

# **Recovery from hypoxia-induced internalization of cardiac Na<sup>+</sup>/H<sup>+</sup> exchanger 1 requires an adequate intracellular store of anti-oxidants**

**Gül Şimşek<sup>1</sup>, Richard D. Vaughan-Jones<sup>2</sup>, Pawel Swietach<sup>2</sup>, Hilmi Burak Kandilci<sup>1\*</sup>**

<sup>1</sup>Department of Biophysics, Faculty of Medicine, Ankara University, Ankara, Turkey

<sup>2</sup>Department of Physiology, Anatomy, and Genetics, Oxford University, Oxford, UK

Conflict of interest: None to declare

Additional supporting information can be found in the  
online version of this article

Contract grant sponsor: Ankara University Scientific Research Projects Unit.

Contract grant sponsor: The Scientific and Technological Research Council of Turkey.

Contract grant sponsor: British Heart Foundation.

\*Correspondence to: Hilmi Burak Kandilci, Department of Biophysics,

Faculty of Medicine, Morphology Campus, Ankara University, 06100 Sıhhiye/Ankara/Turkey

E-mail: kandilci@medicine.ankara.edu.tr

## ABSTRACT

The heart is highly active metabolically but relatively under-perfused, and therefore vulnerable to ischemia. In addition to acidosis, a key component of ischemia is hypoxia which can modulate gene expression and protein function as part of an adaptive or even maladaptive response. Here, using cardiac-derived HL-1 cells, we investigate the effect of various hypoxic stimuli on the expression and activity of Na<sup>+</sup>/H<sup>+</sup> exchanger-1 (NHE1), a principal regulator of intracellular pH. Acute (10 minutes) anoxia produced a reversible decrease in sarcolemmal NHE1 activity attributable to NHE1 internalization. Treatment with either 1% O<sub>2</sub> or dimethyloxaloylglycine (DMOG; 1mM) for 48 hours stabilised hypoxia-inducible factor 1 (HIF1) and reduced sarcolemmal NHE1 activity by internalization, but without a change in total NHE1 immunoreactivity or message levels of the coding gene (*SLC9A1*) determined in whole-cell lysates. Unlike the effect of DMOG, which was rapidly reversed upon washout, re-oxygenation after a prolonged period of hypoxia did not reverse the effects on NHE1, unless media were also supplemented with a membrane-permeant derivative of glutathione (GSH). Without a prior hypoxic episode, GSH supplementation had no effect on NHE1 activity. Thus, post-hypoxic NHE1 re-insertion can only take place if cells have a sufficient reservoir of reducing agent. We propose that oxidative stress under prolonged hypoxia depletes intracellular GSH to an extent that curtails NHE1 re-insertion once the hypoxic stimulus is withdrawn. This effect may be cardioprotective, as rapid post-ischaemic restoration of NHE1 activity is known to trigger reperfusion injury by producing an intracellular Na<sup>+</sup>-overload which is pro-arrhythmogenic.

## INTRODUCTION

Hydrogen ( $H^+$ ) ions are highly reactive and generated by cells in large quantities by metabolism. To maintain a favourable intracellular pH ( $pH_i$ ), cells must remove excess acidity, ultimately by means of membrane transport. In most cells,  $Na^+/H^+$  exchangers (NHE) are a major mechanism for extruding excess  $H^+$  ions from the cytoplasm. Indeed, cardiac myocytes, which are exquisitely sensitive to  $pH_i$  (Allen and Orchard, 1983; Bountra and Vaughan-Jones, 1989), can produce considerable pH-regulatory fluxes through isoform NHE1. These fluxes can reach several mM of  $H^+$  ions per minute when activated by low  $pH_i$ . Since acid-extrusion by NHE1 is coupled to an influx of  $Na^+$ , cells can become overload with  $Na^+$  ions; indeed,  $Na^+$ -overload is part of the pathophysiology associated with cardiac ischemia-reperfusion (I/R) injury (Williams et al., 2007). Clinical trials using NHE1 inhibitors were unable to demonstrate compelling evidence for a cardioprotective effect against myocardial infarction (MI) (Avkiran and Marber, 2002), but issues related to drug accessibility to ischemic zones may have contributed to this. Notwithstanding this outcome, NHE1 remains an attractive target for reducing the extent of myocardial remodelling in heart failure following MI (Kılıç et al., 2014). To ascertain the therapeutic utility of NHE1 inhibition, it is first necessary to characterise the activity of this protein under conditions that are characteristic of ischemia.

