Functional Role of Glucose Metabolism, Osmotic Stress, and Sodium-Glucose Cotransporter Isoform-Mediated Transport on Na⁺/H⁺ Exchanger Isoform 3 Activity in the Renal Proximal Tubule

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ABSTRACT

Na⁺-glucose cotransporter 1 (SGLT1)-mediated glucose uptake leads to activation of Na⁺-H⁺ exchanger 3 (NHE3) in the intestine by a process that is not dependent on glucose metabolism. This coactivation may be important for postprandial nutrient uptake. However, it remains to be determined whether SGLT-mediated glucose uptake regulates NHE3-mediated NaHCO₃ reabsorption in the renal proximal tubule. Considering that this nephron segment also expresses SGLT2 and that the kidneys and intestine show significant variations in daily glucose availability, the goal of this study was to determine the effect of SGLT-mediated glucose uptake on NHE3 activity in the renal proximal tubule. Stationary in vivo microperfusion experiments showed that luminal perfusion with 5 mM glucose stimulates NHE3-mediated bicarbonate reabsorption. This stimulatory effect was mediated by glycolytic metabolism but not through ATP production. Conversely, luminal perfusion with 40 mM glucose inhibited NHE3 because of cell swelling. Notably, pharmacologic inhibition of SGLT activity by Phlorizin produced a marked inhibition of NHE3, even in the absence of glucose. Furthermore, immunofluorescence experiments showed that NHE3 colocalizes with SGLT2 but not SGLT1 in the rat renal proximal tubule. Collectively, these findings show that glucose exerts a bimodal effect on NHE3. The physiologic metabolism of glucose stimulates NHE3 transport activity, whereas, supraphysiologic glucose concentrations inhibit this exchanger. Additionally, Phlorizin-sensitive SGLT transporters and NHE3 interact functionally in the proximal tubule.


The kidney proximal tubule (PT) is the site where the reabsorption of approximately 70% of filtered sodium bicarbonate occurs. It is mainly performed by the Na⁺/H⁺ exchanger isoform 3 (NHE3).¹ The physiologic importance of NHE3 became evident after the development of NHE3 knockout mice, which presented mild metabolic acidosis and volume depletion with reduced BP, underscoring the role of NHE3 in volume homeostasis.²

It has been shown that NHE3 physically and functionally interacts with dipeptidyl-peptidase IV, an enzyme that degrades and inactivates the incretin hormone glucagon-like peptide-1.³ The inhibition of dipeptidyl-peptidase IV and the action of glucagon like peptide-1 were shown to inhibit NHE3 and promote natriuresis.³–⁵ Additionally, various conditions and substances related to glucose metabolism, including diabetes, insulin, ATP, and glucose, modulate NHE3 in different tissues.

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showing a close relationship between carbohydrate homeostasis and NHE3 activity.\textsuperscript{9–12}

Plasma glucose concentration is maintained at a constant level by a complex system, in which the kidneys perform a pivotal role by reabsorbing all the filtered glucose in the PT.\textsuperscript{13} In addition, the kidneys and liver are the only organs that express the glucose-6-phosphatase enzyme, thus enabling them to perform gluconeogenesis.\textsuperscript{14,15} This enzyme is only expressed in the PT,\textsuperscript{16} highlighting the importance of this kidney segment in carbohydrate metabolism.

It has been shown that the kidneys metabolize 20% of the glucose consumed in a meal.\textsuperscript{14} The PT has a low expression of hexokinase but the highest concentration and activity of glucose-6-phosphate dehydrogenase, indicating that this segment is able to metabolize glucose.\textsuperscript{16,17} However, it is currently believed that the PT uses noncarbohydrate compounds as energy sources.\textsuperscript{17}

With relation to glucose uptake, the majority of filtered glucose is reabsorbed by the low-affinity, high-capacity sodium-glucose cotransporter isoform 2 (SGLT2). Some glucose is also reabsorbed by the high-affinity, low-capacity sodium-glucose cotransporter isoform 1 (SGLT1).\textsuperscript{13} Recently, SGLT2 inhibitors have been approved for the treatment of hyperglycemia in diabetic patients. The use of these inhibitors has been shown to decrease blood glucose, glycated hemoglobin, postprandial glucose, insulinemia, and body weight.\textsuperscript{18–20}

The role of glucose uptake in the modulation of NHE3 activity in the small intestine has been extensively studied. Experiments have shown that glucose uptake through SGLT1 promotes intracellular NHE3-dependent alkalinization.\textsuperscript{21–26} However, functional differences between intestinal and renal NaHCO\textsubscript{3} reabsorption have not been established. These two systems differ physiologically, because the gastrointestinal system is exposed to fluctuations in glucose concentration between the periods of fasting and after meals.\textsuperscript{13} The presence of large amounts of solutes within the intestinal cells after meals modulates membrane transporters, such as glucose transporter 2 (GLUT2) and NHE3,\textsuperscript{21,27} an important process for nutrient absorption.

