-2 (human colonic adenocarcinoma) cells, which express only SGLT1 (Kipp et al. 2003).

Cell Surface Biotinylation

Biotinylation of cell surface proteins was performed on HEK-293 cells grown in six-well plates. Experiments were performed on control or chronically treated cells with or without H-89 (10^{-6} M) for 30 min at 37°C. Then the HEK-293 cells were incubated with 1.5 mg/ml EZ-Link-Sulpho-NHS-SS-Biotin (Pierce) in buffer (in mM: NaCl-150, triethanolamine-10, $CaCl_2$ 2, pH 7.4) for 60 min at 4°C and then incubated for 40 min at 4°C in quenching solution (in mM: glycine 100, NaCl 150, NaH_2PO_4 0.9, $CaCl_2$ 0.1, $MgCl_2$ 1, pH 7.4) to stop cell biotinylation. Then the cells were lysed with radioimmunoprecipitation buffer assay (RIPA) buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100; pH 7.4), supplemented with protease and phosphatase inhibitors for 60 min at 4°C, and centrifuged at $14{,}000 \times g$ for 10 min at 4°C. The lysates were incubated with 100 μl of streptavidin beads overnight at 4°C. The pellets were washed three times with cold RIPA buffer, resuspended in 50 μl of Laemmli sample buffer (Tris–HCl 62.5 mM, pH 6.8, SDS 2%, glycerol 20%, β-mercaptoethanol 1.96%, bromophenol blue 0.05%, plus protease inhibitor cocktail) and centrifuged at $14{,}000 \times g$ for 2 min. The resulting supernatant (biotinylated fraction) was separated by SDS-PAGE and immunoblotted with SGLT1 (1:3000) or anti-SGLT2 (1:1000) antibodies.

Statistical Analysis

The results related to pH_i recovery rate are presented as means ± standard error (SE) of 4–10 experiments. Data were statistically analyzed by one-way analysis of variance, followed by the Bonferroni post hoc test. Differences were considered significant if $P < 0.05$.

Results

Measurement of pH_i by Fluorescence

Our results indicate that HEK-293 cells in a HCO_3^--free solution have a mean pH_i baseline of 7.18 ± 0.01 ($n = 182$). Figure 1 shows a representative experiment of pH_i recovery (Fig. 1a) and the mean of pH_i recovery rate in the first 2 min after acid loading (Fig. 1b). Our results indicate that in the control situation (glucose 5 mM), the mean pH_i recovery rate after acid loading was

