SODIUM/HYDROGEN EXCHANGE is a widespread and fundamental mechanism for the translocation of these ions across membranes and represents an important component of transepithelial ion transport in a variety of tissues. In the mammalian kidney, four isoforms of the Na/H exchanger (NHE1, NHE2, NHE3, and NHE4) have been identified. Isoform 3 (NHE3) has been identified as the major Na/H exchanger in the apical membrane of the S1 and S2 segments of the proximal tubule and in the apical membrane of cortical thick ascending limb of the loop of Henle (2, 3, 7, 9, 17). More than 85% of the filtered load of NaHCO3 is reabsorbed in the proximal tubule, and NHE3 contributes up to ~60% of the total reabsorption of this segment (1, 7). Because of its distribution and functional role, one would predict that NHE3 exerts a major influence on overall fluid and electrolyte balance by mediating the bulk reabsorption of sodium and water in the early segments of the proximal tubule as well as in the thick ascending limb of Henle’s loop.

The present study uses a recently developed mouse model in which the gene encoding NHE3 (Nhe3, gene symbol Slc9a3) has been ablated using gene-targeting strategies (22). Initial studies evaluating this mouse revealed that the homozygous null mouse has a decreased arterial blood pressure and a modest acidosis. homozygous null animals also have increased levels of kidney renin mRNA and circulating aldosterone, suggesting that they are somewhat volume depleted. Experiments performed in perfused proximal tubule segments in these mice demonstrated that fluid reabsorption and absorptive HCO3 flux are both decreased by more than 60% (22). In addition to its expression in the kidney, NHE3 is also heavily expressed in intestinal epithelial cells, and in accordance with this, Nhe3 null mice display a marked intestinal phenotype consisting of modest diarrhea, enlarged intestinal tract, and markedly alkaline intestinal contents (22). This intestinal phenotype likely contributes to the presumed volume depletion in these animals.

The purpose of the present study was to evaluate single-nephron function in situ in NHE3-deficient mice. Experiments were conducted in heterozygous knockouts as well as in homozygous and wild-type animals to evaluate the possible quantitative contribution of NHE3 to proximal tubular and loop of Henle function. Late proximal and early distal collections were used to evaluate proximal tubule and loop of Henle fluid transport, as well as the possible contribution of macula densa-mediated processes on nephron filtration rate, which together may contribute to the overall sodium balance achieved in these animals. Our results indicate that NHE3 deficiency is associated with the predicted reduction in proximal tubule fluid reabsorption, but that reductions in single-nephron glomerular filtration rate (SNGFR) and/or increases in loop of Henle trans-
port prevent major increases in fluid delivery into the collecting duct system.

METHODS

Animals. Wild-type, heterozygous, and homozygous NHE3-deficient mice were obtained from an established colony that was generated by targeted gene disruption as previously described (22). All animals were derived from heterozygous crosses, and genotypes were determined by polymerase chain reaction analysis of DNA from tail biopsies. Mice were maintained on normal rodent chow and tap water in a barrier facility until the time of the experiment.

Free-flow micropuncture experiments. Male mice of each genotype (Nhe3+/+, n = 5; Nhe3+/−, n = 4; and Nhe3−/−, n = 4) weighing between 30 and 40 g were prepared for micropuncture according to conventional techniques modified for use in the mouse as previously described (16, 21). Mice were anesthetized with separate intraperitoneal injections of ketamine (50 µg/g body wt) and thiobutabarbitial (1 ncatin, 100 µg/g body wt; Research Biochemicals International, Natick, MA) and placed on a thermally controlled surgical table. Following tracheotomy, the right femoral artery and vein were cannulated with polyethylene tubing hand-drawn to a fine tip over a flame (0.3–0.5 mm OD). The arterial catheter was connected to a fixed-dome pressure transducer (model CDX11; COBE Cardiovascular, Arvada, CO) for measurement of arterial blood pressure, and the venous catheter was connected to a syringe pump for infusion. The bladder was also cannulated with a flared PE-10 polyethylene tubing for the collection of urine. Blood pressure and heart rate were monitored throughout the experiment using a MacLab data acquisition system (AD Instruments, Boston, MA) with a sampling rate of 200 samples/s. In some experiments, data were collected using a conventional chart recorder. Body temperature was maintained at 37.5°C, and animals were provided with a steady stream of 100% O2 to breathe. The left kidney was then exposed via a flank incision, carefully dissected free of adherent fat and connective tissue, placed in a Lucite cup, and covered with mineral oil. A 4 µl/g body wt bolus infusion of isotonic saline containing 2.25 g/100 ml BSA and 1.00 g/100 ml glucose was then administered, followed by a maintenance infusion of the same solution at 0.2 µl·min−1·g body wt−1. The BSA-containing solution was contained either 0.75 µl/100 ml of FITC-inulin (Sigma) or 12% iothalamate at an activity of ~20 μCi/ml (Glofil; Cypros Pharmaceutical, Carlsbad, CA). A previous study from our laboratory (16) demonstrated that these two markers yield nearly identical measurements of SNGFR and tubular fluid/plasma ratios [subsequently, this ratio will be referred to as (TF/P)h].