Although the synergistic activation between SGLT1 and NHE3 has been observed in the intestine,\textsuperscript{21} it is not known if this process also occurs in the kidneys. Considering that the kidneys also express SGLT2 and the particularities of glucose availability in this organ, the goal of the present work was to determine the effect of glucose and SGLT activity on NHE3 in the renal PT.

RESULTS

Glucose Modulates NHE3-Dependent JHCO\textsubscript{3} in the Renal PT

As an initial approach to study the effect of glucose on NHE3-mediated bicarbonate reabsorption, Wistar rats were subjected to stationary microperfusion \textit{in vivo}, and their PTs were perfused with solutions containing different concentrations of glucose or a control solution (CTRL; solution without any transported sugar) (Concise Methods).

As shown in Figure 1A, the rate of bicarbonate flux (JHCO\textsubscript{3}) in the PTs perfused with 5 mM glucose (GLU) was significantly higher than in CTRL-perfused tubules (1.903±0.083 versus 2.779±0.093 nmol/cm\textsuperscript{2} per second). A progressive inhibition of JHCO\textsubscript{3} was found after the perfusion of higher glucose concentrations (1.125±0.13 and 1.25±0.16 nmol/cm\textsuperscript{2} per second for GLU40 and GLU60, respectively). Because the stimulatory effect was observed only on perfusion of GLU5 and the maximum inhibitory effect occurred on GLU40 perfusion, these concentrations were used in all the following experiments. Representative curves of pH changes are given in Supplemental Figure 1.

![Figure 1](image-url)
To confirm that the glucose-dependent $\mathrm{JHCO}_3^-$ modulation was NHE3-dependent, we performed experiments in the presence of the specific NHE3 inhibitor S3226. The addition of 10 $\mu$M S3226 markedly inhibited $\mathrm{JHCO}_3^-$ in all groups (from $1.903 \pm 0.083$ to $0.701 \pm 0.041$ nmol/cm$^2$ per second in CTRL; from $2.779 \pm 0.094$ to $0.521 \pm 0.070$ nmol/cm$^2$ per second in GLU5; from $1.125 \pm 0.130$ to $0.538 \pm 0.069$ nmol/cm$^2$ per second in GLU40) (Figure 1B). The S3226-insensitive bicarbonate reabsorption component was equivalent among all different groups, indicating that NHE3 is the only $\mathrm{H}^+$ transporter in the PT that is modulated by glucose.

The Glucose-Dependent Stimulatory Effect on NHE3 Occurs through Glucose Metabolism

To evaluate whether the glucose-dependent, NHE3-mediated $\mathrm{JHCO}_3^-$ modulation was caused by the intrinsic properties of glucose, we performed a set of experiments, in which glucose was replaced by galactose. The perfusion of different galactose concentrations failed to modulate NHE3 activity (Figure 2A).

Because galactose is metabolized by galactokinase, an enzyme with expression and activity that decrease after weaning, we postulated that differences in the metabolism of glucose and galactose could be responsible for the differences in NHE3 modulation in the PT. To test this hypothesis, we performed experiments in which the nonmetabolizable glucose analog, $\alpha$-methyl-$\mathrm{D}$-glucopiranoside ($\alpha$-MG), was perfused in the tubules.

As depicted in Figure 2B, $\alpha$-MG5 was not able to stimulate NHE3. This result suggests that glucose metabolism is required for 5 mM glucose-dependent NHE3 stimulation. However, $\alpha$-MG40 produced an inhibitory effect on NHE3 similar to the effect produced by GLU40, showing that the glucose inhibitory effect occurs by a metabolically independent mechanism ($1.113 \pm 0.10$ nmol/cm$^2$ per second for $\alpha$-MG40 versus $1.903 \pm 0.083$ nmol/cm$^2$ per second for CTRL).