A major component of ischemia is hypoxia, which switches metabolism to glycolysis and produces lactic acidosis (Neely et al., 1975). Several studies - mostly on cell lines - have shown that low  $O_2$  tension can affect NHE1 activity both acutely and chronically, producing knock-on effects on the cell's acid-base homeostasis and  $Na^+$  handling. In fibrosarcoma HT1080 cells, for example, 4 hours of hypoxia (1%  $O_2$ ) increased NHE1 activity without altering NHE1 mRNA and protein levels (Lucien et al., 2011) and independently of hypoxia inducible factor (HIF). In contrast, 3 hours of hypoxia (1%  $O_2$ ) decreased NHE1 activity in colorectal HCT116 cells (Hulikova et al., 2013), which was not related to NHE1 downregulation but could be mimicked by pharmacological stabilizers (2-oxoglutarate analogs) of HIF signaling. In that same study, prolonged hypoxia (48 hours at 2%  $O_2$ ) reduced NHE activity by protein downregulation. A study on rat primary pulmonary artery smooth muscle reported that 48 hours of mild hypoxia (4%  $O_2$ ) increased both NHE activity and protein (Shimoda et al., 2006) through a HIF-dependent process. Collectively, these findings argue that hypoxia is likely to affect NHE1 in a tissue-specific manner.

Depending on its severity and duration, hypoxia can trigger metabolic changes that could indirectly influence NHE1 function. For example, intracellular acidification during

hypoxia can evoke mitochondrial  $\text{Ca}^{2+}$  overload (Stone et al., 1989), which disturbs intracellular [ATP] ( $[\text{ATP}]_i$ ) production. Additionally, the oxidative stress can increase the production of reactive oxygen species (ROS) (Kim et al., 2006). Mitochondrial ROS can profoundly decrease the cellular antioxidant reserve through the loss of reduced glutathione (GSH) (Brookes et al., 2004). Ultimately, these cascades can trigger apoptosis (Crompton, 1999; Halestrap, 1999), but any intermediary effects on NHE1 activity are poorly understood (Demaurex et al., 1997; Snabaitis et al., 2002).

The aim of this study is to investigate the effect of acute anoxia and longer-term hypoxia on NHE1 activity in heart-derived HL-1 cells, a widely used model for studying cardiomyocyte biology (Claycomb et al., 1998). Importantly, HL-1 cells express NHE1 activity at a similar level to primary cardiomyocytes, but can be cultured for extended periods of time, as required for measuring the effects of chronic hypoxia. We find that hypoxic exposure decreases NHE1 activity at the surface membrane (sarcolemmal) through an internalization process which could be reversed upon re-oxygenation, unless the hypoxic period was sufficient to deplete GSH stores. Thus, acute anoxia can produce a rapidly reversible inhibition of NHE1 activity but longer hypoxic episodes may evoke sustained NHE1 inhibition lasting beyond the point of re-oxygenation. Thus, we propose that the cumulative redox changes that take place during prolonged hypoxia can critically influence the kinetics of NHE1 re-insertion into the sarcolemma, and hence determine the reversibility of hypoxia-triggered inhibition.



## **MATERIALS AND METHODS**

### **Cell culture and hypoxia**

The HL-1 (non-beating) mouse cardiomyocyte cell line was a kind gift from Professor Enn Seppet (Tartu, Estonia) and was maintained as described previously (Claycomb et al., 1998). Cells were grown in supplemented Claycomb medium (Sigma) including  $\text{HCO}_3^-$ , in an atmosphere of 5%  $\text{CO}_2$  for 24-36 hours until 70% confluent. For hypoxic stimuli, medium was replenished and cells were incubated in a hypoxia chamber (Billups-Rothenberg, Inc.) for 48 hours. Oxygen partial pressure, measured by an  $\text{O}_2$  sensitive probe (Oxymicro, WPI) was set to 7 mmHg (1%  $\text{O}_2$ ) and  $\text{CO}_2$  was held at 5%  $\text{CO}_2$  by delivering 95%  $\text{N}_2$  and 5%  $\text{CO}_2$  gas mixture. After 24 hours, the chamber was re-flushed with a fresh mixture of gas. To activate hypoxic pathways using pharmacology, cells were incubated in 21% (normoxia)  $\text{O}_2$  (145 mm Hg) in the presence of 1 mM 2-oxoglutarate-dependent dioxygenase inhibitor dimethyloxalylglycine (DMOG, Sigma) for 48 hours. Control cells were incubated in 21%  $\text{O}_2$  for 48 hours. Metabolism will tend to acidify media, but the extent of this was diluted by using large volumes of media in 60 mm petri dishes containing 6 ml Claycomb medium. For NHE activity measurements, HL-1 cells were first grown on coverslips, which were then transferred to 6 well plates containing 2 ml medium.

