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Expression of Nox1 in 3T3 cells increases cellular acid production but not proton conductance

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Abstract

The role of the NADPH oxidase homolog 1 (Nox1) in plasma membrane H^+ conductance and cellular H^+ production was investigated in 3T3 cells stably expressing Nox1 (Nox1 3T3) compared to vector-expressing control cells (mock 3T3). In whole cell patch clamp experiments both Nox1 and mock 3T3 expressed a similar H^+ conductance (Nox1 3T3, 13.2 ± 8.6 pS/pF; mock 3T3, 16.6 ± 13.4 pS/pF) with a number of similar characteristics (e.g., current-voltage relations, current activation kinetics, Zn^{2+} -sensitivity). When the intracellular pH of cells was alkalinized with NH_4Cl , rates of intracellular acidification were significantly higher in Nox1 3T3 compared to mock 3T3. Nox1 3T3 showed a time course of acidification that followed a double exponential function with a fast and a slow component of, on average, $\tau = 165$ s and 1780 s, whereas mock 3T3 showed only a single slow τ of 1560 s. Expression of Nox1 also caused cells to acidify the extracellular medium at higher rates than control cells; Nox1 3T3 released 96 ± 19 fmole- h^{-1} -cell $^{-1}$ of acid equivalents compared to 19 ± 12 fmole- h^{-1} -cell $^{-1}$ in mock 3T3. These data show that expression of Nox1 results in a mechanism that has the capacity to rapidly acidify the cytosol and generate significant amounts of acid. No significant effect of Nox1 expression on the plasma membrane H^+ conductance was found.

INTRODUCTION

Nox1 was the first homolog of the NADPH oxidase gene family found in non-myeloid cells. It shares 58% identity with the phagocytic NADPH oxidase Nox2 and is expressed mainly in the epithelium of the colon [1;2]. Owing to the structural similarity to Nox2 and the prominent expression in the colon epithelium, the function of Nox1 may similarly be in host defense [3], although a function in cellular signaling has also been proposed [2;4].

In phagocytes, the NADPH oxidase moves electrons from intracellular NADPH across the plasma membrane to form extracellular superoxide ($O_2^{\bullet-}$). During this process H^+ is released from intracellular NADPH according to $NADPH-H^+ \rightarrow NADP^+ + 2 H^+ + 2$ electrons, and additional H^+ are released during metabolic recovery of NADPH [5]. This process has been shown to significantly contribute to intracellular acidification in phagocytes [5;6]. To counteract the cytosolic acidification during NADPH oxidation, it has been suggested that NADPH oxidase functions at the same time as a H^+ channel to support H^+ release from cells [7], and recombinant expression of several NADPH oxidase isoforms have been shown to result in a plasma membrane H^+ conductance, including Nox2 [8], Nox5 [9], and a truncated form

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of Nox1 [1]. However, this conclusion was challenged early on owing to i) the expression of the Nox constructs in cell systems that already expressed native H^+ currents, ii) a lack of H^+ selectivity of the resulting currents, and iii) the much shorter time constants of current activation recorded after Nox expression in these studies when compared to time constants of native H^+ currents [10;11]. Several additional studies then presented evidence that expression of Nox2 did not correlate with H^+ channel function, but suggested a parallel non-Nox H^+ channel (summarized in [11]). Thus, the identity of the H^+ channel and the H^+ conduction by NADPH oxidase has been discussed controversially. Because the putative H^+ permeation pathway across Nox2 involves conserved histidine residues [8;12;13], this argument can be extended to Nox1

H^+ -selective, voltage-activated currents have been found in many cell types [14]. Common characteristics of H^+ currents are strong outward rectification, activation by an intra-to-extracellular H^+ gradient, and activation by depolarizing potentials. Owing to these biophysical characteristics significant H^+ currents are carried only outwardly, and as a result H^+ channel activity results in an alkalization of the cytosol [14]. In addition, H^+ currents typically are blocked by Zn^{2+} , and show slow time constants (several seconds) of current activation during depolarizing pulses. Recently, a distinct H^+ channel gene (HVCN1) was identified based on a structural relation to voltage-gated K^+ channels [15;16]. Recombinant expression of HVCN1 resulted in plasma membrane H^+ currents with characteristics resembling native H^+ currents.