After a 30- to 45-min equilibration period, micropuncture collections and urine collections were begun. Surface convolutions of the same nephron were identified by injecting a small volume of stained saline (0.25% Fast Green dye, Sigma) into a random proximal segment. Late proximal puncture sites were identified as the last surface segment to fill with green dye before it disappeared into the loop of Henle. In a small population of nephrons (10–20%), an early distal puncture site could be identified when the green dye returned to the kidney surface. During two consecutive clearance periods lasting 30–60 min, at least seven timed proximal collections were made, and usually two to three paired distal collections were made. In those nephrons having both proximal and distal collection sites, the distal tubule collection was performed before the proximal collection. In these nephrons, late proximal flow rate (Vprox) in the presence of flow to the macula densa was estimated from the distal measurement of SNGFR (SNGFRdist) and the proximal measurement of (TF/P)h (TF/Pprox) using the following equation: Vprox = SNGFRdist(TF/Pprox). Blood samples (5–10 µl) were taken in heparinized tubes before and after each clearance period. Sharped glass micropipettes used for dye injection were 2–3 µm in diameter, and those used for fluid collection were 6–7 µm. At the end of each experiment, tubular fluid samples were transferred individually to 1-µl constant-bore micropipets for determination of volume and FITC-inulin concentration or [125I]iothalamate activity as described previously (16, 21). Blood samples were centrifuged, and plasma aliquots were transferred into 1-µl microcaps for inulin or iothalamate determination. Urine samples were also evaluated for inulin or iothalamate for the determination of whole kidney GFR.

Stop-flow pressure measurements. Separate male Nhe3+/+ (n = 4) and Nhe3−/− (n = 3) mice weighing 30–40 g were prepared, and proximal tubule segments for micropuncture were identified as described above. Identifed tubules were blocked with wax, and a micropipette attached to a nanoliter infusion pump was inserted into the last superficial proximal segment for loop of Henle perfusion. Another micropipette, attached to a servo-null pressure device (WPI, New Haven, CT), was then inserted into an early proximal segment recognizable from the widening of the tubular lumen. When stop-flow pressure (Pst) stabilized, loop of Henle perfusion rate was altered from 0 to 45 nl/min, and responses in Pst were recorded. Perfusion fluid contained (in mM) 136 NaCl, 4 Na2CO3, 4 KCl, 2 CaCl2, 7.5 urea, and 100 mg/ml Fast Green.

Statistics. Statistical analysis was performed by ANOVA using a single factor design or a mixed factorial design with repeated measures on the second factor. Where necessary, individual comparisons of group means were accomplished using individual contrasts. Data are expressed as means ± SE, and differences are regarded as significant at P < 0.05.

RESULTS

Table 1 summarizes whole animal data obtained from free-flow micropuncture experiments on wild-type (Nhe3+/+), heterozygous (Nhe3+/−), and homozygous knockout mice (Nhe3−/−). As previously reported (22), mean arterial blood pressure was significantly lower in Nhe3−/− mice compared with Nhe3+/+ mice. Furthermore, blood pressure in the Nhe3−/− mice was also significantly lower than in the Nhe3−/− mice. Although the pressure in the Nhe3−/− was somewhat lower than in the Nhe3+/− mice, the difference was not significant (P = 0.18). These data suggest a direct correlation between NHE3 expression and mean arterial blood pressure. Whole kidney GFR was highest in the Nhe3−/− mice.