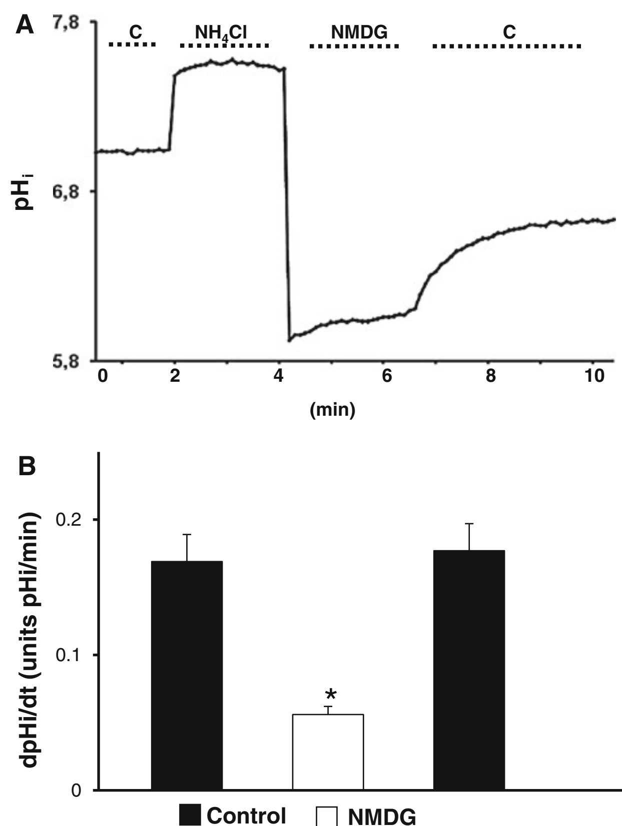

Fig. 1 (**a**) Representative experiment of pH_i recovery in the first 2 min after acid loading HEK-293 cells in the presence of Na^+-control solution or Na^+-free solution. (**b**) pH_i recovery rate of 6 experiments in the same conditions as mentioned above. C = Na^+-control solution (138 mM Na^+). N-methyl-D-glucamine (NMDG) 134 mM = Na^+-free solution. * $P < 0.05$ vs. controls

0.169 ± 0.020 ($n = 6$) pH units/min. In the presence of Na^+-free solution, this value was significantly decreased (0.056 ± 0.006 [$n = 6$] pH units/min) and returned to basal level in the presence of extracellular Na^+ solution (0.177 ± 0.020 [$n = 6$] pH units/min].

Effect of Acute Treatment with Glucose on the pH_i Recovery Rate

Considering that glucose can modulate the activity of NHE1 (Ganz et al. 2000), after acid loading, we treated the cells with different concentrations of glucose. The acute treatment with glucose modified the activity of NHEs only at 25 mM, at which point a significant decrease ($P < 0.05$) in the pH_i recovery rate was observed (Fig. 2 and Table 1).

Effect of Acute Treatment with Mannitol on the pH_i Recovery Rate

To confirm the effect of glucose (25 mM) on the activity of NHEs, we used mannitol, an impermeable and not

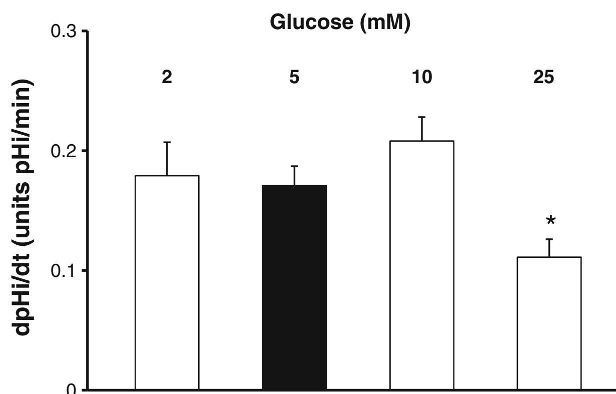

Fig. 2 Effect of acute treatment with glucose (2, 5, 10 and 25 mM) on the pH_i recovery rate after acid loading in HEK-293 cells. * $P < 0.05$ vs. control (glucose 5 mM)

Table 1 Effect of different glucose concentrations (2, 10, and 25 mM) on pH_i recovery rate of HEK-293 cells

Glucose (mM)	dpHi/d*t* (pH units/min)
2	0.179 ± 0.016 (7)
5	0.171 ± 0.020 (7)
10	0.208 ± 0.015 (10)
25	0.111 ± 0.011 (8)*

Values are means ± SE; number of experiments is shown in parentheses. dpH_i/dt, recovery rate in the first 2 min after acid load

* $P < 0.05$ vs. glucose 2, 5, and 10 mM

metabolized solute. As shown in Fig. 3, treatment with 5 mM mannitol did not modify the pH_i recovery rate when compared with 5 mM glucose control (control: 0.179 ± 0.016; $n = 7$ vs. mannitol: 0.197 ± 0.024; $n = 8$). However, the effect of 25 mM mannitol was different from the observed effect of treatment with 25 mM glucose (glucose: 0.111 ± 0.011; $n = 8$ vs. mannitol: 0.173 ± 0.024; $n = 4$), thus confirming that glucose regulation was not related to its osmotic effect.

Effect of Chronic Treatment with 25 mM Glucose on the pH_i Recovery Rate

Once high glucose treatment acutely decreased the pH_i recovery rate, we analyzed the effect of chronic treatment with high glucose (25 mM) on NHE activity in different periods. As shown in Fig. 4 and Table 2, a time-dependent progressive increase of pH_i recovery rate was observed, which reached a maximal effect in 20 days.

