Am J Physiol Gastrointest Liver Physiol
280: G694–G700, 2001.

Targeted disruption of the *Nhe1* gene fails to inhibit β_1-adrenergic receptor-induced parotid gland hypertrophy

JAMES E. MELVIN,[1,2] HA-VAN NGUYEN,[1] KEITH NEHRKE,[1,2] CLAIRE M. SCHREINER,[3] KELLY G. TEN HAGEN,[1] AND WILLIAM SCOTT[3]
[1]*Center for Oral Biology, Aab Institute of Biomedical Sciences,* [2]*Eastman Department of Dentistry, University of Rochester Medical Center, Rochester, New York 14642; and* [3]*Division of Developmental Biology, Children's Hospital Research Foundation, Cincinnati, Ohio 45229*

Received 31 July 2000; accepted in final form 25 October 2000

Melvin, James E., Ha-Van Nguyen, Keith Nehrke, Claire M. Schreiner, Kelly G. Ten Hagen, and William Scott. Targeted disruption of the *Nhe1* gene fails to inhibit β_1-adrenergic receptor-induced parotid gland hypertrophy. *Am J Physiol Gastrointest Liver Physiol* 280: G694–G700, 2001.—Chronic β_1-adrenergic receptor activation results in hypertrophy and hyperplasia of rodent salivary gland acinar cells. Na^+/H^+ exchanger isoform 1 (NHE1) regulates cell volume and the induction of cell proliferation in many tissues. To investigate the relationship between NHE1 and the response of parotid glands to β_1-adrenergic agonists, we examined by Northern blot analysis NHE1 expression in saline-treated mice and mice 30 min and 2, 6, and 24 h after isoproterenol injection. NHE1 transcripts increased ~50% by 2 h, and a more than twofold increase was noted at 24 h. Isoproterenol did not acutely increase Na^+/H^+ exchanger activity; however, exchanger activity was significantly elevated by 24 h. To test whether NHE1 activity is essential for inducing salivary gland hypertrophy in vivo, mice with targeted disruption of *Nhe1* were treated with isoproterenol. Na^+/H^+ exchanger activity was absent in acinar cells from *Nhe1*$^{-/-}$ mice, nevertheless, the lack of NHE1 failed to inhibit isoproterenol-induced hypertrophy. These data directly demonstrate that acinar cell hypertrophy induced by chronic β_1-adrenergic receptor stimulation occurs independently of NHE1 activity.

Na^+/H^+ exchanger activity; salivary gland; acinar cells

IN RESPONSE TO CHRONIC β_1-adrenergic receptor stimulation, rodent parotid glands undergo a 3- to 5-fold increase in mass (6, 34). Gland enlargement is due to both hyperplasia and hypertrophy of the secretory acinar cells (2, 34). The early responses to β_1-adrenergic receptor stimulation include increased expression of transcription factors and signal transduction molecules involved in RNA transcription (21, 43), DNA synthesis (3, 8, 42), and RNA synthesis of salivary gland-specific proteins (1, 9). However, the mechanism by which β_1-adrenergic receptor activation initiates gland hypertrophy remains unclear.

An increase in the intracellular pH of mammalian cells is often mediated by stimulation of Na^+/H^+ exchanger (NHE) activity. This enhanced Na^+/H^+ exchange may be necessary for initiating proliferation in many, but not all, tissues and cell lines (16, 17, 25, 30, 37, 38). Cells lacking NHE activity fail to grow in media of low pH (19) or when NHE activity is inhibited (12). In addition to its role in cell proliferation, activation of Na^+/H^+ exchanger activity has also been linked to cell hypertrophy (15). Although considerable evidence supporting the involvement of Na^+/H^+ exchange in the initiation of cell proliferation/hypertrophy has been generated, this relationship is primarily based on indirect evidence derived from experiments that (necessarily) employed solutions lacking a HCO_3^--CO_2 buffering system and/or, in some cases, inhibitors to infer the role of Na^+/H^+ exchanger function (17). Therefore, the correlation of enhanced Na^+/H^+ exchanger gene expression and activity to cell proliferation and hypertrophy remains to be proven.

The mammalian NHE gene family consists of six isoforms (10, 23). Of these, the ubiquitously expressed NHE1 isoform is the major regulator of the intracellular pH in rodent salivary gland acinar cells (14, 20, 22, 24, 28). NHE1 is involved in cell volume regulation (18, 19) and is a target for growth factor-induced cell proliferation (31, 41). Indeed, cell proliferation correlates with increased levels of NHE1 mRNA (13, 26), which is likely due to direct activation of the NHE1 promoter (5). Thus NHE1 may play a key role in initiating both the hypertrophy and hyperplasia of salivary acinar cells associated with chronic β_1-adrenergic receptor activation, although this relationship has never been directly tested.

To examine the potential connection among β_1-adrenergic receptor stimulation, intracellular pH homeostasis, and gland hypertrophy, we studied by Northern blot analysis NHE1 expression and the effects of *Nhe1* gene disruption on mouse parotid gland hypertrophy. An early response to β_1-adrenergic receptor activation was enhanced expression of NHE1 tran-

Address for reprint requests and other correspondence: J. E. Melvin, Center for Oral Biology, Univ. of Rochester, Medical Center Box 611, 601 Elmwood Ave., Rochester, NY 14642 (E-mail: james_melvin@urmc.rochester.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

scripts, and this increase correlated with increased Na^+/H^+ exchanger activity. Nevertheless, the extent of salivary gland enlargement in *Nhe1*$^{-/-}$ mice in response to β_1-adrenergic receptor stimulation was comparable to that observed in wild-type mice, clearly demonstrating that functional NHE1 protein is not required for in vivo induction of acinar cell proliferation and/or hypertrophy.