In this report, we investigated the function of Nox1 in cellular H^+ production and plasma membrane H^+ conduction using 3T3 cells recombinantly expressing Nox1. We found that expression of Nox1 resulted in a distinct, high-activity acid-generating mechanism that significantly increased the production and release of H^+ from the cell. At the same time, the expression of Nox1 in 3T3 cells showed no significant effects on the characteristics of the plasma membrane H^+ conductance.

METHODS

Cells

Mouse 3T3 fibroblasts stably expressing human Nox1 (Nox1 3T3) or vector (mock 3T3) originated in the laboratory of Dr. J. David Lambeth [2] and were kindly provided by Dr. Jason Eiserich, Univ. of California at Davis. Cells were maintained in DME-H21 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 μ g/ml pyromycin. Recently it has been noted that Nox1 3T3 cells may express in addition the V12-*ras* mutation [17] which could be expected to affect cell growth and metabolism. To investigate whether the cell lines used in this study expressed a *ras* mutation, we sequenced *ras* (Genbank BC011083) from two batches of Nox1 3T3 and mock 3T3, respectively, to cover the cell passages used for the experiments during the course of this study. We found the sequence of *ras* (corresponding to the fragment of amino acid positions 1 to 70) in all cell isolates identical to normal *ras*. In addition, no phenotypical changes that have been previously associated with *ras*-expression (such as focus formation or enhanced cell growth) or an alkalization of intracellular pH owing to an increased activity of the Na^+/H^+ exchanger [18;19] were noted in our study.

Nox1 PCR amplification

Total RNA was prepared from 2×10^5 3T3 fibroblasts and reverse transcribed using random hexamer primers. First strand cDNA was used as template in PCR reactions with Nox1-specific oligonucleotids (forward, tatgaagtggctgtgctggt; reverse, gaggttggtgctgcacactg) resulting in a 105 base pair fragment. Actin was used as a control (forward, gatcattgctcctcctgagc; reverse, tactctgcttgctgatcca). PCR was performed using 35 cycles. One tenth of the amplification products were analyzed on a 2% agarose gel.

Recording of proton currents

Cells were whole cell patch-clamped as previously described [20]. Briefly, cells were bathed in (in mM): 120 N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (Hepes), 70 gluconic acid, 100 trimethylammonium hydroxide (TMAOH), 2 Ca-gluconate, 1 Mg-gluconate, pH = 7.3. Pipettes were filled with (in mM): 100 2-(N-morpholino)-ethanesulfonic acid (MES), 60 gluconic acid, 90 TMAOH, 10 EGTA, 1 glucose, 1 Mg-gluconate, 3.3 Mg-ATP, 0.07 Li-GTP, pH = 5.3. Note that the solutions were Cl⁻ free in order to exclude the contribution of Cl⁻ currents during the measurements of the relatively small H⁺ currents. We allowed electrodes to equilibrate in these solutions for at least 30 min and found that electrode potentials were then quite stable (± 2 mV) over the period of recordings. The amplifier was zeroed with the pipette immersed in the bath, and the pipette interior was maintained under positive pressure to prevent entry of bath solution into the pipette. Leak currents were not subtracted and all recordings shown represent original currents. After establishing the whole cell configuration, the membrane potential was continuously clamped to -50 or -60 mV. The access resistance (R_a) and the cell membrane capacitance (C_m) were measured from current transients caused by a 10-mV pulse. Steady-state current-voltage (I/V) relationships were recorded during voltage pulse protocols from -80 mV to +40 mV. Open channel I/V relations were recorded after a 4.5-s depolarizing pulse to +40 mV followed by a fast 170-ms step ramp from +60 mV to -100 mV. Whole cell conductance was calculated from steady-state currents as the chord conductance of the voltage-activated currents measured between -50 mV and +20 mV. For the calculation of the specific membrane conductance (G_m in pS/pF) the whole cell conductance was corrected for R_a (Nox1 3T3, 21.6 ± 14.1 M Ω ; mock 3T3, 17 ± 8.8 M Ω) and normalized to C_m (Nox1 3T3, 26 ± 21 pF; mock 3T3, 23.6 ± 13.7 pF). Current activation during depolarizing pulses were fitted with single exponential functions resulting in estimates for time constants of current activation (τ_{act}).