### **Immunocytochemistry and microscopy**

HL-1 cells were incubated in either 1%  $\text{O}_2$  or in the presence of DMOG (in 21%  $\text{O}_2$ ) for 48 hours, as described above. After incubation, cells washed thrice in PBS and fixed in 2% (wt/vol) paraformaldehyde for 20 minutes. Cells were permeabilized with 0.1% Triton X-100 for 15 minutes, then washed in PBS and blocked with 1% BSA and 0.1% Triton X-100 for 1 hour. Later, cells were rinsed in PBS and incubated with the primary mouse monoclonal HIF-1 $\alpha$  antibody (Novus Biologica, 1:500) for 1 hour. For NHE1 visualization, cells were treated in cold 100% methanol for 5 minutes, blocked in 1% BSA and incubated with primary mouse monoclonal NHE1 antibody (Santa Cruz, 1:50). After washing thrice in PBS, cells were incubated with the goat anti-mouse secondary antibody labelled with Alexa Fluor 594 (Jacksons Immuno Research, 1:100) for 1 hour. Labelled cells washed thrice in PBS, incubated with the nucleic acid stain Yo-Pro-1 (Molecular Probes, 2.5  $\mu\text{M}$ ) for 40 seconds. Cells were mounted in Diamond Antifade reagent (Life Tech). HIF-1 $\alpha$ , NHE1 immunofluorescence and Yo-pro-1 signal was simultaneously acquired using confocal microscope (TCS SP5, Leica). HIF-1 $\alpha$  accumulation in nuclear regions, visualized using nucleic acid stain Yo-pro-1. Cytoplasmic and

nuclear HIF-1 $\alpha$  staining was determined by measuring the intensity of immunofluorescence. NHE1 distribution in monolayers was assessed visually by 2 independent researchers (blinded). The total number of cells was visualized by the nuclear stain Yo-Pro-1 and the subcellular distribution of NHE1 was imaged by immunofluorescence. NHE1 internalization was determined as the combined area of cells demonstrating no sarcolemmal pattern of staining, divided by the total area of all cells in the field of view. For NHE1 internalization experiments, control group cells were kept in normoxia (21% O<sub>2</sub>) for 48 hours. For acute anoxia, 1 mM sodium dithionite was added directly to the control cell medium for 10 minutes. Long-term hypoxia was imposed by incubating cells in a hypoxic atmosphere (1% O<sub>2</sub>) for 48 hours. The DMOG-treated group included 1 mM DMOG in media for 48 hours in normoxia. Immunofluorescence procedures were carried out on cells. For washout (W/O) groups, sodium dithionite and DMOG treated cells were washed thrice with fresh Claycomb medium. Re-oxygenation after 48 hours of hypoxia was achieved by incubation in normoxia. For the GSH-supplemented group, GSH was added during the last 30 minutes of 2 hours normoxic incubation.

### **Measurement of total intracellular ATP**

CellTiter-Glo- 2.0 Assay (Promega) was used to measure total ATP in cells incubated in 48 hours of long-term hypoxia. ATP measurements were performed immediately after 48 hours hypoxic incubation or after 2 hours of re-oxygenation in normoxia (21% O<sub>2</sub>). ATP luminescence was measured with multi-label reader (Perkin Elmer, Envision 2104). Measurements were normalized to cell amount and expressed as a ratio to measurements performed in 48 hours normoxic controls.

### **Total glutathione measurements**

The glutathione (GSH) content of the cells was measured immediately in HL-1 cell lysates after being incubated in 48 hours of long-term hypoxia using a kit (Invitrogen, Glutathione colorimetric detection kit) according to manufacturer's instructions. Absorbance at 405 nm was measured with a spectrophotometer (Molecular Devices, Spectra Max). Measurements were normalized to total cell protein and expressed as a ratio of measurements under normoxia.

### **Western Blot Analysis**

Cells were washed with ice-cold PBS, scraped and pelleted at 1000 rcf for 5 minutes at 4°C. Pelleted cells lysed with lysis buffer (50 mM HEPES (pH:7.6), 150 mM NaCl, 1 mM EDTA,

1.5 mM MgCl<sub>2</sub>, 0.5% NP-40, plus protease inhibitor cocktail [Complete, Roche]) at 4°C, mixing regularly for 15 minutes. Supernatant proteins were collected by centrifugation at 13572 rcf for 10 minutes at 4°C. Proteins were resolved by SDS-PAGE and transferred to a PVDF membrane (Santa Cruz) and were detected using primary mouse monoclonal antibodies, NHE1 (Santa Cruz, 1/200) and  $\beta$ -actin (Santa Cruz, 1/1000). Protein expressions were visualized by ECL using HRP-conjugated rabbit anti mouse antibodies (Abcam, 1/25000). Densitometric analysis was performed using ImageJ software. NHE1 protein expression levels were normalized to  $\beta$ -actin.

