

NHE2-mediated bicarbonate reabsorption in the distal tubule of NHE3 null mice

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NHE3^{-/-} mice display a profound defect in proximal tubule bicarbonate reabsorption but are only mildly acidotic owing to reduced glomerular filtration rate and enhanced H⁺ secretion in distal nephron segments. *In vivo* microperfusion of rat distal tubules suggests that a significant fraction of bicarbonate reabsorption in this nephron segment is mediated by NHE2. Two approaches were used to evaluate the role of distal tubule NHE2 in compensating for the proximal defect of H⁺ secretion in NHE3^{-/-} mice. First, renal clearance experiments were used to assess the impact of HOE694, an inhibitor with significant affinity for NHE2, on excretion of bicarbonate in NHE3^{-/-} and NHE2^{-/-} mice. Second, *in vivo* micropuncture and microperfusion were employed to measure the concentration of bicarbonate in early distal tubule fluid and to measure distal bicarbonate reabsorption during a constant bicarbonate load. Our data show that HOE694 had no effect on urinary bicarbonate excretion in NHE3^{+/+} mice, whereas bicarbonate excretion was higher in NHE3^{-/-} mice receiving HOE694. HOE694 induced a significant increase in bicarbonate excretion in mice given an acute bicarbonate load, but there was no effect during metabolic acidosis. Bicarbonate excretion was not affected by HOE694 in bicarbonate-loaded NHE2^{-/-} mice. *In vivo* micropuncture revealed that early distal bicarbonate concentration was elevated in both bicarbonate-loaded and NHE3^{-/-} mice. Further, microperfusion experiments showed that HOE694-sensitive bicarbonate reabsorption capacity was higher in acidotic and NHE3 null animals. We conclude that NHE2 contributes importantly to acidification in the distal tubule, and that it plays a major role in limiting urinary bicarbonate losses in states in which a high luminal bicarbonate load is presented to the distal tubule, such as in NHE3 null mice.

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Several isoforms of the sodium–hydrogen exchanger (NHE) have been identified in the mammalian kidney (Biemesderfer *et al.* 1992, 1997; Amemiya *et al.* 1995; Chambrey *et al.* 1998; Peti-Peterdi *et al.* 2000; Goyal *et al.* 2002). NHE3 is recognized as the predominant NHE in the apical membrane of the proximal tubule and thick ascending limb of Henle (TAL), accounting for the major part of bicarbonate reabsorption in these segments (Capasso *et al.* 1991; Wang *et al.* 2001). The distal tubule reabsorbs between 5 and 8% of the filtered bicarbonate load (Gottschalk *et al.* 1960; Malnic *et al.* 1972; Capasso *et al.* 1987), and *in vivo* microperfusion studies suggest that about half of this is mediated by sodium–hydrogen exchange located in the early distal tubule (Wang *et al.* 1993). Immunohistochemical studies identify NHE2 in

the apical membrane of the rat distal convoluted tubule and connecting tubule (Chambrey *et al.* 1998). Studies using HOE694, an agent that inhibits NHE2 but less so NHE3 (Counillon *et al.* 1993), confirm functional expression of NHE2 in the rat distal tubule (Wang *et al.* 2001). Proximal tubule reabsorption of bicarbonate and fluid is reduced by ~60% in mice with a non-functional mutation of NHE3 (Schultheis *et al.* 1998b; Wang *et al.* 1999). In absolute terms, however, fluid (and by inference, sodium) delivery into and out of the loop of Henle is similar in NHE3 null and wild-type mice (Lorenz *et al.* 1999). This reflects a compensatory response involving reduction in single-nephron glomerular filtration rate mediated by activation of tubulo-glomerular feedback.

Bicarbonate excretion has not been measured in NHE3^{-/-} mice. We hypothesize that compromised reabsorption in both the proximal tubule and loop of Henle results in delivery of bicarbonate-rich fluid into the early distal tubule. Since bicarbonate reabsorption in the distal tubule is load dependent (Capasso *et al.* 1987), manoeuvres that increase delivery, such as bicarbonate loading (Malnic *et al.* 1972; Capasso *et al.* 1987) and administration of carbonic anhydrase inhibitors (Vieira & Malnic, 1968; Malnic *et al.* 1972; Capasso *et al.* 1987), promote increased distal reabsorption. Enhancement of the activity of H⁺-ATPase and H⁺,K⁺-ATPase (Nakamura *et al.* 1999) in isolated collecting tubules of NHE3^{-/-} mice removed from the animal is consistent with increased intrinsic capacity for H⁺ ion secretion possibly as a consequence of metabolic acidosis. However, the possible adaptive response of the distal convoluted tubule in NHE3^{-/-} mice has not yet been explored.

In the present study we measured the effect of HOE694, an inhibitor with strong specificity for NHE2-mediated H⁺ ion secretion, on renal bicarbonate excretion. We find that NHE2 contributes importantly to acidification in the distal tubule, that it is up-regulated by metabolic acidosis and in NHE3 null mice, and that it has a major role in limiting urinary bicarbonate excretion in states where distal bicarbonate delivery is increased above normal.

Methods

Animals

Experiments were performed on three groups of animals. Mice with a null mutation in NHE3 were bred locally, as previously described (Schultheis *et al.* 1998b). Wild-type littermates (Black Swiss background) were used as

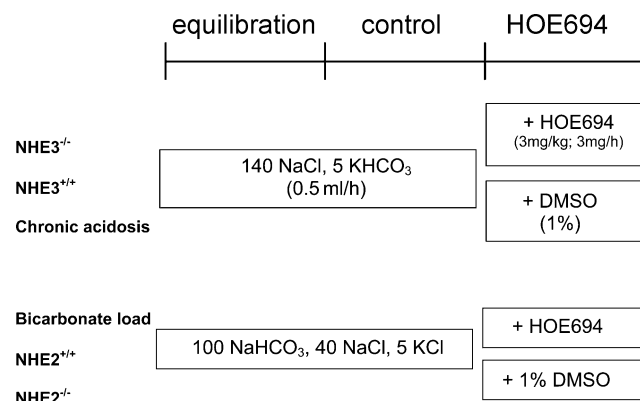


Figure 1. Infusion protocols for renal clearance studies

All concentrations expressed in mmol l⁻¹. Infusions were maintained for 3 h. After a 60 min equilibration period, baseline measurements were made during the control period followed by infusion of either the NHE2 inhibitor HOE694 or the time control vehicle (1% DMSO) during the experimental period.