Measurement of intracellular acid production

Measurements of intracellular pH (pH_i) were done as described [20]. Briefly, BCECF-AM (2', 7'-bis-(2-carboxyethyl)-5-(and-6)- carboxyfluorescein, acetoxymethyl ester)-loaded cells were investigated on an inverted microscope at 400x magnification, excited at 440 nm and 495 nm and emission was collected between 525 nm and 550 nm with a cooled CCD camera (Photometrics CoolSnap HQ, Roper Scientific) controlled by a computerized imaging system (Metafluor, Universal Imaging Corp.). pH_i was calculated from excitation ratios using a 4-point pH calibration in presence of 20 μ M nigericin in KCl solution. Calibration points were pH 7.5, 7, 6.5, and 6.0. Single cells were individually calibrated by fitting 3rd-order polynomials in pH-vs.-ratio plots, and fitted parameters were used to calculate pH_i for individual cells. All solutions were nominally bicarbonate free. NaCl solution composition was (in mM) 145 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 12.5 Hepes, pH = 7.4. pH calibration was performed in (in mM) 5 NaCl, 145 KCl, 1 CaCl₂, 1 MgCl₂, and Hepes and/or MES (total buffer 12.5 mM).

As an identifying characteristic of the activity of the cellular acid producing mechanism we used the time constants (τ) of re-acidification after an NH₄⁺ pulse. τ was determined by fitting an exponential function of the form $pH_i = a \cdot \exp(-t/\tau) + B$ to the data, where a is the amplitude, t is the time, and B a constant offset. Acidification in Nox1 3T3 cells was fitted with a corresponding double exponential function. Fits of pH_i were done for individual cells using the curve fitting routine incorporated in Sigmaplot 8 (Sysstat Software Inc., Richmond, CA).

Determination of extracellular acid production

Cells were seeded in 6-well plates with 2 ml medium per well. After 24, 48, 72, and 96 hours in culture the pH of the culture medium was measured at 37°C and 5% CO₂ using a pH electrode and meter with temperature correction (Corning 430). The number of cells per well was

determined using a particle counter (Coulter Z2, Beckman Coulter Inc.). The buffer capacity of the culture medium (β) was determined using pH titration with NaOH and HCl. We determined the growth rate ($\mu = \ln(N_1/N_0) / t$) of cultured cells assuming exponential growth following $N_1 = N_0 \cdot e^{\mu t}$, where N_0 and N_1 are the cell number at 24 and 96 hours, respectively, and t is 72 hours. Acid release per cell (expressed in $\text{fmole} \cdot \text{h}^{-1} \cdot \text{cell}^{-1}$) was then calculated from

$$J_{\text{H}} = \Delta H_0 \cdot \beta \cdot V_0 / \int_{t=24}^{t=96} (N_0 \cdot e^{\mu t} dt)$$

where ΔH_0 is the difference of the free H^+ concentration in the medium at $t = 96$ hours and 24 hours, and V_0 is the volume of growth medium per well (2 ml).

Statistics

Results are given as mean $\pm$ SD, n refers to the number of cells or cultures investigated. Measures from the two cell lines were compared using unpaired t tests or factorial ANOVAs followed by Bonferroni-corrected t tests. The level of significance p is reported and $p < 0.05$ was considered significant. n refers to the number of experimental runs. Calculations were done with StatView version 4.57 (Abacus Concepts, Berkeley, CA).

RESULTS

Effect of Nox1 expression on proton conductance

3T3 cells stably transfected with Nox1 (Nox1 3T3) or vector (mock 3T3, ref. [21]) were routinely tested for Nox1 expression using RT-PCR. Fig. 1 shows an example of Nox1 expression (and as control the expression of actin) in Nox1 and mock 3T3. All used batches of cell cultures showed clear expression of Nox1 transcripts in Nox1 3T3 and no detectable Nox1 mRNA in mock 3T3.