### **Intracellular pH measurement and superfusion of cells**

Adhered cells on fractured coverslips were incubated with pH sensitive probe BCECF-AM (5  $\mu$ M) for 30 minutes at 37°C temperature. Loaded cells were then put on a Perspex block chamber with a coverslip base and placed on an epifluorescence inverted microscope system (Nikon Eclipse TE 2000, PTI D104). To measure intracellular pH, cells were excited at 440 and 490 nm using a monochromator and emission signals were collected at 535 nm. Raw signal ratios were converted to pH using a calibration curve determined in separate experiments using the nigericin method (10  $\mu$ M, Sigma). Cells were superfused with Hepes buffered Tyrode solution containing (in mM): 135 NaCl, 20 Hepes, 11 Glucose, 4.5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub> and pH of solution was adjusted to 7.4 with saturated NaOH. HCO<sub>3</sub><sup>-</sup> buffered Tyrode contained (in mM): 125 NaCl, 22 NaHCO<sub>3</sub>, 11 Glucose, 4.5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub> and were continuously bubbled with 5% CO<sub>2</sub>. For ammonium (20 mM) containing or Na<sup>+</sup>-free solutions, NaCl was replaced isosmotically with NH<sub>4</sub>Cl or N-methyl-D-glutamine chloride, respectively. In some experiments zoniporide (1 or 30  $\mu$ M, Tocris) was used to specifically target NHE1 activity in HL-1 cells. Solutions were delivered to superfusion chamber with a peristaltic pump at 3 ml/min. Chamber solution temperature was set to 37°C with a feedback control system (CL-100, Warner Instruments). NHE1 and HCO<sub>3</sub><sup>-</sup>-dependent fluxes were measured within 2 hours in normoxia (21% O<sub>2</sub>) after the removal of cells from the hypoxia chamber or within an hour after DMOG removal. For anoxia, sodium dithionite (1 mM, Merck) was added to superfusion solutions immediately prior to measurements and bubbled with 100% N<sub>2</sub>. In some experiments, after 48 hours of long-term hypoxia, cells were removed from the hypoxia chamber and incubated with membrane-permeant glutathione monoethyl ester (1mM, Calbiochem) for 30 minutes.

### **Flux calculation and statistics**

Acid-extrusion across the cell membrane ( $H^+$ -flux) was calculated from the product of the rate of change of intracellular pH ( $dpH_i/dt$ ) during recovery and the intrinsic buffering capacity ( $\beta_i$ ). When calculating  $H^+$ -equivalent flux in the presence of  $CO_2/HCO_3^-$ -buffered solutions, the computed value of  $CO_2$ -dependent buffering was added to  $\beta_i$  (see e.g. Leem et al., 1999). To trigger acid-extrusion, cells were acid-loaded by means of an ammonium prepulse solution maneuver (Boron and De Weer, 1976).  $\beta_i$  values were determined by stepwise ammonium removal, and data were fitted to a previously described model (Leem et al., 1999). The  $Na^+$  independent residual activity was calculated from  $H^+$ -flux measured in  $Na^+$ -free (NMDG substituted) Hepes buffered Tyrode solution. NHE-1 flux is calculated by subtracting the zoniporide-insensitive component of flux from the total flux measured in Hepes buffered solution. Data were fitted with a least squares method. Statistical analysis was done using two-tailed Student's  $t$  test and  $p$  value of  $< 0.05$  was considered as significant.

## RESULTS

### **NHE1 is the principal acid-extruding isoform in normoxic cardiac HL-1 cells.**

Message levels for the major acid-extruding proteins was measured in HL-1 cells by qPCR (see Supplementary Methods, Table S1 for primers). mRNA coding for  $\text{Na}^+/\text{H}^+$  exchangers (NHE) 1, -3, and -8 and  $\text{HCO}_3^-$ -dependent NBCe1 (Fig. 1A) and only low levels of NHE2 (Supplementary Fig. S2D) was confirmed. NHE isoforms that are normally associated with intracellular compartments or have not been associated with cardiac tissue were not assayed (Andersen et al., 2009). To determine the activity of these acid-extruders, cells were acid-loaded by the ammonium prepulse manoeuvre, performed in a superfusion chamber (e.g. Fig. 1B). The time course of the subsequent  $\text{pH}_i$  recovery provides a readout of acid extrusion. In the absence of  $\text{CO}_2/\text{HCO}_3^-$ , i.e. in Hepes-buffered superfusates (Fig. 1B),  $\text{HCO}_3^-$  dependent transporters such as NBCe1 are inactivated. Under these conditions, the recovery of  $\text{pH}_i$  was blocked in  $\text{Na}^+$ -free superfusates (replacing  $\text{Na}^+$  with NMDG, Fig. 1B), indicating that  $\text{Na}^+$ -independent activity (e.g. by primary active  $\text{H}^+$  ATPase pumps) was negligible ( $\sim 0.15$  mM/min at  $\text{pH}_i$  6.75) in HL-1 cells. We next examined the contribution of various NHE isoforms to  $\text{pH}_i$  recovery in Hepes-buffered conditions using the inhibitor zoniporide. This drug selectively blocks NHE1 and NHE2, but only the former is detected at high levels in HL-1 cells (Tracey et al., 2003). Fig. 1C shows paired measurements performed in the presence and absence of zoniporide (1 or 30  $\mu\text{M}$ ). The drug markedly slowed  $\text{pH}_i$  recovery in a dose-dependent manner, indicating NHE1 the main contributor to  $\text{HCO}_3^-$  independent pH-regulatory acid-extrusion flux in HL-1 cells.