controls. NHE2 null mice (Schultheis *et al.* 1998a) and wild-type littermates (Non-Swiss Albino background) were provided by Dr G. E. Shull (Molecular Genetics, University of Cincinnati). The chronic acidosis experiments and those involving bicarbonate loading were performed on C57/Bl6 mice (The Jackson Laboratory, Bar Harbour, ME, USA). Most mice were maintained on a regular diet and tap water *ad libitum* until the day of the experiment. Metabolic acidosis in mice was induced by giving drinking water containing 1.5% ammonium chloride and 5% sucrose for 10 days prior to experimentation. All data reported were obtained under anaesthesia and animals were killed by an overdose of anaesthetic in accordance with Yale University guidelines.

Surgical protocols

The methods for preparing mice for renal clearance measurements, for monitoring mean arterial blood pressure (MABP), and for collecting urine samples have been previously described (Wang *et al.* 1999). Mice were anaesthetized by intraperitoneal injection of [5-ethyl-5-(L-methylpropyl)-2-thiobarbituric acid (Inactin, 100 mg kg⁻¹; Sigma, USA). A tracheotomy was performed and the jugular vein and carotid artery were catheterized for intravenous infusion and blood sampling. Following surgery, a saline solution (see below) was infused at 0.5 ml h⁻¹. For the clearance experiments only, [³H]inulin was included in the infusate (10 μCi ml⁻¹; 10 μCi primer). For the microperfusion experiments, the left kidney was exposed by flank incision and immobilized in a Perspex cup clamped to the operating table. During this period, mice were given an additional bolus of saline to compensate for surgical losses.

Renal clearance infusion protocol

Throughout the experiment, each animal received an intravenous infusion of 140 mM NaCl and 5 mM KHCO₃. The acute increase of bicarbonate load was induced by i.v. infusion of 100 mM NaHCO₃ with 40 mM NaCl and 5 mM KCl. These protocols are shown in Fig. 1. After an equilibration period, renal clearance measurements were initiated for a 60-min control period and a 60-min experimental period during which the animals received either HOE694 (3 mg kg⁻¹ bolus, 3 mg kg⁻¹ h⁻¹ infusion) or the vehicle alone (time control: 1% DMSO in saline). Urine was collected under mineral oil and a 30 μl blood sample was taken at hourly intervals. At the start and end of the experiment a 40 μl blood sample was taken for blood-gas analysis. Blood pressure was measured at the beginning, middle and end of each clearance period.

Microperfusion infusion protocol

Throughout the experiment, each animal received an intravenous infusion of 140 mM NaCl and 5 mM KHCO_3 at a rate of 0.5 ml h^{-1} . For the bicarbonate loading experiments, 100 mM NaHCO_3 , 40 mM NaCl and 5 mM KCl was infused at a similar rate. Mannitol (2%) was added to all infusates.

Stationary microperfusion of the distal tubule

The technique employed in these experiments has been previously described (Fernandez *et al.* 1994). Briefly, a proximal tubule, chosen at random, was impaled with a micropipette (perfusing) containing an FD & C green-coloured solution of 100 mM NaCl and 25 mM NaHCO_3 . In order to minimize fluid reabsorption, this solution had been rendered isotonic through the addition of raffinose pentahydrate (Riedel-de Haën, Hannover, Germany). A small bolus of fluid was injected into the tubule lumen to identify downstream loops of the proximal and early distal tubule. An H^+ -selective micro-electrode (see below) was lowered onto the kidney surface for zero voltage measurement before being inserted into the tubule lumen of the early distal tubule to obtain free-flow recordings of pH. A second (blocking) micropipette containing Sudan black-coloured heavy mineral oil was then inserted into a proximal tubular segment and the original perfusion pipette moved to the last superficial segment of the proximal tubule.

For stationary measurements of tubular H^+ secretion, the control solution was injected into the late proximal tubule thereby perfusing orthogradely the loop of Henle and distal tubule. Perfusion was continued until the early distal microelectrode recorded a large and stable voltage deflection equal to that of the perfusate pH (pH 8.0). At this point, perfusion was stopped and a column of heavy oil injected into the proximal tubule to prevent downstream flow of native tubule fluid or perfusate. Voltage recordings were continued until a stable reading was once again obtained. Following control measurements, the first perfusion pipette was replaced by a second containing HOE694 (100 μM) and again recordings were made. The average from three measurements each of control and HOE694 perfusions was taken to generate one value per tubule thereby allowing for paired comparisons to be made.

Ion-selective microelectrodes

Double-barrelled microelectrodes were pulled and treated as previously described (Bailey, 2004). The mouse was electrically grounded through the tail. The tip of the ion-selective barrel was filled with H^+ ionophore (cocktail B; Fluka, Buchs, Switzerland) and the electrode was

calibrated before and after impalement by superfusion onto the kidney surface of standard solutions kept at 37°C . Standards of pH 6.0, 7.0 and 8.0 were used to measure the mean voltage difference per 10-fold change in H^+ activity. This was $47.6 \pm 0.8 \text{ mV}$ ($n = 39$ electrodes). The reference barrel was used to measure transepithelial potential difference. The voltages from each barrel were measured by a high-impedance electrometer (Model 223, World Precision Instruments, USA). An AD converter (model 104, iWorx, USA), sampling at a rate of 5 Hz, allowed voltages to be recorded onto a PC using LabScribe software (iWorx, USA).