Fig. 2 shows depolarization-activated whole cell H^+ currents recorded from Nox1 and mock 3T3 cells with a pipette-to-bath 5.3-to-7.3 pH gradient. Figs. 2A&D show currents recorded in response to voltage pulses (-80 mV to +40 mV) from a negative (-60 mV) holding potential. In both cell types depolarizing pulses resulted in slow current activation typical for a depolarization-activated H^+ conductance. Figs. 2B&E show current responses to fast voltage step ramps after a depolarizing pulse to activate the H^+ conductance. The resulting openchannel current-voltage (I/V) relations were linear (Figs. 2C&F, *triangles*), while the steady-state I/V relations (Figs. 2C&F, *bullets*) showed outward rectification, i.e., were limited by the inactivation of the H^+ conductance at negative potentials. The H^+ conductance calculated for the current activated during a voltage pulse to 20 mV was on average 15.2 ± 11.7 pS/pF (Nox1 and mock 3T3 pooled) and was not affected by the expression of Nox1 ($p = 0.38$, Fig. 2G).

To further characterize the H^+ currents we investigated the kinetics of current activation during voltage steps. Activation currents elicited by depolarizing pulses were fitted with single exponential functions resulting in fit estimates for time constants (τ_{act}). Figs. 3A&B show examples of fits to sets of currents recorded from both cell types. Note the close fit of the single exponentials and the shortened τ_{act} at increasing potentials indicative for a simple voltage-dependent opening transition of the H^+ channel. Average τ_{act} was not affected by expression of Nox1 (Fig. 3C). In some experiments the deactivating “tail” currents appeared slower in mock than in Nox1 3T3 (i.e., in Figs. 2A&D), however these varied inconsistently.

Zn^{2+} sensitivity of H^+ conductance

Further, we used the dose-dependent block by ZnSO_4 as an identifying characteristic of the measured H^+ conductance. Previously, the sensitivity to Zn^{2+} has been used to distinguish two

types of H^+ conductances in eosinophils [22]. Fig. 4 shows the effects of $ZnSO_4$ on H^+ currents measured in both cell types. In the experiments shown in Figs. 4A&B, the membrane potential was held at -50 mV and pulsed to +20 mV to continuously monitor current activation. In both Nox1 (Fig. 4A) and mock 3T3 cells (Fig. 4B) addition of 200 μM $ZnSO_4$ readily blocked currents during the depolarizing pulses. Figs. 4C&D show effects of 180 μM and 360 μM $ZnSO_4$ on H^+ current activation. Note the qualitatively similar effects in both mock and Nox1 3T3 and the total block of current activation at high $ZnSO_4$ concentrations. Dose-response curves current block by $ZnSO_4$ are shown in Fig. 4E. Fit of data to Michaelis-Menten kinetics (Fig. 4E, *lines*) resulted in halfmaximal block at $276 \pm 50 \mu M$ (mock 3T3) and $265 \pm 114 \mu M$ (Nox1 3T3, $p > 0.5$). Expression of Nox1 had no measurable effects on blocker kinetics by $ZnSO_4$.

Effect of Nox1 on intracellular acid production

It has been shown that Nox2 activity results in an acidification of intracellular pH in neutrophils [6]. Here we investigated whether the expression of Nox1 similarly results in intracellular acid production. Basal pH_i was similar in both cell types (mock 3T3, 6.958 ± 0.157 , $n = 3$; Nox1 3T3, 6.963 ± 0.22 , $n = 6$). To investigate the production of intracellular acid, cells were treated with 30 mM NH_4Cl added to the bath solution (equimolar exchange for NaCl at constant pH). This resulted in a rapid alkalization of the cytosol (Fig. 5A) followed by a spontaneous acidification consistent with intracellular H^+ generation by the cells. Inspection of the time courses of pH_i acidification indicated a faster acidification in Nox1 3T3 compared to mock 3T3 (Fig. 5A). Kinetics of pH_i acidification in presence of NH_4Cl were quantified by determining the time constants (τ) of reacidification for individually measured cells, as shown in Fig. 5B. In Nox1 3T3 kinetics of acidification were best fitted by a double-exponential function (Fig. 5B, *open circles*) with time constants of, on average, $\tau = 165$ s and 1778 s (Fig. 5C) indicative for two different processes of intracellular acidification that were distinguished by their largely different time constants. For comparison, mock 3T3 acidified pH_i slowly and kinetics of acidification were fitted well with a single exponential (Fig. 5B, *filled circles*) resulting in a τ of, on average, 1556 s, which was not different from the slow time constant observed in Nox1 3T3 (Fig. 5C, $p = 0.39$). In mock 3T3 cells, no fast acidifying process was apparent. These kinetic data indicate that the expression of Nox1 resulted in a distinct acid-producing mechanism that is capable of rapidly acidifying the cytosol. The slow acidification present in both cell types may be related to slow NH_4^+ entry or metabolic acid production [23].