METHODS

Materials. Collagenase P was purchased from Boehringer Mannheim (Indianapolis, IN), and 2′,7′-bis(carboxyethyl)-5-carboxyfluorescein-pentaacetoxymethyl ester (BCECF-AM) and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) were from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma Chemical (St. Louis, MO). Six- to seven-week-old male C57BL/6 mice were obtained from Harlan (Indianapolis, IN). Targeted disruption of the murine *Nhe1* gene was performed as previously described (4). Heterozygous offspring were used to establish a breeding colony in the University of Rochester vivarium. Experiments were performed on animals aged between 1.5 and 4 mo. All animals were fed ad libitum on a standard diet and water.

Isoproterenol treatment. Mice were given a single intraperitoneal injection of (±)-isoproterenol hydrochloride (25 mg/kg prepared in 140 mM NaCl). Control mice received vehicle only. After 30 min, 2, 6, and 24 h, and 7 days of isoproterenol exposure, mice were euthanized by exsanguination after CO_2 anesthesia, and the parotid glands were removed and snap frozen in liquid nitrogen for subsequent RNA isolation. For functional studies, parotid glands were removed from mice treated for 24 h or 7 days with either isoproterenol or vehicle, and acinar cells were isolated as previously described (14). For morphological analysis, wild-type, heterozygous, and mice with targeted disruption of the *Nhe1* gene were treated daily with saline or isoproterenol for 7 days. Twenty-four hours after the final injection, mice were euthanized and parotid, submandibular, and sublingual gland weights were measured.

Morphology. Parotid glands were fixed in 10% formalin, paraffin imbedded, sectioned at 10 μm, and stained with hematoxylin and eosin. Images were generated using a SPOT digital camera (Diagnostics Instruments) with a Nikon Plan Apo ×10/0.3 objective or a Nikon Plan Apo 60×/1.4 oil objective and a Nikon Eclipse E800 microscope. The ratio of acinar cells to duct cells was quantitated essentially as previously described (33). In brief, intersecting gridlines were superimposed on randomly selected computer images generated at ×100 magnification, and the intersections over acinar, ductal, and nonparenchymal tissues were recorded. The ratio of acini to ducts in the gland equals the total number of points over acini divided by the total number of points over ducts. To determine the size of the acinar elements, the long and short axes were measured on randomly selected computer images generated at ×600 magnification and converted to cross-sectional area.

Intracellular pH measurements. The acinar cell preparation was loaded with intracellular pH-sensitive fluoroprobe by incubation for 30 min at room temperature with BCECF-AM (2 μM). BCECF-loaded acinar cells were allowed to adhere to the base of a superfusion chamber mounted on a Nikon Diaphot 200 microscope interfaced with an imaging workbench (Axon Instruments, Foster City, CA). Cells were excited at 490 and 440 nm, and emitted fluorescence was measured at 530 nm. Solutions contained (in mM): 135 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 0.33 NaH_2PO_4, 0.8 $MgSO_4$, 1.2 $CaCl_2$, 10 glucose, and 20 HEPES, pH 7.4, with Tris base. To induce an intracellular acid load, 10 mM NaCl was replaced with NH_4Cl (29). Solutions were gassed with 100% O_2.

Intracellular pH was estimated by in situ calibration of the F_{490}/F_{440} fluorescence ratio with the use of the nigericin-high K^+ method of Thomas et al. (39). The high K^+ solution contained (in mM): 120 KCl, 20 NaCl, 0.8 $MgCl_2$, 20 HEPES, and 0.005 nigericin, and the pH was adjusted from 5.6 to 8. Data presented in the figures are from single representative experiments. Values quoted are the means ± SE for the number of acinar aggregates examined. All experiments were performed with three or more separate preparations.

Northern blot analysis. Total RNA was isolated from parotid glands with TRIzol reagent (Life Technologies, Rockville, MD) according to the manufacturer's instructions, fractionated by electrophoresis in a 1% formaldehyde-agarose gel (20 μg per lane), and transferred to Hybond-XL nylon membranes (Amersham Pharmacia, Piscataway, NJ). Parotid glands from the five animals comprising each group were combined to generate the total RNA. The blot was hybridized first with a ^{32}P-labeled cDNA probe containing nucleotides 803–1393 of the mouse NHE1 open reading frame (ORF) in ExpressHyb solution (Clontech Laboratory, Palo Alto, CA) by use of the hybridization and wash conditions recommended by the manufacturer. After autoradiography, the blot was stripped in 0.1% SDS at 90°C for 20 min and then hybridized as above to a ^{32}P-labeled cDNA probe containing nucleotides 2451–2720 of the rat β-actin ORF. Finally, to normalize RNA expression between preparations, the blot was restripped and then hybridized to an end-labeled oligonucleotide that recognizes mouse 18S ribosomal RNA (5′-TATTGGAGCTGGAATTACCGCGGCTGCTGG-3′). Quantitation of the autoradiographs was performed by densitometry using the Alpha Imager system (Alpha Innotech, San Leandro, CA).

RESULTS

Enhanced expression of NHE1 transcripts after β_1-adrenergic receptor stimulation. One potential mechanism for inducing parotid gland hypertrophy in response to isoproterenol stimulation is to increase the expression of NHE1 in acinar cells, the major exchanger isoform expressed in this cell type (14, 20, 22, 24, 28). Northern blot analysis of total RNA with the use of a cDNA probe verified that expression of the major 2.9-kb NHE1 mRNA was enhanced in parotid glands stimulated with isoproterenol (Fig. 1*A, top*). No detectable change was observed in NHE1 expression after 30-min exposure to isoproterenol, but after 2 h an increase was noted, and expression appeared to increase further 24 h after injection of isoproterenol. This blot was then rehybridized with β-actin and 18S probes. Figure 1*A* (*middle*) demonstrates that the level of 1.9-kb transcripts for the structural protein β-actin increased in a comparable fashion to NHE1 in the parotid glands of stimulated mice, whereas 18S expression was stable (±5% of saline treated; Fig. 1*A, bottom*). After normalization of expression of NHE1 to 18S (Fig. 1*B*), no change was observed for NHE1 mRNA expression after 30-min stimulation, but an ~50% increase was noted after 2 h, and NHE1 transcripts increased more than twofold after 24 h of stimulation. Although the mechanism for enhanced expression is unclear, these results demonstrate that NHE1 expres-