The voltage difference between the reference and ion-selective barrels, representing lumen H^+ activity, was converted to pH, and bicarbonate concentration was calculated from this value and from measured arterial P_{CO_2} . For each perfusion, pH fell from the initial value of 8, to a stationary value of ~ 6.6 . A one-phase exponential was fitted to this fall, allowing the half-time ($t_{1/2}$) of acidification to be generated. Bicarbonate reabsorption (J_{HCO_3}) was then calculated using the following equation (Fernandez *et al.* 1994):

$$J_{\text{HCO}_3} = \log 2/t_{1/2} \times (\text{initial}[\text{HCO}_3^-] - \text{stationary}[\text{HCO}_3^-]) \times r/2$$

where J_{HCO_3} is in $\text{nmol cm}^{-2} \text{ s}^{-1}$, $t_{1/2}$ is the acidification half-time, initial $[\text{HCO}_3^-]$ is 25 mmol l^{-1} , stationary $[\text{HCO}_3^-]$ is calculated from pH_{stat} and arterial P_{CO_2} , and r is the lumen radius (μm).

Analysis

Blood gas analysis was performed on freshly drawn arterial blood using a Corning Blood Gas Analyser. Glomerular filtration rate (GFR) was estimated from the clearance of [^3H]inulin, measured in plasma and urine samples by β -emission spectroscopy following dispersal in scintillation cocktail. Sodium and potassium concentrations in plasma and urine were measured by flame photometry (Model 480, Corning, USA). Bicarbonate concentration in urine was measured by a microcalorimetric method (picapnotherm).

Statistics

All data are expressed as the mean \pm s.e.m. For the renal clearance studies, Student's t test was used for comparisons between groups. For multiple comparisons a two-way ANOVA was used with the Bonferroni *post hoc* test for point-to-point comparisons. For the micropuncture data, a paired t test was used to assess the impact of HOE694 on bicarbonate reabsorption within a given group. For comparisons between groups, the non-parametric Kruskal–Wallis test was used since Bartlett's test indicated unequal variances; Dunn's multiple comparison test was

Table 1. Plasma data, mean arterial blood pressure (MABP) and glomerular filtration rate (GFR) in NHE3^{+/+} (*n* = 12) and NHE3^{-/-} (*n* = 13) mice

	NHE3 ^{+/+}	<i>P</i>	NHE3 ^{-/-}
<i>P</i> _{Na} (mmol l ⁻¹)	147.2 ± 1.5	NS	148.6 ± 1.8
<i>P</i> _K (mmol l ⁻¹)	4.54 ± 0.14	NS	4.52 ± 0.22
<i>P</i> _{HCO₃} (mmol l ⁻¹)	22.4 ± 0.7	< 0.05	18.9 ± 1.3
pH	7.36 ± 0.01	< 0.05	7.28 ± 0.02
MABP (mmHg)	108 ± 3	< 0.01	97 ± 2
GFR (ml min ⁻¹ (100 g) ⁻¹)	1.05 ± 0.04	< 0.01	0.62 ± 0.06

Data are means ± s.e.m. Statistical comparisons were made using Student's *t* test.

used for *post hoc* analysis. Differences were considered statistically significant at *P* < 0.05.

Results

Blood electrolytes and kidney function

Blood-gas analysis, summarized in Table 1, indicates that NHE3^{-/-} mice have a moderate metabolic acidosis with a reduction in arterial blood bicarbonate concentration and pH, confirming previous results (Schultheis *et al.* 1998b; Lorenz *et al.* 1999; Wang *et al.* 1999; Brooks *et al.* 2001). GFR and mean arterial blood pressure were significantly lower in NHE3^{-/-} mice than in control mice (Table 1), the latter suggesting volume depletion. The reduction in GFR reduced the filtered sodium load in NHE3^{-/-} mice (44.3 ± 0.3 *versus* 28.6 ± 2.9 mmol min⁻¹; *P* < 0.05). However, sodium excretion was significantly lower (*P* < 0.05) in these animals (Fig. 2A) and fractional sodium excretion was thus similar in both groups (0.54 ± 0.10 *versus* 0.51 ± 0.09%; NS). In contrast, despite the reduction in the filtered bicarbonate load in NHE3^{-/-} mice (6.6 ± 0.4 *versus* 3.5 ± 0.4 mmol min⁻¹; *P* < 0.05), the absolute excretion of bicarbonate was approximately 8 times higher (*P* < 0.01) than in controls (Fig. 2B); ~0.6% of the filtered bicarbonate load thus escapes reabsorption. The rate of potassium excretion was similar in both groups of mice resulting in a significantly greater fractional excretion in NHE3^{-/-} than in control mice (17.1 ± 2.5 *versus* 35.8 ± 8.6%; *P* < 0.05).

Effect of i.v. HOE694

Systemic administration of HOE694 affected neither MABP nor GFR in NHE3^{-/-} or NHE3^{+/+} mice (Table 2A). In NHE3^{+/+} animals, HOE694 was without effect on sodium or bicarbonate reabsorption (Fig. 2A and B). This indicates that under normal physiological conditions, HOE-sensitive, NHE2-mediated hydrogen ion secretion is either negligible or can be effectively compensated by other mechanisms of distal tubule H⁺ secretion, such as electrogenic H⁺ ion transport and/or H⁺,K⁺-ATPase. As shown in Fig. 2B,

Table 2. Mean arterial blood pressure (MABP) and glomerular filtration rate (GFR) in NHE3^{+/+} and NHE3^{-/-} mice during the control period and during a 60-min period of infusion with either HOE694 (3 mg kg⁻¹ h⁻¹) or vehicle (time control 1% DMSO)

	Control	HOE694
A. MABP (mmHg)		
NHE ^{+/+} Time control	107 ± 3	108 ± 3
NHE ^{+/+} HOE694	110 ± 5	112 ± 6
NHE ^{-/-} Time control	99 ± 4	103 ± 2
NHE ^{-/-} HOE694	96 ± 4	97 ± 3
B. GFR (ml min ⁻¹ (100 g) ⁻¹)		
NHE ^{+/+} time control	1.10 ± 0.09	1.16 ± 0.09
NHE ^{+/+} HOE694	1.00 ± 0.04	0.92 ± 0.11
NHE ^{-/-} time control	0.65 ± 0.10	0.59 ± 0.09
NHE ^{-/-} HOE694	0.59 ± 0.10	0.46 ± 0.09

Data are means ± s.e.m. Statistical comparisons were made using ANOVA.