Effect of Chronic Treatment with Glucose on the pH_i Recovery Rate is Mediated by PKA

To investigate the participation of PKA on NHEs activity during chronic treatment with glucose, cells chronically

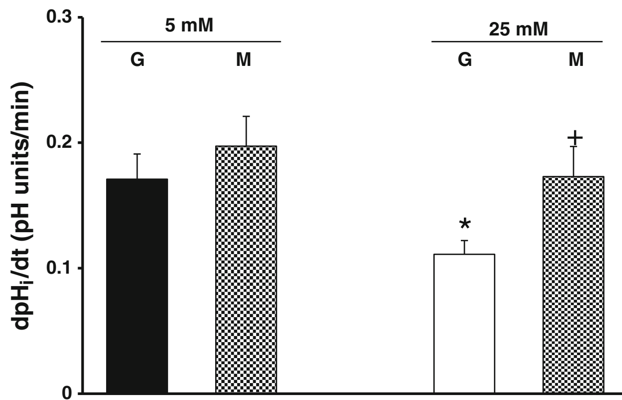

Fig. 3 Effect of acute treatment with mannitol (5 and 25 mM) on the pH_i recovery rate after acid loading in HEK-293 cells. Results are the means of 6–9 experiments. * $P < 0.05$ vs. respective control, and $^+$ $P < 0.05$ vs. glucose (25 mM). M = mannitol, G = glucose

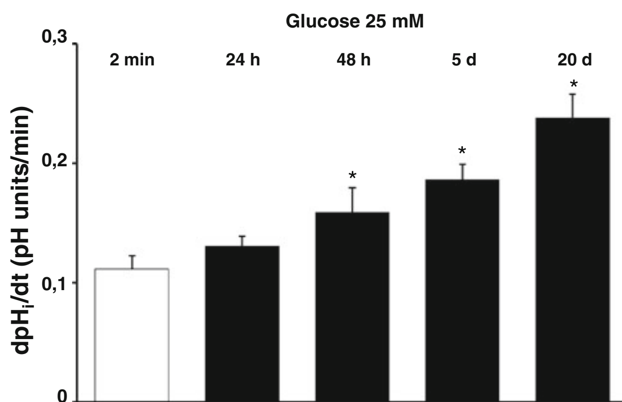

Fig. 4 Time-dependent effect of glucose 25 mM on the pH_i recovery rate after acid loading (24 h: 17.1%, 48 h: 43.2%, 5 days: 67.6% and 20 days: 114.4%) from 2 min. * $P < 0.05$ vs. 2 min

treated with 5 or 25 mM glucose were incubated with H-89 (10^{-6} M), a PKA inhibitor. As shown in Fig. 5 and Table 3, H-89 did not affect the basal pH_i recovery rate. However, it suppressed the stimulatory chronic effect of 25 mM glucose on the same parameter.

Effect of Chronic Treatment with Glucose on NHEs and SGLTs Expression

Considering the stimulatory effect of chronic treatment with glucose on the activity of NHEs, we analyzed the expression of NHEs and SGLTs in the same experimental condition. Chronic treatment with glucose did not affect the expression of NHE1 (Fig. 6a, b) or NHE3 (Fig. 6c, d). Chronic treatment with glucose did not affect the expression of SGLT1 (Fig. 7a, b) when compared to its control, whereas it increased the expression of SGLT2 (Fig. 7c, d) when compared to its control.

Table 2 Time-dependent manner effect of glucose (25 mM) on pH_i recovery rate of HEK-293 cells

Glucose (25 mM)	dpHi/d*t* (pH units/min)
Control (2 min)	0.111 ± 0.011 (8)
24 h	0.130 ± 0.009 (10)
48 h	0.159 ± 0.020 (7)*
5 days	0.186 ± 0.013 (8)*
20 days	0.238 ± 0.020 (8)*

Values are means ± SE; number of experiments is shown in parentheses. dpH_i/dt, recovery rate in the first 2 min after acid load