To evaluate if the mechanism by which GLU5 stimulates NHE3 was by ATP generation, we analyzed if another substrate capable of generating ATP was able to mimic the stimulatory effect of glucose. We perfused tubules with glucose-free solution or GLU5 plus 2-DG in the presence or absence of 10 mM $\alpha$-alanine alone or with 500 $\mu$M 7M DL-cycloserine, an alanine aminotransferase inhibitor. The numbers of perfused tubules are indicated above the bars. The data are the means±SEMs. *P<0.001 versus CTRL; **P<0.001 versus GLU5; ***P<0.01 versus GLU5.

Figure 2. Glucose-dependent NHE3 stimulation is dependent on glucose metabolism. The rate of bicarbonate reabsorption was evaluated by stationary microperfusion and expressed as $\mathrm{JHCO}_3^-$. The dotted horizontal lines indicate $\mathrm{JHCO}_3^-$ in GLU5- and GLU40-perfused tubules. (A) The experiments were performed in the presence of CTRL or galactose (GalAc, 5 and 40 mM), a C-4 epimer of glucose. (B) The tubules were perfused with CTRL or $\alpha$-MG (5 and 40 mM), a nonmetabolizable glucose analog. (C) The experiments were conducted in the presence of CTRL or GLU5 alone or with 2-DG (10 mM), a hexokinase inhibitor. (D) PTs were perfused with glucose-free solution or GLU5 plus 2-DG in the presence or absence of 10 mM $\alpha$-alanine alone or with 500 $\mu$M 7M DL-cycloserine, an alanine aminotransferase inhibitor. The numbers of perfused tubules are indicated above the bars. The data are the means±SEMs. *P<0.001 versus CTRL; **P<0.001 versus GLU5; ***P<0.01 versus GLU5.
effect of glucose on NHE3. We then performed experiments in which the PTs were perfused with amino acids that can be metabolically converted into ATP by specific aminotransferases highly expressed in the kidney.30 Perfusion of 10 mM isoleucine, alanine, or aspartate failed to stimulate NHE3 (data not shown), indicating that ATP generation is not the final step responsible for the stimulatory effect observed on GLU5 perfusion.

To determine the role of glucose metabolism on NHE3 activity, we performed experiments in which the PTs were perfused with CTRL and GLU5 alone or together with 10 mM 2-deoxy-D-glucose (2-DG), a hexokinase inhibitor. Addition of 10 mM 2-DG to GLU5 not only abolished the stimulatory effect of GLU5 on NHE3 but also, produced an additional inhibitory effect that was also observed on the perfusion of CTRL+2-DG (JHCO\textsubscript{3}\textsuperscript{−} from 1.903±0.083 to 1.289±0.054 nmol/cm\textsuperscript{2} per second for CTRL and from 2.779±0.094 to 1.43±0.073 nmol/cm\textsuperscript{2} per second for GLU5) (Figure 2C). Because 2-DG can result in ATP depletion,31 we performed experiments, in which alanine alone or plus DL-cycloserine (an alanine aminotransferase inhibitor) was added to the 2-DG– or GLU5+2-DG–containing solution as an ATP source. Addition of 10 mM alanine abolished the 2-DG–dependent NHE3 inhibition in both the presence and absence of glucose (Figure 2D). The same effect was not observed in the presence of the alanine aminotransferase inhibitor DL-cycloserine, showing that alanine conversion into pyruvate is necessary to abolish the 2-DG–dependent NHE3 inhibition. Therefore, because the addition of alanine to the 2-DG+GLU5 solution did not produce a stimulatory effect, we confirmed that glucose metabolism is absolutely necessary for the NHE3 regulation, even after the addition of another source of energy.

**High Glucose–Dependent NHE3 Inhibition Is Associated with Osmotic Stress**

It is well established that SGLT1-mediated Na\textsuperscript{+}–glucose uptake promotes cell swelling.32 As such, although all the buffers used in this study were osmotically balanced, an increase in cell volume associated with SGLT-mediated glucose uptake was expected. To determine if cell swelling was associated with NHE3 inhibition in the PT, we performed experiments under hypotonic conditions (250 mOsM/kg H\textsubscript{2}O). For comparison, the dotted horizontal line indicates JHCO\textsubscript{3}\textsuperscript{−} in GLU40-perfused tubules. *P<0.001 versus CTRL. (B) The tubules were perfused with CTRL, GLU40, or α-MG40 alone or together with mannitol (40 mM). The dotted horizontal line indicates JHCO\textsubscript{3}\textsuperscript{−} in GLU5-perfused tubules. The data are means±SEMs. The numbers of perfused tubules are indicated in the bars. *P<0.05 versus CTRL; **P<0.01 versus CTRL; ***P<0.001 versus CTRL.