Extracellular acidification by Nox1-expressing cells

From the measurements of intracellular pH it was expected that Nox1 3T3 release more acid and acidify the culture medium faster than mock 3T3. This was investigated by measuring the pH of the medium over a 4-day period every 24 hours. This time frame was chosen because Nox1 and mock 3T3 cell cultures were previously shown to result in similar cell growth up to 6 days in culture [2]. Fig. 6 shows the measured averages of medium pH (Fig. 6A) and cell counts (Fig. 6B) of 8 experiments 24 and 96 hours after seeding (bar charts) and data from one typical experimental run at 24, 48, 72, and 96 hours (*line graphs*). Nox1 3T3 cells (*grey symbols*) acidified the medium significantly stronger than mock 3T3 cells (*black symbols*) with comparable numbers of cells per well (Fig. 6B). After 96 hours in culture the number of Nox1 3T3 cells was slightly lower compared to mock 3T3 cells, however, this did not reach statistical significance ($n = 8$, $p = 0.052$). Cell growth in this time window was not different from growth curves found in a previous study [2]. The acid production per cell was substantially higher in Nox1 3T3 cells (Fig. 6C). On average, expression of Nox1 resulted in an increase in acid production of $76.5 \pm 22.8 \text{ fmole} \cdot \text{h}^{-1} \cdot \text{cell}^{-1}$. This corresponds to a 5-fold increase of acid released per cell compared to mock 3T3 cells indicating a role for Nox1 in cellular acid production.

DISCUSSION

In this study we investigated the role of Nox1 in intracellular H^+ conduction across the plasma membrane and in cellular H^+ production. We found that the expression of Nox1 results in a distinct, kinetically identifiable process of intracellular acid production and a high rate of acid release into the extracellular medium. In contrast, all tested characteristics of the H^+ conductance natively expressed in 3T3 cells were unaffected by the expression of Nox1 suggesting no significant effect of Nox1 expression on H^+ channel function.

Acid production by Nox1

In activated neutrophils the Nox2-based NADPH oxidase has been shown to generate intracellular acid equivalents of $\sim 20 \text{ mM} / 5 \text{ min}$ [6]. For comparison, the expression of Nox1 in our study resulted in intracellular acid production of $3.8 \text{ mM} / 5 \text{ min}$ (i.e., $\sim 19\%$ of neutrophil production, calculated from Fig. 6C). Although the expression levels of the respective Nox isozyme in this comparison might be different, it indicates that recombinantly expressed Nox1 results in substantial acid production. The simplest explanation of our data is that Nox1 generates H^+ during oxidation of NADPH and concomitant release of H^+ . This is supported by the observation that Nox1 expression resulted in a kinetically distinct process of acid production. However, we cannot exclude the possibility that Nox1 expression results in the upregulation of other cellular processes that would result in additional H^+ production, such as increased cellular metabolism or cell growth (as reported in [21]) although no significant effect on cell growth was found in the current study over a 4-day growth period (Fig. 6B).

Previously, Suh et. al. [2] found that Nox1 3T3 cells generate $O_2^{\bullet -}$ at a rate of 18 to 36 $\text{fmole} \cdot \text{h}^{-1} \cdot \text{cell}^{-1}$. In comparison, Nox1 expression in 3T3 cells resulted in an increase in acid production of $76 \text{ fmole} \cdot \text{h}^{-1} \cdot \text{cell}^{-1}$ (Fig. 6C), which is approximately 3 times the rate of $O_2^{\bullet -}$ produced by these cells. The expected stoichiometry during NADPH oxidation and its intracellular recovery by way of the hexose monophosphate shunt is approximately $1.5 H^+$ for every $O_2^{\bullet -}$ released [24;25]. The difference between the expected and the observed stoichiometry is likely explained by differences between the two studies and by technical limitations of the quantitative detection of $O_2^{\bullet -}$ or H^+ , however, different metabolic rates and the associated metabolic acid production between the cell lines might additionally confound these measurements.