* $P < 0.05$ vs. 2 min

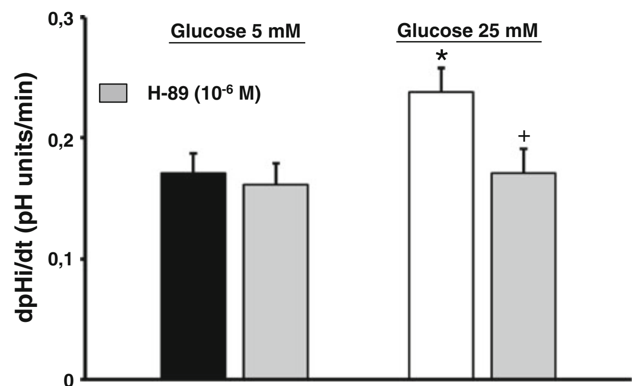

Fig. 5 Effect of H-89 (10^{-6} M) on the pH_i recovery rate in control (glucose 5 mM) or after chronic treatment (glucose 25 mM). * $P < 0.05$ vs. control without H-89, and $^+$ $P < 0.05$ vs. glucose 25 mM without H-89

Table 3 Chronic effect of glucose on the pH_i recovery rate mediated by PKA

Glucose (mM)	dpHi/d*t* (pH units/min)	
	Control	H-89 (10^{-6} M)
5	0.171 ± 0.020 (7)	0.161 ± 0.018 (7)
25	0.238 ± 0.020 (8)*	0.171 ± 0.020 (8)†

Values are means ± SE; number of experiments is shown in parentheses. dpH_i/dt, recovery rate in the first 2 min after acid load

* $P < 0.05$ vs. control (glucose 5 mM)

† $P < 0.05$ vs. glucose 25 mM

PKA Activation Modulates the Amount of SGLTs in the Plasma Membrane

Figure 8 shows the immunoblotting of biotinylated membrane fractions. Treatment with a high glucose concentration increased the expression of SGLT1 in the plasma membrane in comparison to control, and H-89 did not modify this effect. In addition, the same treatment also