### **Acute anoxia reversibly reduces NHE1 flux.**

To characterize the acute effect of low  $\text{O}_2$  on NHE1-flux, Hepes-buffered superfusates were rendered anoxic (0%  $\text{O}_2$ ) by the addition of the oxygen-scavenger sodium dithionite (1 mM) and gassing with 100%  $\text{N}_2$ . 10 minutes of this treatment slowed NHE1-mediated  $\text{pH}_i$  recovery. This inhibition was reversed, within minutes, following sodium dithionite washout (Fig. 2A), as reported previously in adult rat ventricular myocytes (Kandilci et al., 2007; 2009). NHE1-flux, calculated as the product of  $\text{pH}_i$  recovery rate ( $\text{dpH}_i/\text{dt}$ ) and buffering capacity (Supplementary Fig. S1), is plotted as a function of  $\text{pH}_i$  in Fig. 2B. Acute anoxia halved NHE1-flux, which is evident in Fig. 2C, where NHE1-fluxes measured in anoxia and control are plotted at a common  $\text{pH}_i$ . These data points can be fitted with a straight line of slope 0.41, indicative of a two-fold down-scaling of NHE1-flux over the  $\text{pH}_i$  range tested.

### **Prolonged hypoxia produces a sustained inhibition of NHE1.**

To determine the effects of long-term hypoxia on NHE1-flux, cells were kept under 1% O<sub>2</sub> for 48 hours, and subsequent measurements of NHE1 activity were made in atmospheric O<sub>2</sub> within 2 hours of collecting cells from hypoxic incubation. 48 hours of hypoxia inhibited NHE1-flux to a comparable degree as with acute anoxia (Fig. 2D/E, Supplementary Fig. S2B). This effect can be quantified from the linear relationship shown in Fig. 2F (slope: 0.50), suggesting a downscaling of NHE1 activity by a factor of two. Of note, after 48 hours of hypoxia, the zoniporide-insensitive component of acid-extrusion was increased by less than 1 mM/min (Supplementary Fig. S2C). These results indicate that 48 hours of hypoxia inhibited NHE1 activity.

In contrast to the effects of acute anoxia, NHE1 inhibition after 48 hours of hypoxia was sustained and did not promptly reverse upon re-oxygenation. Note that intrinsic buffering capacity,  $\beta_i$ , which is used to compute flux, was somewhat increased by prolonged hypoxia (Supplementary Fig. S1). To explore the mechanism of this sustained hypoxic inhibition, the effect of the prolyl hydroxylase inhibitor dimethyloxaloylglycine (DMOG) was tested under normoxic condition, which is expected to stabilize HIF-mediated signaling. In HL-1 cells, 1 mM DMOG incubation, lasting either 5 or 48 hours, resulted in the accumulation of HIF1 $\alpha$  in the nucleus but not cytoplasm, as observed with 1% O<sub>2</sub> (Fig. 3A-C). 48 hours of DMOG incubation (under atmospheric O<sub>2</sub>) reduced NHE1-flux, probed within an hour of withdrawing cells from DMOG-containing culture media (Fig. 3D). Similar to the linear relationship shown in Fig. 2F, DMOG-treated cells had NHE1-flux by a factor of two (slope: 0.52, Fig. 3E). There was no effect of DMOG on the zoniporide-insensitive component of flux (Supplementary Fig. S2C). The similarity between the effect of DMOG and hypoxia may indicate that NHE1 inhibition involves prolyl hydroxylases.

### **Total NHE1 protein level is not altered after 48 hours of hypoxic exposure.**

In standard incubation vessels containing 4 ml medium/T25 flask hypoxic conditions can often lead to profound medium acidification as a consequence of the accumulation of glycolytic end-products. Thus, measurements performed under these conditions cannot necessarily distinguish between the influences attributable to hypoxic signaling from the effects of acidosis and paracrine/autocrine factors. In the present study, we chose to minimize hypoxia-induced medium acidification in order to dissect the effect hypoxia on NHE1. To achieve this, larger volumes of buffer were used to minimise changes of medium pH. Cells grown in coverslips were transferred to 60 mm petri dishes containing 6 ml medium volume having

standard medium depth (3 ml medium/coverlip), such that at the end of 48 hours of hypoxia, medium remained mildly alkaline (pH 7.4), as determined by pH meter. Using this approach, 48 hours of hypoxia or nDMOG incubation (under normoxia) did not alter the level of mature NHE1 protein detected in whole-cell lysates at 100 kDa (Fig. 4A). In contrast, NHE1 protein was downregulated if medium acidification was allowed to take place during hypoxic incubation (Supplementary Fig. S3). Thus, hypoxia *per se* is unlikely to exert its influence on NHE1 flux by downregulating total NHE1 protein levels. Consistent with the lack of NHE1 downregulation in hypoxia or in DMOG incubation (Fig. 4B), the mRNA level for the gene coding for NHE1 (*SLC9A1*) was not affected by 48 hours of hypoxia (Fig. 4C). At the transcriptional level, two other membrane-bound acid-extruding proteins present in HL1 cells (*SLC9A8*, *SLC4A4*) were also unaffected by 48 hours of hypoxia (Fig. 4C). There was a modest hypoxia-evoked upregulation of *SLC9A3*, the gene coding for NHE3, but this occurred from a low baseline in control. This small induction may explain the small increase in the zoniporide-insensitive flux (Supplementary Fig 2C).