To verify the effect of SGLTs on NHE3, we used the SGLT competitive inhibitor, Phlorizin (Plz). Because of the high variability of Plz concentrations used in different reports21,25,33,34 and because of the fact that SGLTs are able to perform the electrogenic Na\textsuperscript{+} uptake in the absence of a high glucose-dependent inhibitory effect on NHE3 and produced a stimulatory effect similar to the effect observed on GLU5 perfusion (Figure 3B, horizontal dotted line) (JHCO\textsubscript{3}\textsuperscript{−} of 2.802±0.216 nmol/cm\textsuperscript{2} per second for GLU40+40 mM mannitol). As shown earlier, α-MG5 failed to mimic the stimulatory effect. Therefore, we conducted experiments where α-MG40 was perfused alone or together with 40 mM mannitol.

The addition of 40 mM mannitol to α-MG40 abolished the inhibitory effect of α-MG40 on NHE3 (1.113±0.106 versus 1.726±0.119 nmol/cm\textsuperscript{2} per second) but was not able to produce a stimulatory effect. This finding further confirms that the stimulatory effect of glucose on NHE3 transport activity is dependent on glucose metabolism.

**SGLTs Inhibition by Phlorizin Impairs NHE3-Dependent JHCO\textsubscript{3}−**

To verify the effect of SGLTs on NHE3, we used the SGLT competitive inhibitor, Phlorizin (Plz). Because of the high variability of Plz concentrations used in different reports21,25,33,34 and because of the fact that SGLTs are able to perform the electrogenic Na\textsuperscript{+} uptake in the absence of a
sugar,35 we initially performed a Plz dose-response curve starting with the CTRL solution (absence of glucose).

As illustrated in Figure 4A, 10 μM Plz produced a significant decrease of $\text{JHCO}_3^-$ (to $1.388 \pm 0.1283 \text{ nmol/cm}^2$ per second versus CTRL) (Figure 4A, vertical dotted line). The perfusion of higher Plz concentrations caused progressive decreases of $\text{JHCO}_3^-$ with the most pronounced effect observed with 500 μM Plz.

To establish whether Plz was capable of inhibiting NHE3 in the presence of a sugar, we performed a Plz dose-response curve with the GLU5 and GLU40 solutions. As depicted in Figure 4B, the addition of 1 mM Plz to the GLU5 solution caused an inhibition similar to the inhibition observed by the addition of 50 μM Plz to the CTRL solution. Because Plz is a competitive inhibitor, higher concentrations of the inhibitor were required to produce the same effect on NHE3 in the presence of glucose. However, the addition of Plz was not able to produce any additional inhibitory effect when used together with the GLU40 solution (Figure 4C).

Next, we examined the ability of Plz to inhibit $\text{JHCO}_3^-$ in the presence of other sugars. For this purpose, we performed experiments with Plz and 5 mM galactose or α-MG5. The addition of 500 μM Plz to 5 mM galactose (Figure 4D, vertical dotted line) significantly inhibited NHE3-dependent $\text{JHCO}_3^-$. However, only the addition of a higher concentration of Plz (1 mM) to α-MG5 (Figure 4E, vertical dotted line) was able to significantly inhibit $\text{JHCO}_3^-$. Altogether, these results indicate that inhibition of SGLT activity by Plz also inhibits NHE3.

**Systemic Administration of Plz Produces Bicarbonaturia**

To test whether Plz increases $\text{HCO}_3^-$ excretion when administered systemically, rats were treated with 0.4 mg/kg Plz by subcutaneous injection every 12 hours36,37 and placed in metabolic cages. The renal function and other parameters are depicted in Supplemental Table 1. As expected, Plz increased the absolute and fractional excretions of glucose (Figure 5, A and B). As depicted in Figure 5, C and D, Plz markedly increased urinary $\text{Na}^+$ and $\text{HCO}_3^-$ excretion (1.386±0.023 and 1.690±0.038 mmol/d per 100 g body wt, respectively, in vehicle) (Figure 5, C and D, *P<0.05) compared with controls. These findings are in accordance with our microperfusion data, showing that both systemic and local administrations of Plz inhibit NHE3 activity in the PT.