HOE694 caused a significant increase in the renal excretion of both bicarbonate (*P* < 0.01) and sodium (*P* < 0.05) in NHE3^{-/-} mice. These results suggest that NHE2-mediated H⁺ ion secretion contributes importantly to bicarbonate reabsorption following loss of functional activity of NHE3 in the proximal tubule and TAL.

NHE3 null mice differ from wild-type mice in having high distal delivery of bicarbonate as well as metabolic acidosis. We therefore tested whether these parameters would alter the effects of HOE694 in wild-type mice. Mice were either acutely loaded with bicarbonate (blood [HCO₃⁻] = 30.1 ± 2.6 mmol l⁻¹; *n* = 14) or rendered chronically acidotic (blood [HCO₃⁻] = 16.9 ± 0.7 mmol l⁻¹; *n* = 8). Inspection of Fig. 3A shows that HOE694 increased the excretion of sodium bicarbonate in those animals receiving an infusion of sodium bicarbonate. In acidotic mice, bicarbonate excretion was minimal and not significantly different from zero. Thus, HOE694 was without effect on sodium bicarbonate excretion in wild-type mice with acidosis.

In the present study, a total of 0.2 mg HOE694 was administered intravenously. Assuming free distribution in total body water, a blood concentration of about 30 μmol l⁻¹ would be expected. It is reasonable to assume that NHE2 would be inhibited extensively under this condition (*K*_i = 5 μmol l⁻¹), whereas the effect on NHE3 would be small (*K*_i = 650 μmol l⁻¹) (Counillon *et al.* 1993). However, the possibility must be considered that NHE1 (*K*_i = 0.16 μmol l⁻¹) also contributes to the renal effects of HOE694. It would be expected that inhibition by HOE694 of NHE1, which is present in the basolateral membrane of several nephron segments (Biemesderfer *et al.* 1992), should acidify the cytosol and enhance bicarbonate reabsorption. In the rat TAL, however, inhibition of basolateral

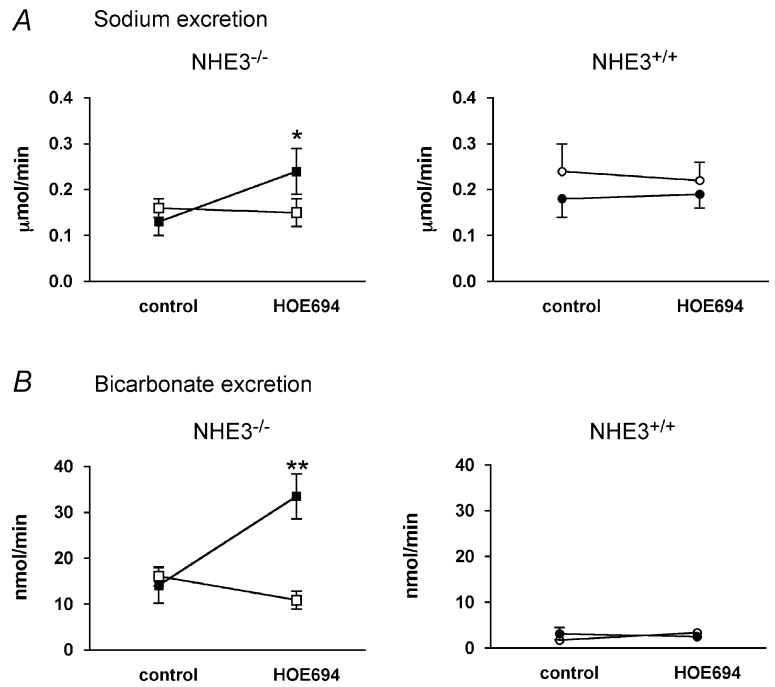


Figure 2. Urinary sodium excretion (A) and bicarbonate excretion (B) in NHE3^{-/-} (squares, n = 13) and NHE3^{+/+} mice (circles, n = 12) during infusion of either the NHE2 inhibitor HOE694 (filled symbols) or vehicle (open symbols)
 The drug had no significant effect in NHE3^{+/+} mice. Two-way ANOVA indicated a significant effect of HOE694 for both sodium and bicarbonate excretion in NHE3^{-/-} mice (see text). Point-to-point comparisons were made using Bonferroni's *post hoc* test; **P* < 0.05, ***P* < 0.01.

NHE1 inhibits HCO₃⁻ reabsorption (Good *et al.* 1995). Moreover, reduced bicarbonate reabsorption is observed in the TAL of NHE1 null mice (Good *et al.* 2004). Accordingly, it is possible that the observed inhibitory effects of HOE694 include a significant contribution of basolateral effects on NHE1-mediated Na⁺-H⁺ exchange.

In order to further demonstrate that the effects of HOE694 reported above were due to inhibition of NHE2, the effect of the drug on sodium and bicarbonate excretion was also assessed in NHE2^{-/-} mice. Inspection of Fig. 4 indicates that sodium and bicarbonate excretion rates in bicarbonate-loaded wild-type mice were again sharply increased by HOE694 (Fig. 4), as previously shown in