### **NHE1 is rapidly internalized by an hypoxic stimulus.**

After 48 hours of hypoxic incubation, NHE1-flux decreases without a matching change in total NHE1 protein (Fig. 4A/B), arguing for a mechanism possibly involving internalization of sarcolemmal NHE1 to internal structures, where the protein no longer regulates cytoplasmic pH. In normoxia, NHE1 is strongly associated with the surface membrane, with only a weaker cytoplasmic signal (Fig. 5A). 10 minutes of anoxia (superfusion with 1 mM sodium dithionite) produced a more homogeneous NHE1 distribution within the cell, including at internal structures, as determined by immunofluorescence. 10 minutes of re-oxygenation (sodium dithionite washout) evoked a re-insertion of NHE1 protein into the surface membrane (Fig. 5A). The internalization of NHE1 triggered by acute anoxia correlated well with the reduction in measured flux (Fig. 2). 48 hours of hypoxia also resulted in NHE1 internalization but, in contrast to acute anoxia, re-oxygenation in a normoxic chamber was not sufficient to restore NHE1 at the sarcolemma, even after 2 hours. These results indicate that the duration of the hypoxic episode has a bearing on NHE1 trafficking between the sarcolemma and internal structures. Interestingly, 48 hours of incubation with DMOG (1 mM) in normoxia produced NHE1 internalization, similar to the effect of 48 hours of hypoxia; however, washout of DMOG evoked a prompt re-insertion of NHE1 (Fig. 5A). Thus, DMOG and long-term hypoxia have a common denominator of evoking NHE1 internalization, likely related to prolyl hydroxylases and HIF-1 $\alpha$  signalling, but differ in how reversible this effect is. Indeed, hypoxia is expected

to evoke a more complex chain of events, including profound changes in ATP turnover and redox state, both of which are plausible reasons why NHE1 re-insertion into the sarcolemma is impaired.

### **Intracellular ATP is partially depleted under long-term hypoxia.**

Hypoxic conditions may affect intracellular ATP (ATP<sub>i</sub>) levels which, in turn, could plausibly affect NHE1-flux. 48 hours of 1% O<sub>2</sub> or 1 mM DMOG incubation reduced cell proliferation, a variable that must be accounted for in calculations of ATP levels in lysates. Thus, cell counts in the microscope field of view were used to normalize ATP levels obtained by the luciferase assay performed on lysates (Fig. 6A). 48 hours of hypoxia decreased normalized [ATP]<sub>i</sub> levels by 60% and remained depleted upon re-oxygenation, arguing for a metabolic remodelling that persisted for at least 2 hours after the hypoxic episode (Fig. 6B). Although the NHE1 transport cycle does not consume ATP *per se*, an allosteric ATP<sub>i</sub> dependency has been postulated for cardiomyocytes (Demaurex et al., 1997) and NHE1-flux is strongly sensitive to phosphorylation status (for review, see Vaughan-Jones et al., 2009). Additionally, adequate ATP levels are necessary for maintaining redox balance and ionic gradients, including [Na<sup>+</sup>]. Therefore, the decline in ATP<sub>i</sub> might be a downstream factor in the hypoxic inhibition of NHE1-flux related to prolyl hydroxylases and HIF-1α signalling.

### **Supplementation of GSH following hypoxic depletion permits NHE1 re-insertion**

An important regulator of redox is intracellular reduced glutathione (GSH) concentration (Mailloux, 2016). GSH pools, particularly in mitochondria, can be depleted by hypoxia (Lluis et al., 2005). In HL-1 cells, 48 hours of hypoxia reduced total GSH levels in lysates (Fig. 6C), which is consistent with constrained energetics. Under depleted GSH, mitochondria may, for example, produce free radicals that could signal onto sarcolemmal NHE1. To test how redox affects NHE1, intracellular GSH levels were manipulated using a membrane-permeable monoethyl ester of reduced glutathione. 48 hours of hypoxia normally produced a sustained inhibition of NHE1 that did not readily reverse upon re-oxygenation, but if cells were treated with 1 mM of the GSH ester for 30 mins after 48 hours hypoxic incubation, NHE1 activity was rapidly restored upon re-oxygenation (Fig. 7A). Furthermore, GSH supplementation allowed NHE1 protein to re-insert into the sarcolemma after prolonged hypoxia (Fig 7C). Additionally, these effects took place without recovering the declined ATP levels in re-oxygenation period after 48 hours of hypoxia (Supplementary Fig. S4). Importantly, GSH supplementation of cells that had not been exposed to hypoxia did not affect NHE1-flux



(Fig. 7B). This finding indicates that the effect of exogenous GSH was attributable to a rescue from depleted levels, rather than a stimulatory effect of GSH *per se*. The dynamics of NHE1 re-insertion thus critically rely on adequate GSH levels, which normally become depleted with prolonged hypoxia.