Table 1. Body and kidney weights, blood pressure, and whole kidney function in Nhe3+/+ (n = 5), Nhe3+/− (n = 4), and Nhe3−/− (n = 4) mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body wt (BW), g</th>
<th>Kidney wt (KW), g</th>
<th>KW/BW ratio</th>
<th>Mean arterial pressure, mmHg</th>
<th>GFR, µl/min</th>
<th>Urine flow, µl/min</th>
<th>Urine osmolality, mosmol/kgH2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>++</td>
<td>37 ± 2</td>
<td>0.53 ± 0.025</td>
<td>0.05 ± 0.015</td>
<td>188 ± 5</td>
<td>364 ± 95</td>
<td>3.8 ± 0.7</td>
<td>924 ± 163</td>
</tr>
<tr>
<td>+/−</td>
<td>33 ± 2</td>
<td>0.06 ± 0.007</td>
<td>0.01 ± 0.001</td>
<td>108 ± 5</td>
<td>335 ± 51</td>
<td>3.1 ± 0.6</td>
<td>988 ± 116</td>
</tr>
<tr>
<td>−/−</td>
<td>38 ± 2</td>
<td>0.12 ± 0.007</td>
<td>0.002 ± 0.001</td>
<td>89 ± 6†</td>
<td>236 ± 41</td>
<td>1.2 ± 0.4†</td>
<td>1034 ± 57</td>
</tr>
</tbody>
</table>

Values are means ± SE. GFR, glomerular filtration rate. *P < 0.05 compared with Nhe3−/−. †P < 0.05 compared with Nhe3−/−.
and lowest in the Nhe3−/−, but none of the differences between groups reached significance. Although body weight and kidney weight did not differ among the three groups of mice, the kidney weight-to-body weight ratio was consistently and significantly lower in Nhe3−/− mice compared with the other two groups, which did not differ from each other. Urine flow rate was significantly reduced in the Nhe3−/− mice compared with the other two groups, but urine osmolality was not different among any of the groups.

The results from late proximal micropuncture collections from these experiments are shown in Fig. 1. Fluid reabsorption in the proximal segment was found to be directly related to the gene copy number: reabsorption was significantly lower in Nhe3+/* mice compared with Nhe3+/− mice, and lower still in Nhe3−/− mice. Fractional reabsorption showed a similar pattern (Nhe3+/+, 42 ± 1%; Nhe3+/−, 33 ± 1%; Nhe3−/−, 28 ± 2%; P < 0.001). Despite these differences in reabsorption, fluid delivery to the late proximal tubule was not significantly different in the Nhe3−/− animals compared with wild type. Interestingly, proximal fluid delivery in the Nhe3−/− animals was significantly higher than in the Nhe3+/− animals. The normalization of late proximal flow rate in the Nhe3−/− mice was primarily due to a significantly lower SNGFR in these animals compared with both Nhe3+/+ and Nhe3+/− (P < 0.05). It should be noted that these collections were made during complete blockade of loop of Henle flow rate and therefore with interrupted tubuloglomerular feedback (TGF). Thus the measured values for SNGFR are probably artificially elevated to some degree.

We found that, on average, only one in four to five nephrons on the kidney surface had an early distal convolution that was accessible for micropuncture. Therefore there were markedly fewer early distal collections obtained in these experiments than proximal collections. Measurements from these collections are summarized in Fig. 2. In contrast to the measurements from proximal collections, distally measured values of SNGFR were significantly lower in the Nhe3−/− mice compared with both Nhe3+/+ and Nhe3+/− mice, which were not different from each other. Early distal flow rate, however, was not different among any of the three groups. When the distal measurement of SNGFR and the proximal measurement of (TF/P)In from the same tubule were used to estimate flow rate to the late proximal tubule in the presence of an intact TGF mechanism, late proximal flow rate was found to be significantly increased in the Nhe3−/− mice compared with the other two groups. Counteracting this increased delivery, fluid reabsorption in the loop segment was significantly elevated in the Nhe3−/− mice compared with the other two groups.

The above observations suggest that the TGF mechanism is largely intact in mice with reduced or absent NHE3 expression and that it plays a major role in maintaining normal fluid flow to the distal nephron. Analysis of SNGFR measurements from proximal and distal collection sites in the same nephron further suggest active participation of the TGF mechanism, as illustrated in Fig. 3. In all three groups of mice, distal measurements of SNGFR were lower than proximal measurements (P < 0.001). Interestingly, in Nhe3−/− mice, the distal/proximal ratio of 70 ± 9% was significantly reduced compared with the other two groups (P < 0.05).