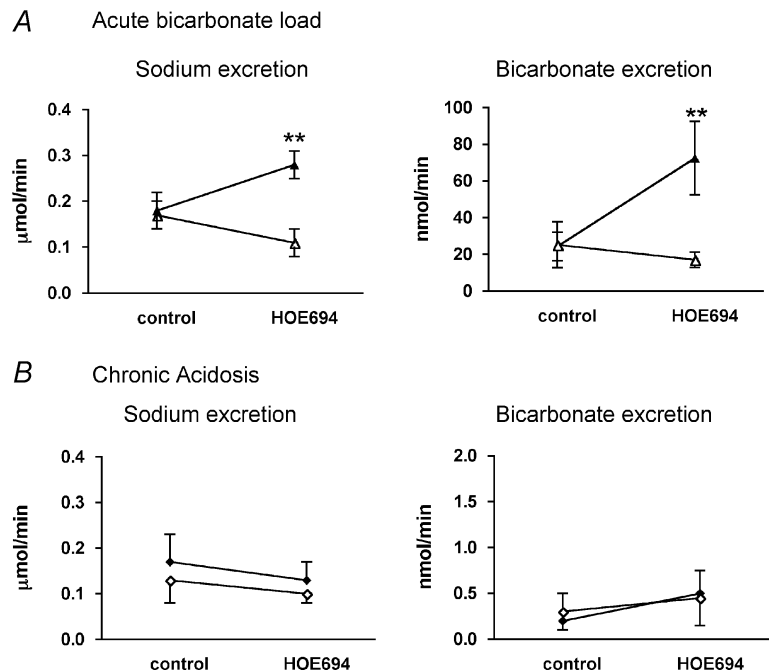


Figure 3. Sodium and bicarbonate excretion in bicarbonate-loaded mice (A) (triangles; n = 7 in each group) and acidotic mice (B) (diamonds; n = 5 for HOE694 and 4 for time controls) during infusion of either the NHE2 inhibitor HOE694 (filled symbols) or vehicle (open symbols)
 Two-way ANOVA indicated a significant effect of HOE694 on both variables in bicarbonate-loaded mice only (see text). Point-to-point comparisons were made using Bonferroni's *post hoc* test; ***P* < 0.01.

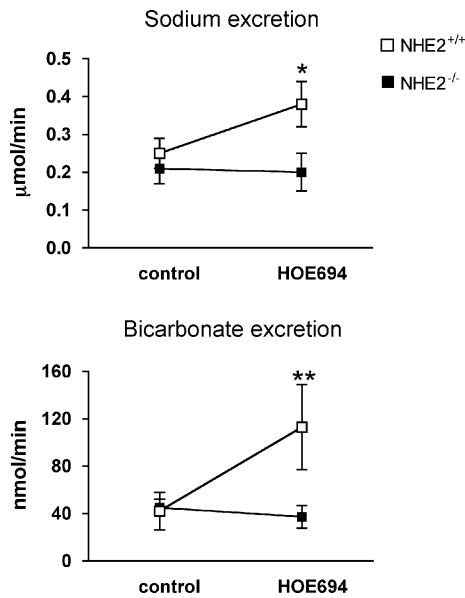


Figure 4. Sodium and bicarbonate excretion in bicarbonate-loaded NHE2^{+/+} mice (open squares, $n = 5$) and NHE2^{-/-} mice (filled squares, $n = 6$) during infusion of the NHE2 inhibitor HOE694

Point-to-point comparisons between groups were made using Bonferroni's *post hoc* test; * $P < 0.05$, ** $P < 0.01$.

Fig. 3. Such an effect, however, was totally absent in NHE2 null mice. These findings verify that the main effect of HOE694 under the conditions of our experiments is to inhibit NHE2-mediated H⁺ secretion and bicarbonate reabsorption.

Urinary potassium excretion was affected in a predictable manner by acid-base perturbations, being suppressed by acidosis and stimulated by alkalosis in wild-type mice (Fig. 5) (Giebisch, 1998). As also shown in Fig. 5, under conditions in which HOE694 had been shown to increase sodium and bicarbonate excretion, namely in NHE3 null mice or with bicarbonate loading in wild-type mice, HOE694 also increased potassium excretion.

Free-flow bicarbonate concentration in the early distal tubule

The free-flow transepithelial potential differences in the four groups of mice are summarized in Table 3. These values, being in the range of -10 mV, confirm the positioning of the electrode in the early to mid, rather than late, distal tubule (De Mello-Aires & Malnic, 2002). The bicarbonate concentrations in early distal tubule

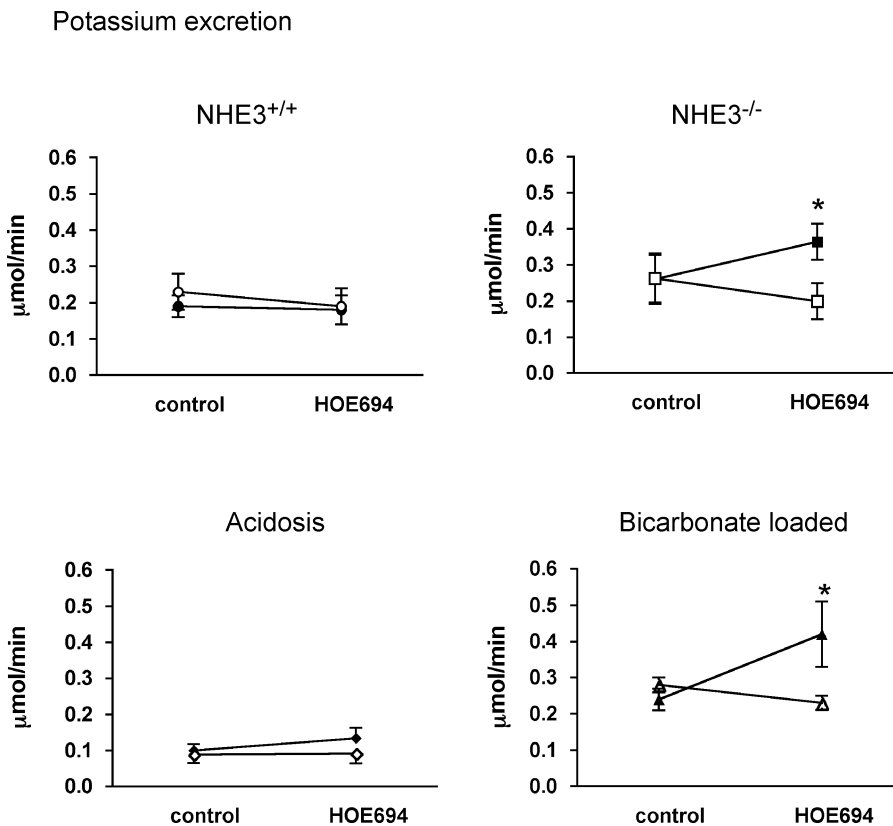


Figure 5. The effect of HOE694 on renal potassium excretion in four groups of mice. Infusion of the NHE2 inhibitor (black symbols) increased potassium excretion compared to time controls (open symbols) in NHE3^{-/-} mice and bicarbonate-loaded animals

Point-to-point comparisons were made using Bonferroni's *post hoc* test; * $P < 0.05$. Data are means \pm s.e.m.