## DISCUSSION

### Validating the use of HL-1 cells to study $\text{pH}_i$ regulation after prolonged hypoxia

Ischemia and reperfusion injury of the heart are associated with episodes of tissue hypoxia and changes to  $\text{pH}_i$ , both of which can exert multifaceted effects on cellular physiology. Myocytes extrude excess acid by NHE1, but the interplay between this  $\text{pH}_i$  regulator and hypoxia is still poorly understood, partly because of difficulties in maintaining tissues adequately viable for extended periods of time. Whole-heart preparations, although invaluable for studying the effects of acute hypoxia, are not tractable models for long-term follow-up experiments, such as measuring the effects of 48 hours of hypoxic incubation. Primary cardiomyocyte cultures have been used to obtain molecular insight into mechanisms, but this approach is confounded by difficulties in long-term culture. For example, alterations in cardiomyocyte morphology are observed overnight, even under normoxic conditions (Louch et al., 2011). An alternative experimental approach is to study hypoxic responses in cardiac-derived cell lines that can be cultured stably for long periods of time but retain a similar phenotype to primary tissue. In this study, we used cardiac derived HL-1 cells that, under normoxic conditions, express NHE1 to a level that produces an ionic flux closely resembling that measured in freshly isolated, adult mammalian myocytes (Fig. 2B/E) (Vaughan-Jones et al., 2009).

Previously, there have been some suggestions that 48 hours of exposure to 1% hypoxia can induce apoptosis and decreases cell viability in HL-1 cells (Hu et al., 2010; Ambrose et al., 2014). This process would run-down all forms of membrane transport activity, potentially obstructing effects on NHE1. However, our measurements of a distinct secondary-active transport process also involved in  $\text{pH}_i$  regulation,  $\text{Na}^+$ -dependent  $\text{HCO}_3^-$  transport, demonstrated an increase in flux (Supplementary Fig. S2A). This effect argues against a general and non-specific decrease in the activity of membrane transporters.

### **Redox balance affects the reversibility of hypoxia-induced NHE1 internalization**

Immunofluorescence data suggest that the decrease in sarcolemmal NHE1 activity in response to hypoxia is due to NHE1 internalization, evoked within several minutes and maintained for the duration of hypoxia (Fig. 5). Hypoxia-dependent internalization of membrane proteins has been documented previously (Bourseau-Guilmain et al., 2016), although the precise mechanisms may differ. Previous studies have described the regulation of NHE3 activity by membrane trafficking (Chow et al., 1999). Re-oxygenation after a short period of hypoxia reversed the internalization process fully, leading to a functional recovery of sarcolemmal NHE1 activity (Fig. 2A and 5). Thus, acute hypoxia-re-oxygenation cycles affect the kinetics of NHE1 turnover between the surface membrane and intracellular structures. In contrast, internalization under 48 hours of hypoxia was not readily reversible by re-oxygenation, indicating that a concurrent and cumulative effect takes place inside cells that gradually hinders the re-insertion process. This factor is likely to relate to the depletion of GSH during prolonged hypoxia, because re-oxygenation of cells supplemented with exogenous GSH was able to restore sarcolemmal NHE1 (Fig. 7C). The importance of restoring redox balance for the recovery of NHE1 activity has been demonstrated previously for pulmonary arterial cells in normoxic conditions (Cutaia and Parks, 1994). Furthermore, 2 hours of reoxygenation following 48 hours of hypoxia in cells supplemented with GSH lead to a re-insertion of NHE1 without a restoration of depleted ATP (Supplementary Fig. S4). These results indicate that ATP *per se* is not responsible from re-insertion process of NHE1 to sarcolemma.

### **NHE1 internalisation involves prolyl hydroxylase-dependent mechanisms**

The prolyl hydroxylase (PHD) enzymes are inhibited at low oxygen tension in cells (Ke and Costa, 2006), and engage pathways such as HIF-1 $\alpha$  signalling. In HL-1 cells, inhibition of PHDs with hypoxia or DMOG resulted in a sustained translocation of HIF-1 $\alpha$  protein to nucleus and a decrease in NHE1-flux (Fig. 3). However, neither NHE1 mRNA nor protein levels were affected by PHD inhibition (Fig. 4), which argues against a HIF-driven change in transcription, its canonical mechanism of action. Therefore, in contrast to the observations made in pulmonary arterial smooth muscle cells (Shimoda et al., 2006), a direct interaction between HIF and the NHE1 gene, *SLC4A9*, is unlikely in cardiac HL-1 cells. NHE1 internalization evoked by either hypoxia or DMOG may relate to previously characterised mechanisms, such as Nedd4-1 E3 ligase-dependent ubiquitination, which has been implicated in the mechanism of NHE1 endocytosis in HEK293 cells (Simonin and Fuster, 2010). Nedd4-1 levels are regulated under ischemia/reperfusion in cardiomyocytes via a process related to HIF (Hu et al., 2017).