**SGLT2 Colocalizes with NHE3**

Plz was able to inhibit NHE3, even in the absence of a sugar, indicating that SGLT-dependent $\text{Na}^+$ uptake can modulate NHE3. Given the functional interaction between SGLTs and NHE3, we then hypothesized that these transporters might be located in the same microdomain within the PT brush border (BB) membrane. Thus, we performed immunofluorescence experiments to examine whether NHE3 colocalizes with SGLT1 and/or SGLT2.

As depicted in Figure 6, the expression of NHE3 (Figure 6A) and SGLT2 (Figure 6B) was restricted to the PT apical BB, and the merged image (Figure 6C) showed a considerable colocalization of these proteins. In contrast, the immunofluorescence staining of SGLT1 (Figure 6F) was more diffuse and could be detected at both BBs and in cytoplasmic vesicles. Accordingly, the merged image of SGLT1 and NHE3 staining (Figure 6G) did not show a significant colocalization of these two proteins. These results suggest that the inhibition of NHE3 by Plz is most likely mediated by SGLT2.

**DISCUSSION**

The present work shows that physiologic concentrations of glucose (5 mM) stimulate NHE3 transport activity by a process that is dependent on glycolytic metabolism. Higher glucose concentrations inhibit the transporter because of cell swelling. In addition, we showed that the inhibition of the SGLT-dependent $\text{Na}^+$ uptake inhibits NHE3. Moreover, immunofluorescence experiments showed that NHE3 colocalizes with SGLT2 but not SGLT1. Figure 7 summarizes the major findings of the present work.

With regard to the effects of glucose metabolism on NHE3, the results presented in this work are significantly different from the results reported in a previous study using the intestinal cell line CaCo-2.21 In this intestinal cell model, SGLT1-mediated $\text{Na}^+$-glucose uptake led to NHE3-dependent cytoplasmic alkalinization that was not dependent on glucose metabolism. In contrast, we showed that glycolytic metabolism is necessary for the stimulatory effect of GLU5 on NHE3, because the perfusion of nonmetabolizable sugars did not produce the same stimulation and the addition of 2-DG to GLU5 abolished the stimulatory effect. The generation of intermediary compounds of glycolytic metabolism may be involved in this effect, because the generation of these compounds has been reported to modulate intracellular proteins.38

In agreement with our findings, some studies have shown that glucose metabolism modulates other members of the NHE family.9,31,39–44 These transporters have been shown to be inhibited by glycolytic inhibitors, such as 2-DG,9,45–47 and have also been observed to be more sensitive to glycolytic inhibitors than oxidative phosphorylation inhibitors.48–50 Additionally, our study suggests, for the first time, that the apical uptake of glucose is important for maintaining NHE3 activity, because glucose was perfused only apically in the tubules, and that glucose metabolism occurs in the PT and is important for maintaining NHE3 activity.

NHE3 can also be modulated by ATP, and it is believed that the ancillary proteins bound to the C-terminal tail of NHE3 are responsible for its modulation by ATP.41 Because alanine, a noncarbohydrate source of energy, was able to abolish the inhibitory effect produced by the perfusion of 2-DG only in the absence of DL-cycloserine (an alanine aminotransferase inhibitor), we can assume that (1) 2-DG perfusion produced
ATP depletion, which has been observed previously,\textsuperscript{41} and (2) alanine is converted into pyruvate and can be used by the PT cells as an energy source. In addition, these data suggest that decreased glycolytic metabolism as well as ATP depletion might inhibit NHE3 in the PT.

Experiments conducted in absorptive epithelial cells and other heterologous systems have shown that SGLTs are capable of transporting water together with GLU.\textsuperscript{51–53} Thus, although all solutions used in this work were isotonic, cell swelling associated with SGLT-mediated glucose uptake was expected. In the present study, we found that hypotonic shock inhibits NHE3, supporting the hypothesis that the GLU40- or α-MG40–dependent inhibitory effect on NHE3 is caused by an increase in cell volume. The abolishment of this inhibitory effect by the addition of mannitol confirmed that the osmotic flow of water was responsible for the GLU40-dependent inhibitory effect on NHE3.