The activity of $O_2^{\bullet -}$ production by Nox1 is regulated by the co-expression of the two cellular cofactors NOXO1 and NOXA1 [26;27]. Significant production of $O_2^{\bullet -}$ by cells that natively express Nox1 and its cofactors [27;28] or in heterologous Nox1 expression systems in presence of these subunits [26;27] has been shown. However, when Nox1 was recombinantly expressed without its subunits, such as in Nox1 3T3 [21;29] or in other expression systems [26], production of $O_2^{\bullet -}$ was lower. Nevertheless, Nox1 3T3 release $O_2^{\bullet -}$ at levels of 5 to 10% of levels generated by activated human neutrophils [2] indicating a significant NADPH oxidase activity in these cells.

H^+ conductance and Nox1

Previously, the expression of several isoforms of the Nox gene family have been shown to result in a plasma membrane H^+ conductance [7;8;9], including a short, unphysiological form of Nox1 [1;30]. However, this work was criticized because the observed H^+ currents in these studies showed little resemblance to H^+ currents observed in cells that express native H^+ channels [11], disease-causing mutations in Nox2 did not affect the H^+ conductive properties [31;32], and Nox2 knock-out cells showed unchanged H^+ currents [10]. Apart from the initial report on the H^+ channel function of a short form of Nox1 [1], there have been no reports on the ability of Nox1 to form a H^+ channel or to affect properties of H^+ channels.

Similar to many other cell types 3T3 cells express a native plasma membrane H^+ conductance characterized by i) activation by depolarization, ii) outward rectification of steady-state I/V relations, iii) linear open channel I/V relations, iv) slow rates of current activation, and v) block by Zn^{2+} . The recombinant expression of Nox1 in 3T3 cells did neither affect the total H^+ currents nor the voltage-dependent kinetics of the measured H^+ currents indicating that Nox1 itself did not contribute in a measurable way to the plasma membrane H^+ conductance. Thus, likely all recordings in this study investigated the native H^+ conductance of 3T3 cells. However, in some recordings we noticed subtle kinetic differences of the deactivating tail currents between Nox1 and mock 3T3 (compare Figs. 2A & 2D). However, because of their considerable variability these were not used as functional measures to distinguish H^+ currents. Variability of tail currents may be caused by inconsistent intracellular depletion of protonated buffers during the prepulse and the associated dissipation of the transmembrane pH gradient, and by the relatively small driving forces for H^+ during the repolarizing pulse.

Our data are most consistent with the notion that the H^+ conductance in 3T3 cells is governed by a channel unrelated to Nox1 expression. Recently a H^+ channel gene (HVCN1) has been identified owing to its molecular similarity to voltage-gated K^+ channels [15;16]. When recombinantly expressed, its electrophysiological characteristics resemble those of the native H^+ conductance found in many cells. Whether HVCN1 is the basis for the H^+ currents measured in 3T3 cells has not been investigated.

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Figure 1. Nox1 expression in 3T3 cells

RT-PCR gel for transcripts of Nox1 (resulting in a PCR product of 105 bp) and actin (resulting in a PCR product of 530 bp) of Nox1-expressing cells (Nox1 3T3) and control cells (mock 3T3).

Figure 2. Plasma membrane H^+ currents in 3T3 cells

Mock 3T3 (A,B,C) and Nox1 3T3 (D,E,F). **A&D.** Whole cell currents measured during voltage pulse protocols from -80 mV to 40 mV with a pipette-to-bath 5.3-to-7.3 pH gradient. **B&E.** Fast voltage stair ramps after an activating, depolarizing prepulse. Voltage pulse protocols are shown in the *bottom graphs*. Note break in time axis. **C&F.** Steady-state current-voltage (I/V) relationships (*filled circles*) and open-channel I/V relations (*open triangles*). **G.** Specific plasma membrane H^+ conductance was not affected by expression of Nox1 (n given in parentheses, $p = 0.38$); corresponding membrane capacitance was $C_m = 26 \pm 21$ pF (Nox1 3T3) and 24 ± 14 pF (mock 3T3, $p > 0.5$).