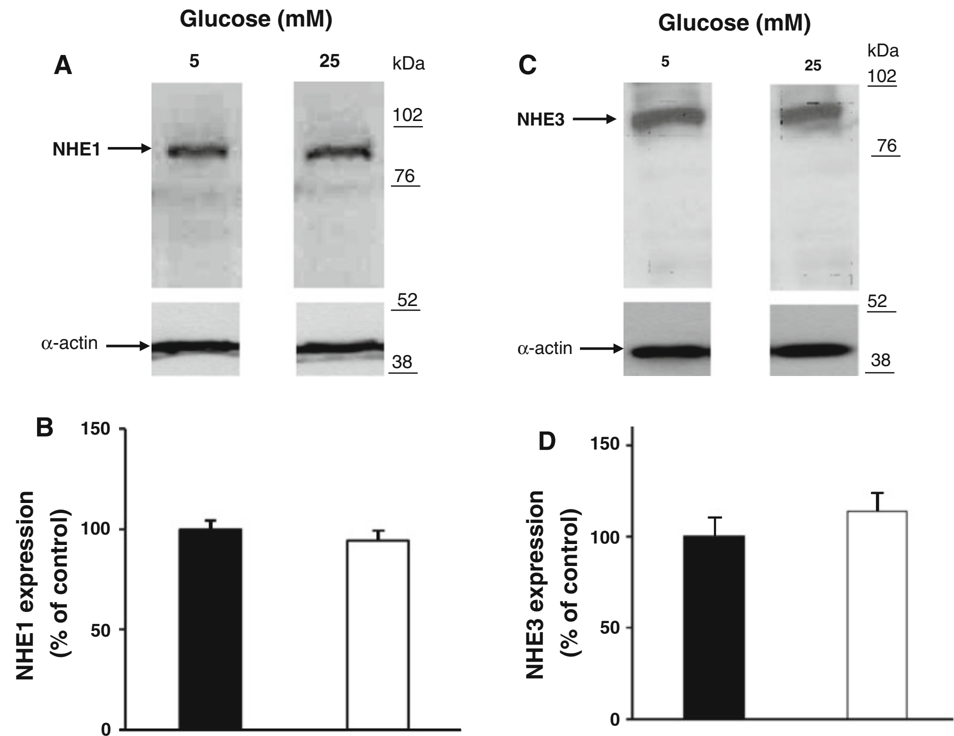

Fig. 6 Effect of chronic treatment with glucose on NHEs expression. (**a** and **c**) Immunoblotting with 50–100 μg total protein from HEK-293 cells. Blots were probed with NHE1, NHE3 and α-actin antibodies, which recognized the endogenous NHE1 (protein with 91 kDa), NHE3 (protein with 90 kDa) and α-actin with 45 kDa, respectively. (**b** and **d**) Graphs with the mean of three experiments are shown

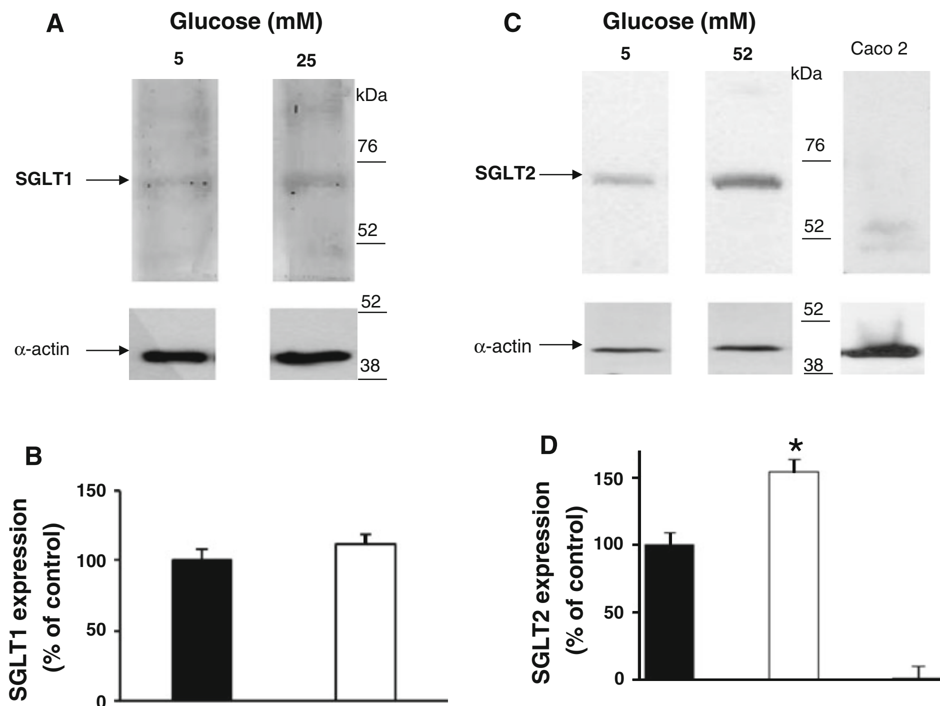

Fig. 7 Effect of chronic treatment with glucose on SGLTs expression. (**a** and **c**) Immunoblotting with 50–100 μg total protein from HEK-293 cells. Blots were probed with SGLT1 (1:3000) or SGLT2 (1:1000) or α-actin (1:2000) antibodies, which recognized the endogenous SGLT1 (protein with 70 kDa), SGLT2 (protein with 73 kDa) and α-actin with 45 kDa, respectively. The negative control of SGLT2 expression was analyzed in Caco 2 cells (an intestinal cell line that express only SGLT1). (**b** and **d**) Graphs with the mean of three experiments are shown. * $P < 0.05$ vs. control (glucose 5 mM)