The NHE family members, especially the housekeeping isoforms, are involved in the control of cell volume. Therefore, cell swelling might induce NHE3 inhibition as a cell survival response, although NHE3 itself is not considered a housekeeping protein. Alternatively, an increase in cell volume could signal excessive sodium uptake, leading to the inhibition of this transporter to maintain volume homeostasis.

In the literature, there is no consensus regarding the effect of increased cell volume on NHE3 activity. Several studies have found that an increase in volume promotes NHE3 stimulation.\textsuperscript{54–57} However, other studies have found that the addition of an extracellular hypotonic solution inhibits or has no effect on NHE3.\textsuperscript{21,40,58} The differences in NHE3 activity caused by hypo-osmotic shock might be because of the involvement of different intermediaries responsible for NHE3 modulation.\textsuperscript{1,57,59,60}

We showed that the pharmacological inhibition of SGLT by Plz is accompanied by marked NHE3 inhibition, indicating that SGLT activity plays a role in NHE3-dependent sodium bicarbonate reabsorption. Because the CTRL solution does not

\begin{figure}[h]
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\caption{The SGLTs inhibitor, Plz, inhibits NHE3-dependent HCO\textsubscript{3}\textsuperscript{−} reabsorption in the renal proximal tubule. The graphs show the dose–response curves obtained for Plz treatment under different conditions. (A) The tubules were perfused with control solution in the presence of Plz (5, 10, 50, 100, 500 μM, or 2 mM). The dotted horizontal lines indicate JHCO\textsubscript{3}\textsuperscript{−} in GLU5- and GLU40-perfused tubules. (B) The experiments were performed in the presence of GLU5 instead of CTRL solution. The following concentrations of Plz were used: 50 μM, 500 μM, 1 mM, 2 mM, or 3 mM. The dotted horizontal lines indicate JHCO\textsubscript{3}\textsuperscript{−} in CTRL- and GLU40-perfused tubules. (C) Plz was added to GLU40 solution in the following concentrations: 500 μM, 1 mM, or 2 mM. The dotted horizontal lines indicate JHCO\textsubscript{3}\textsuperscript{−} in CTRL- and GLU5-perfused tubules. (D and E) The tubules were perfused with Plz (50 μM, 500 μM, or 1 mM) together with GALAC5 and α-MG5, respectively. The dotted horizontal lines indicate JHCO\textsubscript{3}\textsuperscript{−} in CTRL- and GLU5-perfused tubules. The dotted vertical lines indicate the concentration of Plz that significantly inhibited JHCO\textsubscript{3}\textsuperscript{−}: aP<0.05 versus no Plz; bP<0.001 versus no Plz; cP<0.001 versus no Plz.}
\end{figure}
contain any transportable sugar, the NHE3 inhibition by Plz cannot be attributed to abolition of glucose uptake. In the absence of glucose, SGLT1 and SGLT2 are still able to produce currents, which are attributable to Na⁺ leak.⁳⁵ Then, NHE3 inhibition by Plz might occur through dissipation of this trans-epithelial Na⁺ current. However, a direct action of Plz in the exchanger cannot be discarded.

Noteworthy, a recent report has shown that dapagliflozin, an SGLT2 inhibitor, promotes diuresis and reduces BP.¹⁸ Moreover, Plz prevents hypertension development in an experimental model of diabetes.⁶¹ Our results suggest that one possible mechanism by which dapagliflozin and Plz could induce diuresis and attenuate hypertension development is through NHE3 inhibition of Na⁺/HCO₃⁻ reabsorption.

As expected, acute systemic administration of Plz increases the fractional excretion of glucose. We have found that Plz-induced glycosuria is accompanied by natriuresis and bicarbonaturia. Interestingly, Plz also promoted an increase in GFR estimated by creatinine clearance. Although creatinine clearance may not be an optimal filtration marker in rodents, the effect of Plz on GFR has been previously shown in earlier studies, in which Plz was chronically administered to different animal models.⁶²,⁶³ Therefore, although the increase of urinary excretion of sodium bicarbonate induced by Plz may have a glomerular component, the differences of the magnitude of changes (35% increase of GFR and 87% and 100% increase of sodium urinary excretion, respectively) allied with the microperfusion finding that intraluminal perfusion of Plz inhibits NHE3 activity strongly suggest that there is an important proximal tubular component responsible for the Plz-mediated sodium bicarbonate urinary loss.