To confirm operation of the TGF system in these animals, we performed a separate series of experiments in Nhe3+/+ and Nhe3−/− mice to directly evaluate Psf during loop of Henle perfusion. These data are summarized in Fig. 4A, which shows the individual Psf responses to changing loop of Henle flow rate from 0 to 45 nl/min. In both Nhe3+/+ and Nhe3−/− mice, there was a significant and consistent decrease in Psf in response to increased loop of Henle perfusion rate. The mean values are shown by the bold lines and indicate that the
magnitude of the response was not different among groups. A representative set of tracings from an Nhe3−/− mouse is shown in Fig. 4B.

**DISCUSSION**

Consistent with previously published results from proximal tubules perfused in situ (22), we found that proximal fluid reabsorption in free-flowing nephrons was decreased by about one-half in Nhe3−/− compared with Nhe3+/+ mice. Furthermore, these results show that Nhe3+/− mice also demonstrate a clear deficit in proximal tubule reabsorption, suggesting that even modest alterations in the expression of this protein can influence overall sodium and water balance. Although the amount of NHE3 protein expression in Nhe3+/− mice has not been evaluated, Northern analysis indicated that NHE3 mRNA is reduced by about one-half in the kidneys of Nhe3+/− animals (22). Our results also demonstrate that the TGF mechanism is largely intact in NHE3-deficient animals and accounts for a considerable portion of the compensation that occurs in these animals to achieve sodium balance.

In the present study, mean arterial blood pressure was significantly lower in the Nhe3−/− compared with both Nhe3+/+ and Nhe3+/− mice. This hypotension is consistent with the notion that these animals are significantly volume depleted, as suggested in our original report (22). Since NHE3 is a major pathway for electrolyte and water reabsorption in both the kidney and small intestine, volume depletion is likely due to a
combination of reduced dietary NaCl absorption and increased renal NaCl excretion. Furthermore, it would seem that the lower pressure is a necessary adaptive response, over and above other salt-conserving mechanisms (such as activation of the renin-angiotensin-aldosterone axis), that enables these animals to achieve sodium balance. Interestingly, we found in the present studies that Nhe3<sup>−/−</sup> also showed a tendency for lowered blood pressure. Whether the more modest decrement in NHE3 expression seen in the heterozygous animals can directly influence blood pressure remains to be determined. Along these lines, it is important to note that neither serum aldosterone nor renal renin mRNA appears to be elevated in these animals (22), suggesting that intrarenal mechanisms are able to largely compensate for the decreased proximal reabsorption observed in these animals.

The proximal micropuncture measurements presented in this study directly evaluated the capacity of the proximal tubule to transport fluid in NHE3-deficient mice. However, to evaluate proximal tubule function in the context of more distal compensatory mechanisms, such as TGF, proximal-distal differences must be analyzed. We found that in Nhe3<sup>−/−</sup> mice, the decreased proximal transport was almost completely compensated through a decrease in SNGFR, such that delivery to the distal tubule was not different from that observed in Nhe3<sup>+/+</sup> mice. Distal micropuncture measurements indicated that SNGFR was ~40% lower in Nhe3<sup>−/−</sup> mice compared with Nhe3<sup>+/+</sup> mice, whereas corresponding proximal measurements indicated that SNGFR was only ~20% lower in Nhe3<sup>−/−</sup> than in Nhe3<sup>+/+</sup> mice. These data suggest that about one-half of the compensatory decrease in SNGFR in these mice is attributable to activation of TGF. That the TGF mechanism is indeed intact, and even robust in Nhe3<sup>−/−</sup> animals, is confirmed by the measurements of P<sub>fr</sub>. It should be cautioned, however, that due to the substantially different volume and pressure conditions which almost certainly exist in these two groups of animals, quantitative interpretation of these TGF responses cannot be reliably made. One could argue, for instance, that volume depletion, as well as the associated increase in the renin-angiotensin system observed in Nhe3<sup>−/−</sup> animals, might be expected to significantly increase the magnitude of the TGF response in these animals.