Table 3. Transepithelial potential difference (V_{te}), measured under free flow conditions, in NHE3^{+/+} ($n=5$), NHE3^{-/-} ($n=2$), chronically acidotic ($n=7$) and acutely bicarbonate loaded ($n=3$) C57/Bl6 mice

	V_{te} (mV)	n (tubules)
NHE3 ^{+/+}	-10.7 ± 0.8	18
NHE3 ^{-/-}	-8.9 ± 1.2	9
Acidotic	-10.3 ± 1.4	8
Bicarbonate-loaded	-10.3 ± 1.5	14

Data are means \pm S.E.M. of individual tubules. Statistical comparisons indicated no difference between the groups (Kruskal–Wallis test).

fluid, estimated from tubule fluid pH and arterial P_{CO_2} , are summarized in Fig. 6. In control animals, the mean bicarbonate concentration was ~ 5 mmol l⁻¹, similar to that obtained from the rat using direct measurement of tubule fluid bicarbonate (Capasso *et al.* 1986). Chronic acidosis reduced early distal bicarbonate concentration by $\sim 50\%$ ($P < 0.05$), whereas acute bicarbonate loading elicited a 3-fold increase ($P < 0.01$) in distal bicarbonate concentration. In NHE3^{-/-} mice, tubule fluid bicarbonate concentration was significantly elevated, ~ 13 mmol l⁻¹, supporting the interpretation that impaired reabsorption in the proximal tubule and thick ascending limb of Henle leads to delivery of a bicarbonate-rich fluid into the distal nephron.

Bicarbonate reabsorption in the early distal tubule

H⁺-selective microelectrodes were used to measure HCO₃⁻ reabsorption in the early distal tubule of control, acidotic and NHE3 null mice. Since the concentration of HCO₃⁻ perfused into the tubule was the same in all groups of mice, this technique allowed the evaluation of

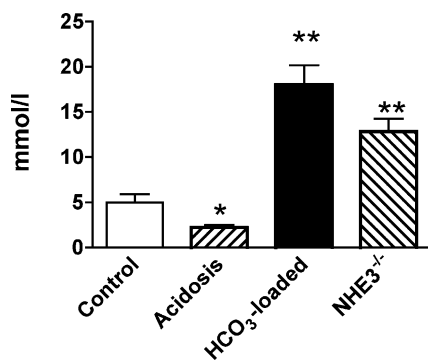


Figure 6. Bicarbonate concentration in the early distal tubule of control ($n=18$ tubules/5 mice), chronically acidotic mice ($n=14/7$), bicarbonate loaded ($n=8/3$) or NHE3^{-/-} ($n=9/2$) mice

The Kruskal–Wallis test indicated a significant difference between the 4 groups ($P < 0.001$). Point-to-point comparisons were made using Dunn's *post hoc* test; * $P < 0.05$, ** $P < 0.01$ versus control. Data are means \pm S.E.M.

the reabsorptive capacity of the distal tubule independent of variations in the luminal bicarbonate load. Inspection of Fig. 7 shows that J_{HCO_3} was different in the three groups of mice ($P < 0.01$). Specifically, J_{HCO_3} was increased by $\sim 90\%$ in the acidotic mice ($P < 0.01$) and increased by $\sim 50\%$ ($P < 0.05$) in NHE3 null mice. These findings thus indicate that there is up-regulation of distal tubule H⁺ secretion in NHE3 null mice, as in metabolic acidosis.

Impact of HOE694 on J_{HCO_3} in the early distal tubule

As shown in Fig. 8, HOE694 significantly reduced J_{HCO_3} in the three groups of mice. The net effect of this inhibitor, shown in Fig. 9, was significantly greater in both acidotic ($P < 0.01$) and NHE3 null mice ($P < 0.05$) than in control animals. These findings demonstrate that administering bicarbonate directly into the distal tubule induces appreciable HOE694-sensitive, NHE2-mediated bicarbonate absorption even under control conditions when an effect of the drug on urinary sodium and bicarbonate excretion in the intact animal was not detected. Importantly, the capacity of the distal tubule for HOE694-sensitive, NHE2-mediated bicarbonate absorption was up-regulated both in NHE3 null mice and in wild-type mice with metabolic acidosis. Under the latter condition, the effect of HOE694 on urinary sodium and bicarbonate excretion was not detected, presumably because of the low distal bicarbonate load in metabolic acidosis. Taken together, these findings demonstrate that NHE2 contributes importantly to acidification in the distal tubule, that it is up-regulated by metabolic acidosis and in NHE3 null mice, but that it only affects urinary bicarbonate excretion in states where distal bicarbonate delivery is increased above normal.

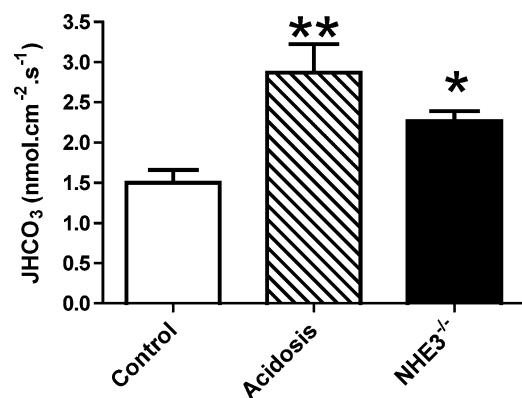


Figure 7. Bicarbonate reabsorption during perfusion of the early distal tubule with a control perfusate (25 mm HCO₃) in either control ($n=11$ tubules/5 mice), chronically acidotic ($n=14/7$) or NHE3^{-/-} ($n=6/2$) mice

The Kruskal–Wallis test indicated a significant difference between the 3 groups ($P < 0.01$). Point-to-point comparisons were made using Dunn's *post hoc* test; * $P < 0.05$, ** $P < 0.01$ versus control. Data are means \pm S.E.M.