Figure 3. Time constants of current activation (τ_{act})

A. Activation of currents by voltage pulses from -50 mV to potentials given, recorded from mock 3T3 cells. Data were fitted with single exponential functions (*line*) resulting in τ_{act} of (top to bottom) 0.49 s, 0.74 s, 1.18 s, and 2.96 s. **B.** Recordings from Nox1 3T3 resulted in τ_{act} of (top to bottom) 0.33 s, 0.52 s, 0.89 s, and 1.88 s. **C.** Average τ_{act} were not different between cell types (*black symbols*, mock 3T3; *grey symbols*, Nox1 3T3, $p > 0.5$) but τ_{act} at -20 mV was significantly longer compared to other potentials ($n = 10$ for each symbol, $p < 0.0001$, two-way ANOVA) indicative for a voltage-dependent activation step.

Figure 4. Effects of Zn²⁺ on H⁺ currents

A&B. Continuous recordings of depolarization-activated H⁺ currents in Nox1 3T3 (A) and mock 3T3 (B). Voltage was held at -50 mV and pulsed every 10 s for 6 s to 20 mV. ZnSO₄ (200 μM) was added where indicated. **C&D.** Effects of increasing concentrations of ZnSO₄ on H⁺ currents in Nox1 3T3 and mock 3T3. Recordings show three pulses each selected from a continuous recording in presence of ZnSO₄ concentrations as indicated. **E.** Dose-response relationship for ZnSO₄ effect on H⁺ currents. Lines are best fits to Michaelis-Menten kinetics resulting in halfmaximal block at $276 \pm 50 \mu\text{M}$ (mock 3T3) and $265 \pm 114 \mu\text{M}$ (Nox1 3T3, $p > 0.5$) and maximal block of $125 \pm 10\%$ (mock 3T3) and $129 \pm 23\%$ (Nox1 3T3, $p > 0.5$). Effects of ZnSO₄ on depolarization-activated currents were not affected by expression of Nox1.

Figure 5. Intracellular pH and acid production

A. Addition of 30 mM NH_4Cl rapidly alkalized cells followed by a re-acidification. Shown is mean $\pm$ SD of experimental runs with $n = 19$ Nox1 3T3 cells and $n = 28$ mock 3T3 cells each. **B.** Examples of exponential fits (*line*) to pH_i acidification of single cells; fit results are $\tau = 2371$ s (mock 3T3, *filled circles*), and double-exponential fit resulted in $\tau = 151$ s and 1688 s (Nox1 3T3, *open circles*). **C.** Log-transformed τ showed normal distribution and were used for statistical comparison. A slow process, which was not different between cell types ($p = 0.39$), was on average $\tau = 1556$ s (mock 3T3, $n = 89$ cells, $\log(\tau) = 3.18 \pm 0.65$) and $\tau = 1778$ s (Nox1 3T3, $n = 118$ cell, $\log(\tau) = 3.25 \pm 0.28$). These averages are the inverse logarithms of the averaged log-transformed τ values. Nox1 3T3 expressed an unique process with a significantly ($p < 0.001$) faster time constant of, on average, $\tau = 165$ s ($\log(\tau) = 2.22 \pm 0.12$), which was not present in mock 3T3.

Figure 6. Effect of Nox1 expression on acid release

A. Acidification of the cell culture medium by Nox1 3T3 (grey bars and symbols) and mock 3T3 cells (black bars and symbols). Bar chart on the left shows pH of the culture medium (pH_o) 24 hours after seeding, and bar chart on the right after 96 hours in culture, $n = 8$ cultures per time point, $p < 0.0001$. Line graph shows one representative experimental run. Time-axis labels as in B. **B.** Growth curves. Number of cells per well at 24 hours and 96 hours was not significantly different between cell types ($n = 8$, $p = 0.052$). **C.** Nox1 3T3 released significantly more acid than mock 3T3, $p < 0.0001$, $n = 8$.