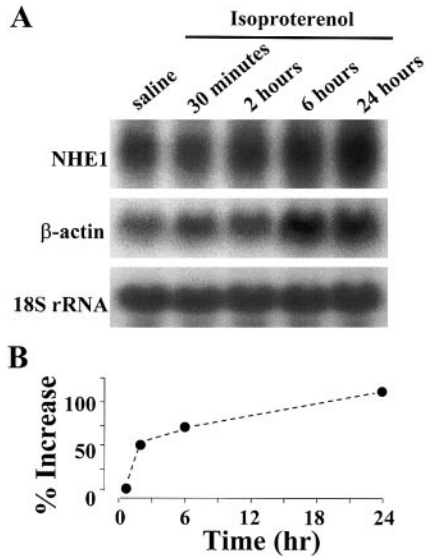

Fig. 1. Enhanced expression of Na^+/H^+ exchanger isoform 1 (NHE1) transcripts in response to β_1-adrenergic receptor stimulation in mouse parotid gland. Total RNA was isolated from the parotid glands of mice as described in METHODS after stimulation with 25 mg isoproterenol/kg body wt for 30 min or 2, 6, or 24 h. *A, top*: twenty micrograms of total RNA were loaded per lane and probed with a mouse NHE1-specific cDNA. *Middle*: the blot in the *top row* was stripped and probed with a rat β-actin-specific cDNA. *Bottom*: the blot was then restripped and probed with a mouse 18S-specific oligonucleotide. *B*: time course of the β_1-adrenergic receptor-induced increase in the expression of NHE1 as normalized between preparations by the expression of 18S ribosomal RNA.

sion in mouse parotid glands is upregulated within 2 h after a single injection of isoproterenol.

β_1-adrenergic receptor stimulation increases Na^+/H^+ exchanger activity in parotid acinar cells. The increased expression of NHE1 mRNA suggests that this Na^+/H^+ exchanger may be required for the isoproterenol-induced gland hypertrophy (Fig. 1). Consequently, if NHE1 plays such a role, it was predicted that enhanced expression would result in increased activity. In agreement with this hypothesis, the Na^+/H^+ exchanger activity in acinar cells isolated from isoproterenol-treated mice was greater than the exchanger activity in cells from saline-treated animals (Fig. 2*A*). The rectangular area in Fig. 2*A* was enlarged (*inset*) to show clearly that the initial rate of the recovery on extracellular Na^+ addition was about twofold faster for isoproterenol-treated mice than for saline-treated controls (Fig. 2*B*). Figure 2*C* also shows that the intracellular pH "set point" was raised ~0.15 pH unit in acinar cells 24 h after isoproterenol stimulation. The aforementioned results are consistent with previous studies in which NHE1 activity was enhanced in a similar fashion after exposure to mitogenic agents in a heterologous expression system (41). Thus acinar cells were exposed to isoproterenol for 5 min to determine whether activation of acinar Na^+/H^+ exchange occurs acutely or requires chronic β_1-adrenergic receptor stimulation. Figure 3*A* shows that acute exposure to β_1-adrenergic agonist did not increase Na^+/H^+ exchanger activity in vitro, indicating that chronic exposure is required for the isoproterenol-induced response. Indeed, under these experimental conditions, isoproterenol acutely inhibited activity ~15%. In contrast, the Ca^{2+}-mobilizing agonist carbachol or cell shrinkage acutely upregulated NHE1 activity in mouse parotid acinar cells (14), and this response is comparable in magnitude to that detected during chronic isoproterenol treatment (Fig. 2).

To verify that the increased Na^+/H^+ exchanger activity shown in Fig. 2 was associated with NHE1 expression, NHE1-deficient mice were treated with isoproterenol. Figure 3*B* shows that 24 h after a single injection and after treatment for 7 days with isoproterenol, parotid acinar cells isolated from *Nhe1*$^{-/-}$ mice lacked Na^+/H^+ exchanger activity (>95% loss of activity). Thus these results clearly demonstrate that increased Na^+/H^+ exchanger activity correlated with NHE1 expression and that chronic β_1-adrenergic receptor stimulation did not induce expression of another Na^+/H^+ exchanger in the *Nhe1*$^{-/-}$ mice to compensate for the loss of this pH regulatory pathway.