Discussion

The main finding of the present study is the emergence of NHE2 as a mechanism of renal bicarbonate reabsorption in circumstances of elevated delivery to the early distal tubule. This is based upon the observation that systemic HOE694 produced a significant enhancement of bicarbonate excretion in (1) NHE3 null mice having sharply impaired proximal bicarbonate transport and (2) in wild-type mice receiving an acute bicarbonate load. The increased bicarbonate reabsorption capacity occurring in response to elevated bicarbonate delivery to the early distal tubule is best explained by activation of NHE2 since HOE694

was without effect in bicarbonate-loaded NHE2 null mice.

Although most of the filtered bicarbonate is reabsorbed along the proximal tubule, significant reabsorption is also observed in the thick ascending limb of Henle (Good, 1993; Capasso *et al.* 2002); in both sites, NHE3 is the predominant apical mechanism of H⁺ secretion. The superficial distal tubule reabsorbs ~5% of the filtered bicarbonate under physiological conditions (De Mello-Aires & Malnic, 2002). Reabsorption is load dependent and rises sharply with increased bicarbonate delivery (Capasso *et al.* 1987; Chan *et al.* 1989). Both electrogenic H⁺ secretion and Na⁺-H⁺ exchange (Wang *et al.* 1993; Fernandez *et al.* 1994) have been shown to mediate bicarbonate transport in the distal tubule. The role of NHE2 is further underscored by immunological studies demonstrating NHE2 expression in the apical membrane of the distal tubule (Chambrey *et al.* 1998). *In vivo* microperfusion studies provided pharmacological evidence to suggest that it is this isoform, not NHE3, that mediates an important fraction of distal tubule bicarbonate reabsorption (Wang *et al.* 2001).

The clearance experiments reported herein suggest that NHE2-mediated bicarbonate reabsorption is, under physiological circumstances, of small magnitude. It is not surprising therefore that NHE2 knockout mice have normal acid-base status (Ledoussal *et al.* 2001a,b). Given that ~50% of distal tubule bicarbonate reabsorption is mediated by NHE-dependent H⁺ secretion, demonstrated here in the mouse and previously in the rat (Wang *et al.* 1993, 2001; Fernandez *et al.* 1994; Wesson, 1998), the failure of HOE694 to increase bicarbonate excretion in control mice appears to be counterintuitive. This discrepancy can be accounted for on the basis of variations in bicarbonate load. In the rat, perfusion with a solution containing 25 mM bicarbonate results in

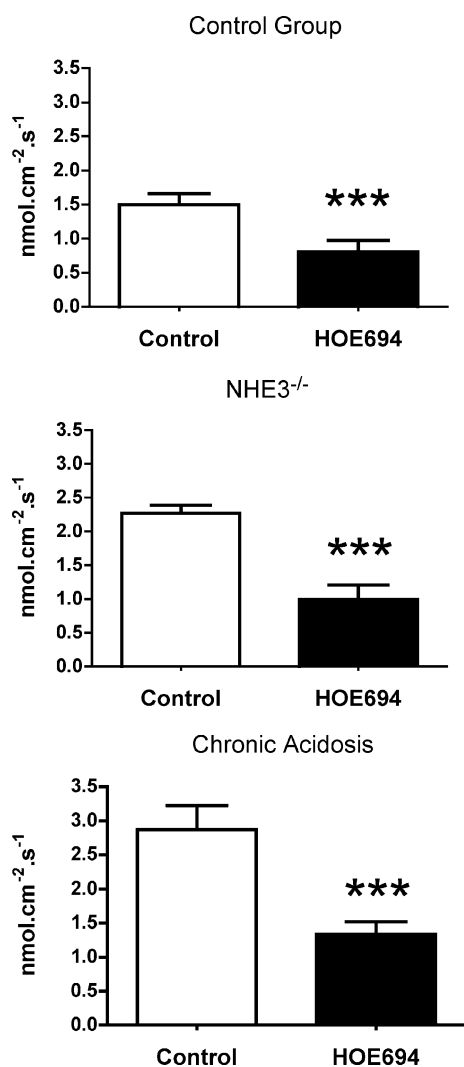


Figure 8. Bicarbonate reabsorption during perfusion of the early distal tubule with a control perfusate (25 mM HCO₃⁻) and the same solution to which HOE694 (100 μmol l⁻¹) had been added in either control (*n* = 11 tubules/4 mice), chronically acidotic (*n* = 14/7) or NHE3^{-/-} (*n* = 6/2) mice

The drug evoked a significant inhibition of bicarbonate reabsorption in each of the 3 groups, as indicated. Comparisons were made using Student's paired *t* test.

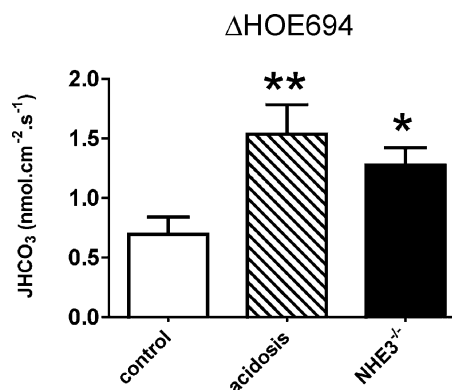


Figure 9. The net effect of HOE694 (100 μmol l⁻¹) on early distal bicarbonate reabsorption in either control (*n* = 11 tubules/5 mice), chronically acidotic (*n* = 14/7) or NHE3^{-/-} (*n* = 6/2) mice. The Kruskal-Wallis test indicated a significant difference between the 3 groups (*P* < 0.01). Point-to-point comparisons were made using Dunn's *post hoc* test; **P* < 0.05, ***P* < 0.01 versus control. Data are means ± S.E.M.

a J_{HCO_3} of $\sim 70 \text{ pmol min}^{-1} \text{ mm}^{-1}$ (Wang *et al.* 2001), about $\sim 50\%$ of which is mediated by $\text{Na}^+ - \text{H}^+$ exchange. With lower bicarbonate concentrations (7.5 mM), J_{HCO_3} is $15 \text{ pmol min}^{-1} \text{ mm}^{-1}$ (Wesson, 1998), yet the proportion effected by $\text{Na}^+ - \text{H}^+$ exchange remains about the same. In the present study therefore the concentration of luminal bicarbonate observed in control mice under free-flow conditions (5 mM) would limit the absolute magnitude of NHE2-mediated reabsorption and thus render a systemic effect of HOE694 too small to discern.