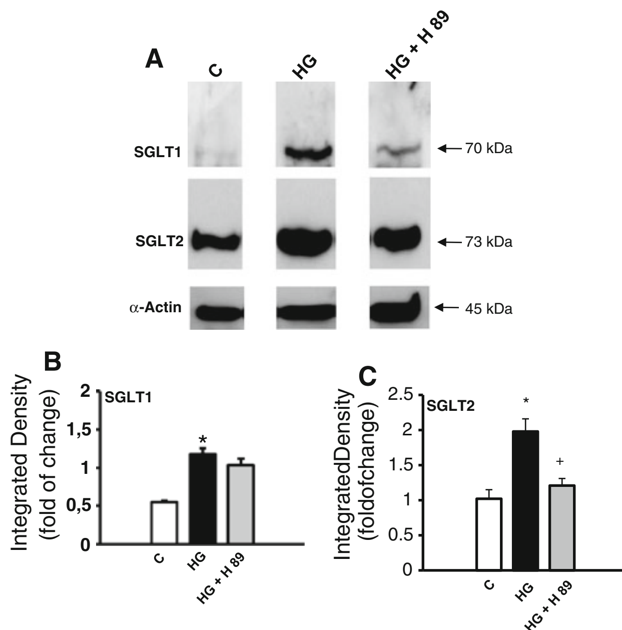

Fig. 8 Effect of chronic treatment on plasma membrane and biotinylated fractions of HEK-293 cells. Cells were treated with high glucose and/or H-89 (10^{-6} M) and after biotinylation experiments, 50 µg of proteins were resolved by 10% SDS-polyacrylamide gel and the resultant blots were probed with SGLT1 (1:3000) or SGLT2 (1:1000) antibodies, which recognized the SGLT1 (70 kDa), SGLT2 (73 kDa). The positive control was obtained using samples of supernatant to probe with α-actin antibody 1:2000, which recognized α-actin (45 kDa) (**a**). Graphs (**c** and **b**) with the mean of three experiments are shown. * $P < 0.05$ vs. control (glucose 5 mM) and $^+$ $P < 0.05$ vs. glucose (25 mM)

increased the expression of SGLT2 in the plasma membrane, which was reversed with H-89.

Discussion

The particular role of PKA on NHEs activity or SGLT1 expression and/or recycling has been extensively suggested (Khoursandi et al. 2004; Kipp et al. 2003; Wright et al. 1997, 2003; Subramanian et al. 2009; Pontoglio et al. 2000; Freitas et al. 2008; Lee et al. 2008; Cano et al. 1993; Casavola et al. 1992; Helmle-Kolb et al. 1993). However, PKA activation during high glucose conditions, as well its participation in coordinate cell signaling to both SGLTs and NHEs, is not completely elucidated. Thus, we analyzed the effect of chronic treatment with glucose on the activity of NHEs and/or SGLTs expression mediated by PKA in HEK-293 cells. To our knowledge, the present report is the first study concerning the direct chronic effect of glucose on the activity of NHEs and SGLTs modulated by PKA signaling.

Our data demonstrate that HEK-293 cells, which endogenously express NHE1 and NHE3, maintain a mean baseline pH_i of 7.18 ± 0.01 ($n = 182$) in the presence of HCO_3^--free solution in the same way as other cell lines, such as MDCK cells, endogenously express NHE1 protein (Sardet et al. 1989; Oliveira-Souza and Mello-Aires 2000).

The activity of NHEs was estimated by measuring the pH_i recovery rates in the first 2 min after acid loading with NH_4Cl solution because in HEK-293 cells this parameter was mostly dependent on Na^+/H^+ exchanger. However, only a slight pH_i recovery rate was observed, probably due to Na^+-independent proton extrusion mechanisms, including H^+-ATPase (Lang et al. 2003).

Our results indicate that in HEK-293 cells, a high glucose concentration (25 mM) acutely decreased the pH_i recovery rate. The mechanism by which HEK-293 cells sense and respond to variations in glucose concentrations remains unclear. Nevertheless, the activity of many Na^+/H^+ exchangers can be associated with many cell signaling factors, including osmolarity, growth factors, protein kinase C and PKA (Shigekawa and Pouyssegur 1997). It is known that G protein-coupled receptors (GPCRs) are important for various cellular responses induced by different agents. In addition, a new class of nutrient-sensing GPCR has been identified and designated as Gpr1 receptor. In the yeast *Saccharomyces cerevisiae,* Gpr1 is a glucose-sensing receptor that can couple to Gpa2, a G-protein alpha subunit, and activate the cAMP-PKA pathway (Van Dijck 2009). Considering that renal cells express endogenous GPR1 (Tokizawa et al. 2000), we suggest that the acute and inhibitory effect of glucose on the activities of NHEs depends on the GPR1/PKA activity, which we will analyze in future research.