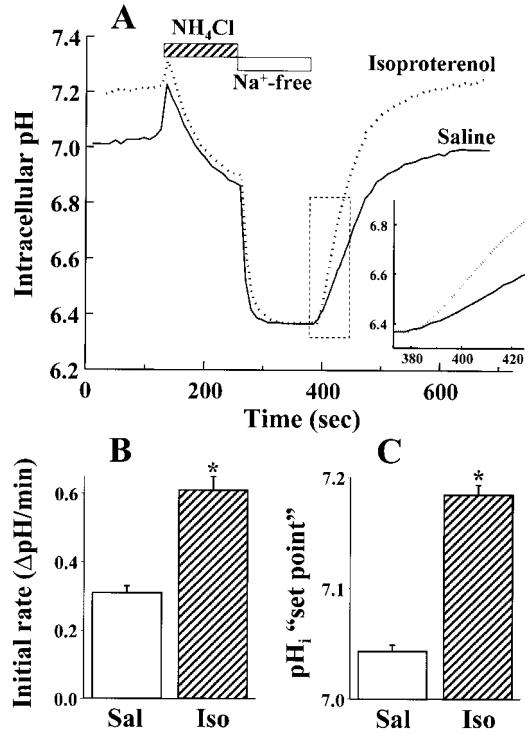

Fig. 2. β_1-Adrenergic receptor stimulation for 24 h increases NHE1 activity in acinar cells from mouse parotid gland. Parotid glands were isolated from mice 24 h after treatment with either saline (Sal; solid line) or 25 mg isoproterenol (Iso)/kg body wt (dotted line), collagenase digested, and loaded with the pH-sensitive dye 2′,7′-bis(carboxyethyl)-5-carboxyfluorescein-pentaacetoxymethyl ester (BCECF-AM). The intracellular pH was determined as described in METHODS. *A*: an intracellular acid load was induced by an NH_4Cl pulse during the time period indicated by the hatched rectangle. Recovery from the acid load was Na^+ dependent (time period indicated by the dotted rectangle) and amiloride-sensitive (data not shown). *B*: summary of the effects of isoproterenol treatment on the initial rate of the intracellular pH recovery. *C*: summary of the effects of isoproterenol treatment on the set point of the intracellular pH. (*$P < 0.05$, $n \geq 44$).

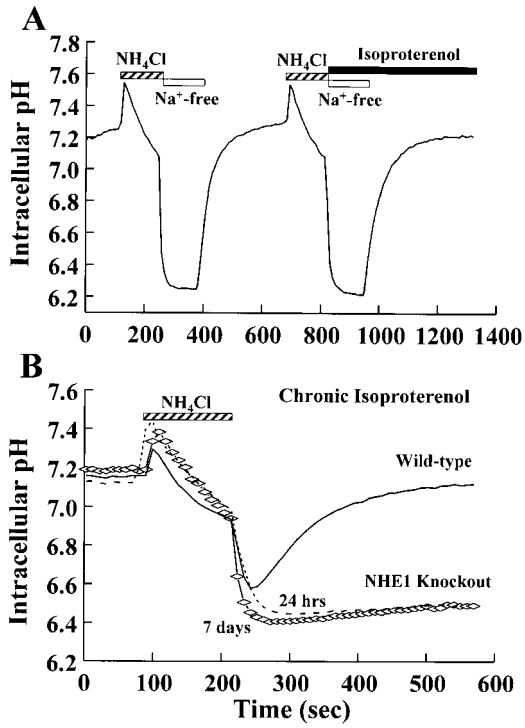

Fig. 3. Chronic β_1-adrenergic receptor stimulation-induced increases in parotid acinar cell Na^+/H^+ exchanger activity are dependent on NHE1 expression. Parotid gland acinar cells were isolated from *Nhe1*$^{+/+}$ mice and loaded with the pH-sensitive dye BCECF. *A*: to compare Na^+/H^+ exchanger activity of the acinar cells in the presence or absence of β_1-adrenergic receptor stimulation, an intracellular acid load was induced by an NH_4Cl pulse, and the Na^+-dependent pH recovery was recorded. A paired-pulse protocol was used wherein the cells were acid loaded, and after recovery, the same cells were acid loaded a second time after pretreatment for ~5 min with 10 μM isoproterenol. A representative trace is shown ($n \geq 10$). *B*: parotid glands were isolated from *Nhe1*$^{+/+}$ (solid line), and *Nhe1*$^{-/-}$ mice were treated for 24 h (dashed line) or 7 days (diamond symbols) with 25 mg isoproterenol/kg body wt and loaded with the pH-sensitive dye BCECF, and an intracellular acid load was induced by the NH_4Cl pulse technique. Na^+-dependent recovery from the acid load was nearly absent in parotid acinar cells from *Nhe1*$^{-/-}$ mice ($n \geq 6$).

Loss of Nhe1 gene expression fails to disrupt β_1-adrenergic receptor stimulation-induced gland hypertrophy. To examine the effects of *Nhe1* gene disruption on the β_1-adrenergic receptor-stimulated gland hypertrophy, salivary gland wet weights from isoproterenol- and saline-treated null mutant, heterozygous, and wild-type mice were determined. On the basis of the enhanced Na^+/H^+ exchanger activity in parotid glands of isoproterenol-treated mice, our prediction was that hypertrophy would be inhibited in knockout mice. However, Fig. 4 illustrates that the wet weights of parotid and submandibular glands (Fig. 4, *A* and *B*, respectively) from NHE1-deficient mice were comparable to those in wild-type and heterozygous littermate mice after treatment with isoproterenol for 7 days. In agreement with previous results (32), no significant change in sublingual gland weight was induced by chronic isoproterenol treatment (Fig. 4*C*).

Morphology of the parotid gland in NHE1-deficient mice after isoproterenol treatment. It has previously been reported that chronic isoproterenol treatment increases gland mass by increasing both the number and the size of acinar cells (2, 3, 6, 32, 34). One possibility is that the increase in the wet weight observed in NHE1-deficient mice represents expansion of a nonacinar cell type. To test this hypothesis, parotid glands of *Nhe1*$^{+/+}$ and *Nhe1*$^{-/-}$ mice were examined by light microscopy.

In agreement with previous studies (4, 14), homozygous *Nhe1*$^{-/-}$ mice exhibited decreased rates of postnatal growth, resulting in significantly lower body weights than their wild-type littermates. In the present experiments, the mean body weight was 27.0 ± 1.3 g for wild-type animals ($n = 6$), 26.2 ± 1.3 g for heterozygous mice ($n = 6$), and 17.2 ± 0.9 g for *Nhe1* mutant mice ($n = 7$; $P < 0.01$ vs. *Nhe1*$^{+/+}$ or *Nhe1*$^{+/-}$, Student's *t*-test). However, the reduced body weight of NHE1-deficient mice was not associated with a decrease in parotid gland weight (Fig. 4): parotid weights were 48.2 ± 5.6 (*Nhe1*$^{+/+}$, $n = 12$), 49.7 ± 2.8 (*Nhe1*$^{+/-}$, $n = 12$) and 50.5 ± 3.4 mg (*Nhe1*$^{-/-}$, $n = 14$). No obvious morphological differences were observed between *Nhe1*$^{+/+}$ or *Nhe1*$^{-/-}$ parotid glands (Fig. 5, *A* and *C*, respectively) from saline-treated mice. Morphometric analyses were performed to verify these observations. Table 1 shows that the ratios of acinar to