Systemic administration of HOE694 does, however, evoke significant bicarbonate excretion under conditions in which bicarbonate delivery to the early distal tubule (confirmed by free-flow micropuncture) was elevated, as observed in mice receiving an acute bicarbonate load and in NHE3 null mice. Taken together, our data suggest that NHE2 reclaims normally only a small proportion of the filtered bicarbonate load but is part of the compensatory up-regulation of apical transporters that guard against base loss under circumstances in which proximal reabsorption is overwhelmed or compromised.

It is of interest that we did not observe increased urinary bicarbonate excretion in acidotic animals in response to systemic HOE694. This may be taken to suggest that acidosis does not stimulate distal tubule activity of NHE2. Yet previous reports of micropuncture studies in which high bicarbonate concentrations were used suggest that distal tubule bicarbonate reabsorption rises in metabolic acidosis (Wesson, 1996, 1997). Similarly, the intrinsic reabsorptive capacity of the early distal tubule, measured in the present study during perfusion with a high (25 mM) bicarbonate solution, was almost doubled in acidotic mice compared to control conditions. Importantly, the absolute effect of luminal HOE694 was also found to be significantly greater in acidotic mice than in untreated control animals. Since our free-flow measurements show that metabolic acidosis markedly reduced the delivery of bicarbonate into the distal tubule, it is likely that limiting bicarbonate delivery under free-flow conditions countervails the stimulatory effects of metabolic acidosis on bicarbonate reabsorption in general, and NHE2 activity in particular.

Our micropuncture analysis also showed that early distal tubule bicarbonate reabsorption was higher in NHE3^{-/-} mice, as was the absolute inhibitory effect of HOE694. Thus, it is reasonable to suggest that NHE2-mediated bicarbonate reabsorption is stimulated by the moderate systemic acidosis observed in NHE3 null mice. Moreover, impaired bicarbonate reabsorption in the proximal tubule (Schultheis *et al.* 1998b; Wang *et al.* 1999) ensures that delivery to the early distal tubule is not limiting and that an effect of HOE694 on whole-kidney bicarbonate excretion can be observed. A note of caution should be mentioned in evaluating the relative importance of compensation by NHE2 in NHE3 null mice. Studies of NHE2/NHE3

double knockout mice (Ledoussal *et al.* 2001b) revealed no additional disturbance of acid–base balance beyond that observed in NHE3^{-/-} mice. In fact, conscious NHE2/NHE3 double knockouts maintained relatively normal blood pH and bicarbonate concentrations. In light of the current studies, which clearly demonstrate the loss of a significant bicarbonate absorptive capacity in the distal tubule of NHE2^{-/-} mice, it seems likely that other renal compensatory mechanisms for maintenance of acid–base balance were activated in the double knockout. In addition to sodium–hydrogen exchange activity, both $\text{H}^+ - \text{ATPase}$ and $\text{H}^+, \text{K}^+ - \text{ATPase}$ -mediated bicarbonate reabsorption are also augmented in outer-medullary and cortical collecting ducts harvested from NHE3^{-/-} mice (Nakamura *et al.* 1999). However, as evidenced by elevated urinary bicarbonate excretion rates in NHE3^{-/-} mice prior to HOE694 administration, the combined effect of these three acidification mechanisms does not prevent the spilling of bicarbonate in the final urine. The continued loss of bicarbonate both through renal and gastrointestinal routes (Schultheis *et al.* 1998b) is thus responsible for the mild metabolic acidosis in these animals.

We have confirmed effects of acid–base derangements upon potassium secretion. Thus, induction of metabolic acidosis curtailed potassium excretion and metabolic alkalosis induced kaliuresis (Giebisch, 1998). NHE3^{-/-} mice maintained renal potassium excretion at a level comparable to that of the wild-type mice despite a substantial reduction in GFR. This dissociation of potassium excretion from filtered load has long been recognized (Berliner & Kennedy, 1948) and is a central tenet of the maintenance of potassium balance. Since sodium delivery to the early distal tubule is adequate in both groups of mice (Lorenz *et al.* 1999), it can be assumed that potassium secretion is elevated in the knockout animals. These data contrast with those of a previous investigation in which NHE3 null mice were hyperkalaemic and had reduced potassium excretion (Brooks *et al.* 2001). It is possible that the net effect on potassium in the present study may reflect the balance between countervailing parameters. Elevated aldosterone (Schultheis *et al.* 1998b) and increased bicarbonate to chloride ratio of tubule fluid are known to stimulate potassium secretion (Carlisle *et al.* 1991; Amorim *et al.* 2003). Metabolic acidosis and increased buffer delivery would, on the other hand, be expected to activate potassium reabsorption by $\text{H}^+, \text{K}^+ - \text{ATPase}$ (Silver *et al.* 1996; Schultheis *et al.* 1998b; Nakamura *et al.* 1999). HOE694 induced a significant kaliuresis in both NHE3 null mice and in alkalotic wild type mice. This may reflect both the diuretic effect of the drug and the inhibition of NHE2-mediated bicarbonate reabsorption in the early distal tubule: increased flow rate and luminal bicarbonate concentration stimulate potassium secretion in the distal tubule (Malnic *et al.* 1989; Amorim *et al.* 2003).

In conclusion, we find that NHE2 contributes importantly to acidification in the distal tubule, and that it plays a major role in limiting urinary bicarbonate losses in states in which a high luminal bicarbonate load is presented to the distal tubule. Up-regulation of NHE2-mediated bicarbonate reabsorption occurs in response to metabolic acidosis. Thus, up-regulated NHE2-mediated bicarbonate absorption in the distal tubule is one of the compensatory mechanisms that are activated in the kidneys of NHE3 null mice.

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