Because glucose has a powerful osmotic effect, we treated the cells with mannitol, which can mimic the osmotic effect of glucose. At high concentration (25 mM), mannitol has no effect on the activity of NHEs, highlighting that the effect of 25 mM glucose was not a consequence of its osmotic effect. Our results with HEK-293 cells extend these findings because under normal tonicity (300 mOsm), high glucose progressively increased the pH_i recovery rate, with a maximal effect in 20 days, although it did not affect the expression of NHE1 or NHE3. These data are in accordance with Ganz et al. (2000), who showed that mesangial cells subcultured for 2 weeks in high glucose media presented a significant increase in NHE1 activity. In addition, altered Na^+/H^+ exchanger activity, without changes in the expression of NHEs, can be explained by subcellular signaling mechanisms, such as nonenzymatic glycation process, oxidative-reductive stress, aldose-reductase activation, and/or GPCR activation, which induce protein kinase C and PKA activation (Kim et al. 2003; Mosley et al. 2003). In the present study, treatment with H-89 (a PKA inhibitor) prevented the stimulatory effect of chronic high glucose treatment on the pH_i

recovery rate, revealing that a high glucose concentration modulates the activity of NHEs in a PKA-mediated mechanism. Our findings differ from some previous studies (Casavola et al. 1992; Slepkov et al. 2007) that showed an inhibitory effect of PKA on the activity of NHEs. Nevertheless, they used other cell lines, such as LLCPK1 and OK cells from the proximal tubule with short-time hormonal (calcitonin and vasopressin) treatment. Under these conditions, it is likely that other cell-type-specific regulatory elements were present.

Considering that both NHEs and SGLTs depend on a favorable Na^+ electrochemical gradient, we hypothesized that the stimulatory effect of PKA on the pH_i recovery rate was related to changes in expression or activity of SGLTs. Our results show that chronic treatment with high glucose increased the total levels of SGLT2 but not SGLT1 expression. The glucose-induced increase in SGLT2 expression is in accordance with the observations by Freitas et al. (2008) in the kidneys of diabetic rats. Because the total level of SGLT2 expression was increased during chronic treatment, we hypothesized that the effect of PKA on the pH_i recovery rate might also involve changes in SGLTs translocation, modifying its activity and consequently the NHEs in the plasma membrane. Thus, we performed cell-surface biotinylation experiments to detect SGLTs. Chronic treatment increased the translocation rate of both SGLT1 a SGLT2 to the plasma membrane, highlighting the effective participation of these cotransporters in cellular glucose disposal. Interestingly, H-89 blocked the chronic treatment-induced increase in the plasma membrane levels of SGLT2, indicating that PKA modulates SGLT2 translocation.

Dyer et al. (2003), using intestinal cells that express only SGLT1, demonstrated that high glucose treatment stimulated SGLT1 expression via PKA activation. Nevertheless, our current cell model expresses both SGLT1 and SGLT2, and under this condition, our results suggest a powerful modulatory effect of PKA on SGLT2 expression and/or translocation to plasma membrane.

In conclusion, the present study shows that in HEK-293 cells, high glucose levels modulate the activity of NHEs in a time-dependent manner. Acutely, glucose inhibits but chronically it stimulates the activity of NHEs; the latter is accompanied by increased PKA phosphorylation. Moreover, in addition to the effect on the activity of NHEs, the results show that PKA also modulates expression and translocation of SGLTs, which may be essential to sense the high glucose concentration. The present results reveal mechanisms of glucotoxicity and may contribute to the understanding of diabetes-induced damage of renal epithelial cells.

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