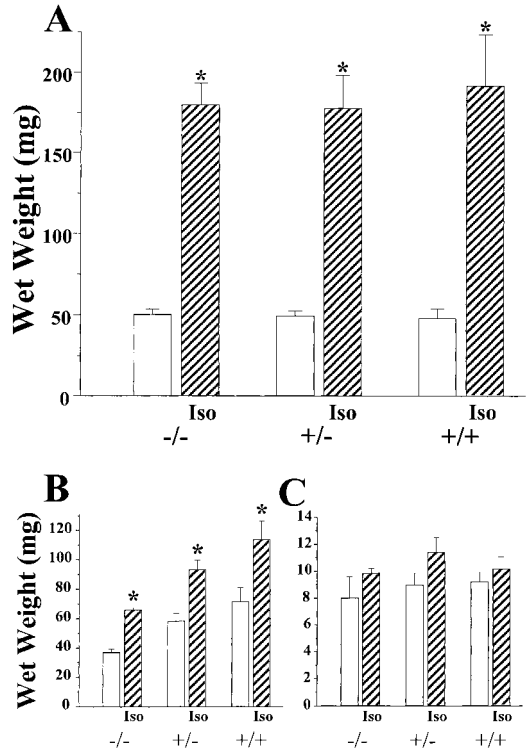

Fig. 4. Chronic β_1-adrenergic receptor stimulation induces parotid and submandibular gland hypertrophy in wild-type and *Nhe1* knockout mice. Parotid (*A*), submandibular (*B*), and sublingual (*C*) glands were isolated from *Nhe1*$^{+/+}$, *Nhe1*$^{+/-}$, and *Nhe1*$^{-/-}$ mice treated for 7 days with saline or with 25 mg isoproterenol/kg body wt, and gland wet weight was determined (*$P < 0.05$, $n \geq 3$).

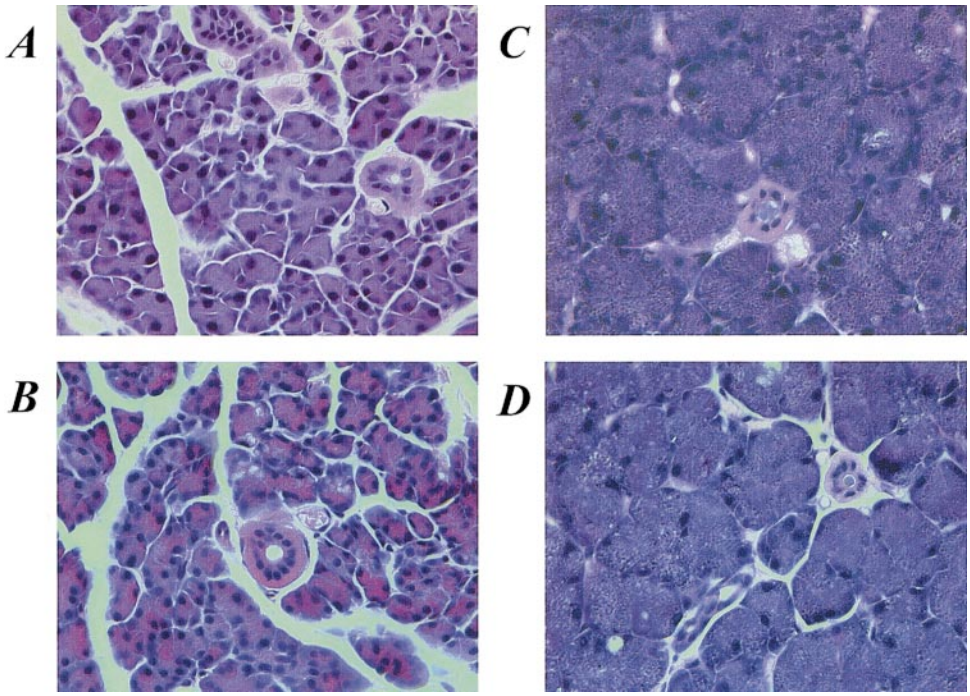

Fig. 5. Morphology of parotid glands from *Nhe1* wild-type and knockout mice after chronic β_1-adrenergic receptor stimulation. Parotid glands were isolated from *Nhe1* wild-type (*A* and *C*) and knockout mice (*B* and *D*), treated for 7 days with saline (*A* and *B*) or with 25 mg isoproterenol/kg body wt (*C* and *D*), and fixed in 10% formalin for 3 days. After dehydration and paraffin imbedding, 10-μm sections were stained with eosin and hematoxalin. The morphology of parotid acinar and ductal cells was comparable in both saline- and isoproterenol-treated mice.

ductal elements in the glands were equivalent. Moreover, a comparable degree of acinar cell hypertrophy was clearly evident in *Nhe1*$^{+/+}$ and *Nhe1*$^{-/-}$ parotid glands after chronic treatment with isoproterenol for 7 days (Fig. 5, *B* and *D*, respectively, and Table 1). Because the acinar-to-duct ratio increased about threefold in both wild-type and knockout mice, this suggests that the number and/or the size of the acinar cells increased dramatically with isoproterenol treatment. In fact, the cross-sectional area of acinar cells increased about fivefold in *Nhe1*$^{+/+}$ and *Nhe1*$^{-/-}$ parotid glands, demonstrating that the increase in the acinar-to-duct ratio was due primarily to an increase in the size of the acinar cells. Consistent with the glandular hypertrophy correlating with acinar cell enlargement, the percentage of nonparenchymal tissue decreased after chronic β_1-adrenergic receptor stimulation.