Having defined a functional interaction between SGLT and NHE3, we next examined whether SGLT1 and/or SGLT2 are expressed in the same PT subcellular sites as NHE3. By immunostaining, we determined that, in adult rats, NHE3 and SGLT2 are localized in the apical membrane of PT and showed a clear colocalization. However, SGLT1 staining was not concordant with NHE3 staining. SGLT1 staining was observed in regions where NHE3 staining was not present, and its intracellular staining was observed in regions of strong apical NHE3 staining. Consistent with these findings, it has been shown that NHE3 is virtually absent in the PT S3 segment,⁶⁴ the region with the highest expression of SGLT1. Additionally, although SGLT1 is expressed throughout the S1 and S2 segments of the PT, its location is markedly intracellular, whereas the localization of NHE3 is mostly apical.⁶⁴,⁶⁵

Having shown that SGLT2 and NHE3 colocalize in the rat renal PT, we hypothesized that these two transporters may be physically associated. Unfortunately, we were unable to validate this hypothesis, because all commercially available antibodies recognizing SGLT2 that we tested were not suitable for immunoprecipitation procedures (data not shown). Although the exact structural and functional features of the interaction of NHE3 and SGLT2 remain to be further characterized, our present data suggest that, because we could not find the apical colocalization of SGLT1 and NHE3, only SGLT2 inhibition is involved in NHE3 inhibition.

In summary, the findings of this study show a bimodal effect of glucose on NHE3 activity in the renal PT. The stimulatory effect of physiologic concentrations of glucose on NHE3 involves glucose metabolism by the glycolytic cascade, whereas the inhibitory effect of supraphysiologic glucose concentrations involves the water influx caused by glucose uptake on high glucose perfusion. We also showed that SGLT2 colocalizes with NHE3 in the PT and that SGLT inhibition is accompanied by NHE3 inhibition. Furthermore, these results provide evidence that the inhibition of NHE3 is the mechanism by which SGLT2 inhibitors cause diuresis.

**CONCISE METHODS**

**Reagents and Antibodies**
All chemicals were obtained from Sigma-Aldrich unless otherwise noted. S3226 was donated by Jurgen Punten (Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany). Anti-SGLT2 polyclonal antibody and anti-NHE3 monoclonal antibody were obtained from Santa Cruz...
Biotechnology. Anti-SGLT1 polyclonal antibody was obtained from Abcam, Inc. Alexafluor-588 and AlexaFluor-488 secondary antibodies were obtained from Life Technologies.

Animal Protocols
Animal procedures and protocols followed the ethical principles in animal research of the Brazilian College of Animal Experimentation and were approved by the Institutional Animal Care and Use Committee. The experiments were performed using male Wistar rats (200–350 g) housed under standardized conditions of constant temperature of 22°C, 12:12-hour dark to light cycles, and relative humidity of 60% at the Institute of Biomedicine of the University of São Paulo animal facility. On the days of the experiment, the rats were anesthetized with ketamine-acepromazine (200 and 1.5 mg/kg intramuscularly, respectively) and Propofol (25 mg/kg intravenously as needed) and prepared for the stationary microperfusion experiments as described below.

Stationary Microperfusion Experiments
The animals were prepared for in vivo microperfusion as described previously. Supplemental Figure 3 represents a model for the technique. In this technique, a double-barreled micropipette was used to puncture a PT. One pipette barrel was filled with the experimental solutions (90 mM NaCl, 25 mM NaHCO₃, 5 mM KCl, 1 mM CaCl₂, and 1.2 mM MgSO₄) stained with FDC green. The glucose concentrations of these solutions varied between 0 (CTRL) and 60 mM or were substituted with 5 or 40 mM galactose or α-MG. Another group

Figure 6. SGLT2, but not SGLT1, colocalizes with NHE3 in the renal proximal tubule. Kidney sections were obtained from adult rats. The kidney tissue was fixed, and the sections obtained were subjected to immunofluorescence staining with antibodies against NHE3, SGLT2, and SGLT1. The images were obtained using a Carl Zeiss confocal microscope. (A–C) SGLT2 (red) and NHE3 (green) staining were only present in the apical membrane of the proximal tubule. (C) The merged image shows the colocalization of SGLT2 and NHE3. (D) The merged staining images including the contrast phase channel of selected tubules and the nuclei staining (blue). (E–G) SGLT1 (red) staining was distributed diffusely in the proximal tubules, whereas NHE3 (green) was only detected in the apical membrane of the proximal tubule. (G) The merged image shows that SGLT1 does not colocalize with NHE3. (H) The contrast phase channel of selected tubules with the nuclei staining (blue).
was perfused with the same CTRL solution and the 5 mM glucose solution with 5 or 10 mM isoleucine or with 10 mM 2-DG alone or plus 10 mM isoleucine. The 40 mM glucose and 40 mM α-MG solution were also perfused together with 40 mM mannitol. The osmolality of solutions ranged from 250 (hypotonic) to 300 (adjusted with mannitol), up to 340 (hypertonic) mosM/kg H2O.