The possible role of Na/H exchange in macula densa signaling has recently been addressed in a series of studies from Bell and coworkers (5, 13, 19). Using the isolated perfused loop of Henle with attached macula densa, these investigators have demonstrated apical and basolateral Na/H exchange activity in macula densa cells, and they have shown that both are responsive to low concentrations of angiotensin II. They hypothesize, therefore, that the modulation of macula densa-mediated signaling events (such as TGF and renin secretion) by angiotensin II may be in part due to its influence on Na/H exchange. If this is indeed the case, then one could speculate that the lack of angiotensin II-sensitive NHE3 in macula densa cells, and they have shown that both are responsive to low concentrations of angiotensin II. They hypothesize, therefore, that the modulation of macula densa-mediated signaling events (such as TGF and renin secretion) by angiotensin II may be in part due to its influence on Na/H exchange. If this is indeed the case, then one could speculate that the lack of TGF potentiation observed in volume-depleted Nhe3<sup>−/−</sup> animals in the present study may be due to the absence of angiotensin II-sensitive NHE3 in macula densa cells. Although there are conflicting data regarding the identity of the Na/H exchangers in macula densa cells, available functional evidence from the rabbit (13) and immunohistochemical evidence from the rat (2) suggests that NHE3 is expressed in the apical membrane. There is also evidence, however, that NHE2 is expressed in the cortical thick ascending limb up to and perhaps including the macula densa (10). Also, NHE1
proximal flow rate in delivery seen in accommodate the modest increase in late proximal higher flow rates (4, 20). It is possible that there is well established that loop of Henle reabsorption is a elucidated, a number of possibilities exist. First, it is underlying this compensatory response remain to be ion transporters in the loop segment are upregu-
ized in Nhe3 by (if left unchecked) that would overload loop transport mechanisms, in turn activating TGF. It is also possible that ion transporters in the loop segment are upregu-
lated in the Nhe3−/− mice. There are numerous studies demonstrating that Na⁺/H⁺ exchange activity and or NHE3 mRNA expression can be significantly altered in response to changes in NaCl intake and acid-base balance (18). For example, chronic metabolic acidosis has been shown to increase NHE3 mRNA levels, NHE3 protein expression, and Na⁺/H⁺ transport activity in medullary thick ascending limb (15). Whether alterations in Na⁺/H⁺ exchange in the loop of Henle can significantly influence net fluid and NaCl reabsorption remains to be seen. Other loop of Henle transporters may also be altered in these animals as a result of NaCl depletion or acidosis. Indeed, it has been shown that high dietary NaCl intake is associated with increased NH₃; absorption (14), as well as increased BSC1 protein expression (12), suggesting that increases in NaCl and fluid delivery to the loop of Henle upregulate the activity of the Na-K-2Cl cotransporter. Increased activity of the Na-K-ATPase has also been reported in salt-loaded rats (24). Unfortunately, the relevance of these findings on NHE3-deficient animals may be difficult to predict, since they have the unusual circumstance of being volume depleted (usually associated with NaCl restriction) and having increased delivery to the loop of Henle (usually associated with NaCl loading).

The proximal tubule micropuncture results presented here bear a strong resemblance to those in previous studies in rats (11, 23) and mice (16) evaluating the effects of carbonic anhydrase inhibition on proximal tubular function. The Na⁺/H⁺ exchange-dependent pathway for fluid reabsorption in the proximal tubule is mediated by a process requiring carbonic anhydrase activity (6). Inhibition of carbonic anhydrase by either acetazolamide or benzolamide has been shown to inhibit proximal tubule fluid reabsorption in the rat by 50–60% or more (11, 23). Furthermore, in a recent study, we showed that carbonic anhydrase inhibition also reduces proximal fluid reabsorption in mice by ~40% (16). These values are consistent with the present findings that proximal fluid reabsorption is 41% lower in Nhe3−/− than in Nhe3+/− mice. Furthermore, the high degree of similarity between these two sets of data in the mouse suggest that there is little, if any, upregulation of other proximal tubule transport pathways in Nhe3−/− mice to compensate for the lack of Na⁺/H⁺ exchange. It is also interesting to note that, in these previous investigations of the rat and the mouse, carbonic anhydrase inhibition results in a decrease in SNGFR of ~30%, which is consistent with the decrease seen in distal collections from Nhe3−/− mice.

In summary, proximal fluid reabsorption in situ was lower in Nhe3−/− mice than in Nhe3+/− mice, and lower still in Nhe3−/− mice. The decrements in proximal tubule function appear to be directly proportional to the level of NHE3 mRNA expression. Despite these observed decreases in proximal reabsorption, the delivery of fluid to the early distal tubule was not different among the three groups of animals. In Nhe3−/− animals, this compensation appeared to be primarily due to TGF-dependent and -independent decreases in SNGFR. Although TGF was shown to be intact in Nhe3−/− animals, more studies will be required to determine whether modulation of the TGF signaling pathway is altered in the absence of NHE3. The TGF-independent decrease in SNGFR may be related to volume depletion in these animals. In contrast, compensation in Nhe3−/− animals seemed to be related more to increased transport in the loop of Henle segment.

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