DISCUSSION

Several lines of evidence suggest that Na^+/H^+ exchange plays an active role in the induction of the hyperplastic response to mitogenic agents in many different cell types (5, 13, 16, 25, 30, 36, 38, 41). The mechanism through which Na^+/H^+ exchangers are thought to regulate this response is to maintain an alkaline cytoplasmic pH (17); however, direct verification of this proposed mechanism has never been reported. Indeed, current evidence supporting the involvement of Na^+/H^+ exchange in the initiation of cell proliferation/hypertrophy relies on indirect evidence in which HCO_3^--CO_2-free buffering systems or amiloride analogs were used to test the role of Na^+/H^+ exchanger function (17). Thus it not clear whether Na^+/H^+ exchange plays an active role in the induction of hyperplasia under physiological conditions or is independent of nonspecific effects of inhibitors on cell proliferation. Thus the objective of the current study was to determine, with the use of knockout mice, the role of NHE1 in the β_1-adrenergic receptor stimulation-induced salivary gland hypertrophy. The functional effects of disrupting the expression of the murine *Nhe1* gene have been described in several tissues (4, 11), including

Table 1. *Morphological analyses of parotid glands from Nhe1*$^{+/+}$ *and Nhe1*$^{-/-}$ *mice after isoproterenol treatment*

	Nhe1$^{+/+}$		*Nhe1*$^{-/-}$	
Treatment	Saline	Isoproterenol	Saline	Isoproterenol
Acinar/duct ratio	10.24 ± 0.99	35.41 ± 4.35*	13.92 ± 3.66	48.67 ± 5.38*
Acinus area	315.5 ± 54.8	1706.5 ± 152.5*	328.3 ± 16.1	1505.7 ± 101.4*
%Nonparenchyma	15.20 ± 1.86	4.93 ± 0.34*	8.73 ± 1.33†	4.77 ± 0.83*

Values are means ± SE. Parotid glands were isolated from *Nhe1* wild-type and knockout mice treated for 7 days with saline or with 25 mg isoproterenol/kg body wt and fixed in 10% formalin for 3 days. After dehydration and paraffin imbedding, 10-μm sections were stained with eosin and hematoxalin. The morphology of parotid acinar and ductal cells was analyzed using a stratified random sampling method (33) as described in METHODS. *Significant difference from saline-treated mice (n = 3 for each group); †significant difference between saline-treated *Nhe1*$^{+/+}$ and *Nhe1*$^{-/-}$ mice.

salivary glands (14, 22). It is interesting to note that NHE1 expression appears not to be critical during early development, but after birth, knockout mice begin to grow slower than their wild-type and heterozygous littermates and seizures and ataxia develop.

β_1-Adrenergic receptor stimulation increased NHE1 mRNA levels in the parotid gland within 2 h (Fig. 1). Many mitogenic agents have been shown to increase Na^+/H^+ exchanger activity and NHE1 expression in other systems as well (5, 7, 13, 25, 27, 35–37, 40, 41). Enhanced NHE1 expression in the parotid gland is possibly due to increased transcription. A similar phenomenon has been noted in NIH/3T3 cells expressing the mouse NHE1 promoter, in which a variety of mitogenic factors activated the NHE1 promoter, linking Na^+/H^+ exchanger activity to cell growth and proliferation (5). Regardless of the mechanism that mediates the increase in NHE1 transcript expression, β_1-adrenergic receptor stimulation also produced an alkaline shift in Na^+/H^+ exchanger activity, generating an increase in the intracellular pH (Fig. 2). This increased Na^+/H^+ exchanger activity was due to upregulation of NHE1, because knockout of the *Nhe1* gene virtually eliminated exchanger activity (Fig. 3). These results are consistent with the observation that the *Nhe1* gene product is the major regulator of intracellular pH in this cell type (14, 22) and also demonstrates that chronic isoproterenol treatment does not induce the expression of another Na^+/H^+ exchanger isoform to compensate for the loss of NHE1. Moreover, muscarinic receptor activation and cell shrinkage induce upregulation of Na^+/H^+ exchanger activity, and this enhanced activity is inhibited in parotid acinar cells isolated from NHE1-deficient mice (14).

In Na^+/H^+ exchanger-deficient Chinese hamster ovary cells, other NHE isoforms can support cell proliferation (19); however, NHE1 appears to be the major, if not the only, regulator of intracellular pH in this cell type (Fig. 4; see Refs. 14, 22). Despite the lack of upregulation of Na^+/H^+ exchanger activity after β_1-adrenergic receptor stimulation in NHE1-deficient mice, chronic isoproterenol treatment produced salivary gland enlargement (Fig. 5), largely due to acinar cell hypertrophy (Table 1). These results clearly demonstrate that upregulation of NHE activity is not necessary for the isoproterenol-induced hyperplasia/hypertrophy. Although Na^+/H^+ exchanger activity may be permissive in this regard in some cell types (12, 15, 19), this is clearly not the case in parotid acinar cells. Activation of other factors that regulate salivary gland-specific gene expression may lead to β_1-adrenergic receptor-stimulated gland hypertropy (21, 42, 43).

In conclusion, chronic β_1-adrenergic receptor stimulation increased the Na^+/H^+ exchanger activity in mouse parotid acinar cells by enhancing the expression of NHE1 transcripts. Nevertheless, *Nhe1* knockout mice clearly demonstrated that, in vivo, isoproterenol-induced salivary gland hypertrophy does not require the intracellular alkalinization associated with expression of this gene. Thus the factor(s) responsible for the hyperplasia/hypertrophy induced by isoproterenol in parotid acinar cells remains unknown. Future studies in this area may benefit from the recent development of microarray technologies that provide a simultaneous quantitative readout of thousands of gene transcripts. This latter approach will likely uncover the transcription factors, signaling pathways, structural proteins, and other elements involved in the development of salivary gland hypertrophy.