The other pipette barrel was filled with castor oil stained with Sudan black. The tubules were perfused with the abovementioned solutions depending on the experimental strategy, and the column of injected fluid was isolated from tubular and glomerular fluid and held stationary until the next perfusion by droplets of castor oil.

To measure the tubular pH, a double-barreled, asymmetric microelectrode was used. The larger barrel was silanized with an H⁺ ion-sensitive ion exchange resin (Sigma Fluka) and contained an H⁺ ion exchange resin (Sigma Fluka). The smaller barrel contained 1 mM KCl colored by FDC green. The voltage, representing tubular H⁺ activity, was measured by the electrode by a WPI electrometer. The voltage was measured continuously and recorded by an analog to digital converter (Lynx). Luminal bicarbonate was calculated from luminal pH and arterial blood PCO₂. The rate of tubular acidification was expressed as the half-time of the exponential reduction of the injected HCO₃⁻ concentration to its stationary level (t₁/₂). The net HCO₃⁻ reabsorption (JHCO₃⁻) per centimeter² of tubule epithelium was calculated using the following equation:

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JHCO₃⁻ = \frac{\ln 2}{t_{1/2}} [\frac{(HCO₃⁻)_0 - (HCO₃⁻)_t}{r} \cdot \frac{r}{2}]
\]

where \(t_{1/2}\) is the half-time of bicarbonate absorption, \(r\) is the tubule radius, and \((HCO₃⁻)_0\) and \((HCO₃⁻)_t\) are the concentrations of the injected HCO₃⁻ and HCO₃⁻ at the stationary level, respectively. The value of \(N\) is the number of perfused tubules. Each experimental group was composed of at least three animals, and each animal had one to four proximal tubules perfused.

**Acute Phlorizin Treatment**

Male Wistar rats were placed in individual metabolic cages for the collection of urine samples, quantification of urine output, and food and water consumption. Animals were placed individually in a metabolic cage for a 48-hour period of adaptation. In the next 24-hour period, animals received Plz dissolved in a solution containing ethanol (2.5:1) in PBS injected subcutaneously at a dose of 0.4 g/kg two times (in 12-hour intervals) or vehicle. At the end of the period, animals were immediately decapitated, and blood samples were collected for analysis.

**Renal Function Analysis**

Urine and blood samples, collected in metabolic cages, were used to measure urine output, HCO₃⁻ excretion, plasma glucose, glucose excretion, fractional glucose excretion, sodium excretion, potassium excretion, and GFR. Creatinine clearance was used to estimate GFR. Serum and urinary creatinine and glucose concentrations were measured by a kinetic method (Labtest) using a ThermoPlate Analyzer Plus (ThermoPlate). Sodium and potassium were measured on an electrolyte analyzer (Roche Diagnostic). Urine HCO₃⁻ was measured in a gasometer Radiometer ABL5 (Radiometer Medical).

**Kidney Preparation for Immunohistochemistry**

The animals were anesthetized as described above, and the kidneys of all animals were perfused with PBS at 37°C through the abdominal aorta for 10 minutes followed by periodate-lysine-paraformaldehyde perfusion fixation for 20 minutes. The kidneys were removed and immediately frozen in Tissue-Tek (Tissue-Tek OCT Compound) using liquid nitrogen.

**Immunohistochemistry**

The kidneys were frozen in Tissue-Tek and sectioned (6 μm) using a Cryostat (Microm HM 505E). After fixation in 4% paraformaldehyde, the sections were rehydrated in PBS for 5 minutes and incubated in 0.5% Triton X-100 solution for 10 minutes at room temperature.
Statistical Analyses
Statistical comparisons were made by paired t test by taking the probability of 0.05 (5%) as the limit of significance. When more than two groups were compared, a one-way ANOVA followed by Tukey post hoc test was used. For each group, a minimum of six tubules was analyzed (N=number of perfused tubules).

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DISCLOSURES
None.

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