We are indebted to Drs. Lawrence Tabak and Art Hand for their input and support during the course of these studies. We thank Linda Richardson and Marlene Balys for technical assistance with genotyping, injection of the animals, and isolation of the salivary gland tissues.

This work was supported in part by National Institutes of Health Grants DE-13539, DE-08921, and DE-09692 (J. E. Melvin).

REFERENCES

1. **Ann DK, Clements S, Johnstone EM, and Carlson DM.** Induction of tissue-specific proline-rich protein multigene families in rat and mouse parotid glands by isoproterenol. Unusual strain differences of proline-rich protein mRNAs. *J Biol Chem* 262: 899–904, 1987.
2. **Barka T.** Induced cell proliferation: the effect of isoproterenol. *Exp Cell Res* 37: 662–669, 1965.
3. **Barka T.** Stimulation of DNA systhesis by isoproterenol in the salivary glands. *Exp Cell Res* 39: 355–364, 1965.
4. **Bell SM, Schreiner CM, Schultheis PJ, Miller ML, Evans RL, Vorhees CV, Shull GE, and Scott WJ.** Targeted disruption of the murine *Nhe1* locus induces ataxia, growth retardation, and seizures. *Am J Physiol Cell Physiol* 276: C788–C795, 1999.
5. **Besson P, Fernandez-Rachubinski F, Yang W, and Fliegel L.** Regulation of Na^+/H^+ exchanger gene expression: mitogenic stimulation increases NHE1 promoter activity. *Am J Physiol Cell Physiol* 274: C831–C839, 1998.
6. **Brown-Grant K.** Enlargement of salivary gland in mice treated with isopropylnoradrenaline. *Nature* 191: 1076–1078, 1961.
7. **Bussolino F, Wang JM, Turrini F, Alessi D, Ghigo D, Costamagna C, Pescarmona G, Mantovani A, and Bosia A.** Stimulation of the Na^+/H^+ exchanger in human endothelial cells activated by granulocyte- and granulocyte-macrophage-colony-stimulating factor. Evidence for a role in proliferation and migration. *J Biol Chem* 264: 18284–18287, 1989.
8. **Bybee A and Tuffery AR.** The short-term effects of a single injection of isoproterenol on proliferation in the submandibular gland, parotid gland and oesophagus in vivo. *Cell Tissue Kinet* 21: 133–141, 1988.
9. **Cooper LF, Elia DM, and Tabak LA.** Secretagogue-coupled changes in the expression of glutamine/glutamic acid-rich proteins (GRPs). Isoproterenol induces changes in GRP transcript expression and changes in isoforms secreted. *J Biol Chem* 266: 3532–3539, 1991.
10. **Counillon L and Pouyssegur J.** The expanding family of eucaryotic Na(+)/H(+) exchangers. *J Biol Chem* 275: 1–4, 2000.
11. **Cox GA, Lutz CM, Yang CL, Biemesderfer D, Bronson RT, Fu A, Aronson PS, Noebels JL, and Frankel WN.** Sodium/hydrogen exchanger gene defect in slow-wave epilepsy mutant mice. *Cell* 91: 139–148, 1997.
12. **Delvaux M, Bastie MJ, Chentoufi J, Cragoe EJJ, Vaysse N, and Ribet A.** Amiloride and analogues inhibit Na^+-H^+ exchange and cell proliferation in AR42J pancreatic cell line. *Am J Physiol Gastrointest Liver Physiol* 259: G842–G849, 1990.
13. **Elsing C, Reichen J, Marti U, and Renner EL.** Hepatocellular Na+/H+ exchange is activated at transcriptional and post-transcriptional levels in rat biliary cirrhosis. *Gastroenterology* 107: 468–478, 1994.
14. **Evans RL, Bell SM, Schultheis PJ, Shull GE, and Melvin JE.** Targeted disruption of the Nhe1 gene prevents muscarinic agonist-induced up-regulation of Na(+)/H(+) exchange in mouse parotid acinar cells. *J Biol Chem* 274: 29025–29030, 1999.

15. **Fine LG, Holley RW, Nasri H, and Badie-Dezfooly B.** BSC-1 growth inhibitor transforms a mitogenic stimulus into a hypertrophic stimulus for renal proximal tubular cells: relationship to Na+/H+ antiport activity. *Proc Natl Acad Sci USA* 82: 6163–6166, 1985.
16. **Fitzgerald RC, Omary MB, and Triadafilopoulos G.** Altered sodium-hydrogen exchange activity is a mechanism for acid-induced hyperproliferation in Barrett's esophagus. *Am J Physiol Gastrointest Liver Physiol* 275: G47–G55, 1998.
17. **Grinstein S, Rotin D, and Mason MJ.** Na+/H+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim Biophys Acta* 988: 73–97, 1989.
18. **Grinstein S, Woodside M, Sardet C, Pouyssegur J, and Rotin D.** Activation of the Na+/H+ antiporter during cell volume regulation. Evidence for a phosphorylation-independent mechanism. *J Biol Chem* 267: 23823–23828, 1992.
19. **Kapus A, Grinstein S, Wasan S, Kandasamy R, and Orlowski J.** Functional characterization of three isoforms of the Na+/H+ exchanger stably expressed in Chinese hamster ovary cells. ATP dependence, osmotic sensitivity, and role in cell proliferation. *J Biol Chem* 269: 23544–23552, 1994.
20. **Lee MG, Schultise P, Yan M, Shull G, Bookstein C, Chang E, Tse M, Donowitz M, Park K, and Muallem S.** Membrane limited expression and regulation of NHE isoforms by P2 receptors in the rat submandibular duct. *J Physiol (Lond)* 513: 341–357, 1998.
21. **Lin HH, Tu ZJ, and Ann DK.** Involvement of nuclear orphan receptor NGFI-B in transcriptional activation of salivary-specific R15 gene by cAMP. *J Biol Chem* 271: 27637–27644, 1996.
22. **Nguyen HV, Shull GE, and Melvin JE.** Muscarinic receptor-induced acidification in sublingual mucous acinar cells: loss of pH recovery in Na^+-H^+ exchanger-1 deficient mice. *J Physiol (Lond)* 523: 139–146, 2000.
23. **Orlowski J and Grinstein S.** Na+/H+ exchangers of mammalian cells. *J Biol Chem* 272: 22373–22376, 1997.
24. **Park K, Olschowka JA, Richardson LA, Bookstein C, Chang EB, and Melvin JE.** Expression of multiple Na^+/H^+ exchanger isoforms in rat parotid acinar and ductal cells. *Am J Physiol Gastrointest Liver Physiol* 276: G470–G478, 1999.
25. **Quinn DA, Dahlberg CG, Bonventre JP, Scheid CR, Honeyman T, Joseph PM, Thompson BT, and Hales CA.** The role of Na+/H+ exchange and growth factors in pulmonary artery smooth muscle cell proliferation. *Am J Respir Cell Mol Biol* 14: 139–145, 1996.
26. **Rao GN, Sardet C, Pouyssegur J, and Berk BC.** Differential regulation of Na+/H+ antiporter gene expression in vascular smooth muscle cells by hypertrophic and hyperplastic stimuli. *J Biol Chem* 265: 19393–19396, 1990.
27. **Ritter M, Woll E, Haller T, Dartsch PC, Zwierzina H, and Lang F.** Activation of Na+/H(+)-exchanger by transforming Ha-ras requires stimulated cellular calcium influx and is associated with rearrangement of the actin cytoskeleton. *Eur J Cell Biol* 72: 222–228, 1997.
28. **Robertson MA, Woodside M, Foskett JK, Orlowski J, and Grinstein S.** Muscarinic agonists induce phosphorylation-independent activation of the NHE-1 isoform of the Na+/H+ antiporter in salivary acinar cells. *J Biol Chem* 272: 287–294, 1997.
29. **Roos A and Boron WF.** Intracellular pH. *Physiol Rev* 61: 296–434, 1981.
30. **Rotin D, Steele-Norwood D, Grinstein S, and Tannock I.** Requirement of the Na+/H+ exchanger for tumor growth. *Cancer Res* 49: 205–211, 1989.
31. **Sardet C, Fafournoux P, and Pouyssegur J.** Alpha-thrombin, epidermal growth factor, and okadaic acid activate the Na+/H+ exchanger, NHE-1, by phosphorylating a set of common sites. *J Biol Chem* 266: 19166–19171, 1991.
32. **Schneyer CA and Shackleford JM.** Accelerated development of salivary glands of early postnatal rats following isoproterenol. *Proc Soc Exper Biol Med* 112: 320–324, 1963.
33. **Scott J, Berry MR, Gunn DL, and Woods K.** The effects of a liquid diet on initial and sustained, stimulated parotid salivary secretion and on parotid structure in the rat. *Arch Oral Biol* 35: 509–514, 1990.
34. **Selye HR, Veilleux R, and Cantin M.** Excessive stimulation of salivary gland growth by isoproterenol. *Science* 133: 44–45, 1961.
35. **Simonson MS, Wann S, Mene P, Dubyak GR, Kester M, Nakazato Y, Sedor JR, and Dunn MJ.** Endothelin stimulates phospholipase C, Na+/H+ exchange, c-fos expression, and mitogenesis in rat mesangial cells. *J Clin Invest* 83: 708–712, 1989.
36. **Sweeney FP, Quinn PA, and Ng LL.** Enhanced mitogen-activated protein kinase activity and phosphorylation of the Na+/H+ exchanger isoform-1 of human lymphoblasts in hypertension. *Metab Clin Exp* 46: 297–302, 1997.
37. **Sweeney FP, Siczkowski M, Davies JE, Quinn PA, McDonald J, Krolewski B, Krolewski AS, and Ng LL.** Phosphorylation and activity of Na+/H+ exchanger isoform 1 of immortalized lymphoblasts in diabetic nephropathy. *Diabetes* 44: 1180–1185, 1995.
38. **Takewaki S, Kuro-o M, Hiroi Y, Yamazaki T, Noguchi T, Miyagishi A, Nakahara K, Aikawa M, Manabe I, and Yazaki Y.** Activation of Na(+)-H+ antiporter (NHE-1) gene expression during growth, hypertrophy and proliferation of the rabbit cardiovascular system. *J Mol Cell Cardiol* 27: 729–742, 1995.
39. **Thomas JA, Buchsbaum RN, Zimniak A, and Racker E.** Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* 18: 2210–2218, 1979.
40. **Vairo G, Argyriou S, Bordun AM, Gonda TJ, Cragoe EJJ, and Hamilton JA.** Na+/H+ exchange involvement in colony-stimulating factor-1-stimulated macrophage proliferation. Evidence for a requirement during late G1 of the cell cycle but not for early growth factor responses. *J Biol Chem* 265: 16929–16939, 1990.
41. **Wakabayashi S, Bertrand B, Shigekawa M, Fafournoux P, and Pouyssegur J.** Growth factor activation and "H(+)-sensing" of the Na+/H+ exchanger isoform 1 (NHE1). Evidence for an additional mechanism not requiring direct phosphorylation. *J Biol Chem* 269: 5583–5588, 1994.
42. **Zeng T, Yamamoto H, Bowen E, Broverman RL, Nguyen KH, and Humphreys-Beher MG.** Cell cycle control in isoproterenol-induced murine salivary acinar cell proliferation. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 115: 271–279, 1996.
43. **Zhou J, Wright PS, Wong E, Jessen K, Morand JN, and Carlson DM.** Cyclic AMP regulation of mouse proline-rich protein gene expression: isoproterenol induction of AP-1 transcription factors in parotid glands. *Arch Biochem Biophys* 338: 97–103, 1997.