Although hypoxia and DMOG have similar effects on NHE1 surface membrane expression and activity, they may not signal through identical pathways. DMOG, as a non-specific 2-oxoglutarate dependent dioxygenase inhibitor, may have additional effects which are not involved in hypoxic signalling. Additionally, there may be HIF-independent effects of hypoxia that are not observed in DMOG (Arsham et al., 2003). Changes to metabolism and redox are among the processes that could be altered differentially by DMOG and hypoxia, the latter tending to produce more profound changes because oxygen deprivation has a plethora of metabolic effects. Indeed, our data show that DMOG wash-out triggered a prompt re-insertion of NHE1 (Fig. 5A/B), which suggests that DMOG treatment may not deplete GSH to the level attained with prolonged hypoxia. This difference could relate to the fact that in DMOG-treated cells still had access to atmospheric oxygen. Another possible explanation for the difference between DMOG and hypoxia may relate to a disparity in experimental procedures between the recovery groups. Wash-out of DMOG may also remove HIF1 $\alpha$ -evoked secretions from the medium, whereas re-oxygenation of incubation media, following long-term hypoxia, did not involve a medium exchange, i.e. any putative factors would remain. However, potentially inhibitory secreted factors may have only marginal importance in the recycling of NHE1 because in experiments with supplemented GSH, where NHE1 flux could be restored fully, media were not refreshed at the end of the hypoxic episode.

### **Oxidative stress may affect NHE1 dimerization**

Sarcolemmal NHE1 exists as a homodimer (Hisamitsu et al., 2006; Moncoq et al., 2008), believed to be formed in endo/sarcoplasmic reticulum (ER/SR) lumen (Hisamitsu et al., 2006) by means of a disulphide bond (Fliegel et al., 1993) between cysteine-794 and -561 (Hisamitsu et al., 2004) and, possibly, interactions involving the carboxyl termini (Li et al., 2003). The lumen of the SR/ER is a normally an oxidizing environment, and altered redox state (e.g. in long-term hypoxia) could plausibly affect disulphide bridge formation (Cumming et al., 2004) and a luminal retention of misfolded protein (Cao and Kaufman, 2014). The onset of re-oxygenation may enhance ROS production; indeed, in a 12-hour ischemia model (2% O<sub>2</sub> and an ischemia-mimetic solution), peak ROS levels were detected after 2 hours of re-oxygenation. This rise in ROS levels was attenuated by the ROS scavenger *N*-acetylcysteine (Miao et al., 2013), arguing for a role of adequate GSH (and hence ATP) in maintaining physiological levels of ROS (Mari et al., 2009). Oxidative stress-induced depletion of GSH may disturb NHE1 dimerization and this could be a plausible mechanism that prevents NHE1 from being re-inserted into the sarcolemma upon re-oxygenation.

A slow rate of GSH depletion under hypoxic conditions may explain why NHE1 internalization can be reversed rapidly after a brief hypoxic episode. During such a short stress, the GSH pool would remain largely intact. Indeed, 6 hours of hypoxia (1.5% O<sub>2</sub>) was needed to deplete half of intracellular [GSH] in HEK293 cells (Mansfield et al., 2004). Moreover, previous studies have demonstrated no significant ROS production (Robin et al., 2007) nor GSH depletion (Mansfield et al., 2004) during the acute hypoxia. In the heart, two hours of myocardial ischemia in anaesthetized dogs was able to induce mitochondrial GSH decrease, but the cardiac dysfunction could be minimised by maintaining mitochondrial GSH concentration by administration of  $\gamma$ -glutamylcysteine ethyl ester (Nishinaka et al., 1991). In adult myocytes, incubation with a cell-permeable glutathione monoethyl ester prevented ROS-induced mitochondrial depolarization during re-oxygenation (Ganitkevich et. al., 2006), although this effect was observed after acute hypoxia. Based on our data, we propose that prolonged hypoxia depletes GSH pools down to levels that can curtail NHE1 re-insertion (Fig. 7). We speculate that this is a cardioprotective strategy for limiting the extent of injurious Na<sup>+</sup>-overload that would otherwise occur upon re-oxygenation. The dependence of NHE1 reinsertion on adequate GSH levels thus ensures that the myocyte's redox state recovers fully before the cell can commit to a restoration of energy-demanding pH<sub>i</sub> regulation and its ensuing Na<sup>+</sup> load.

## **Conclusions and physiological significance**

In conclusion, we have shown that NHE1, the dominant acid-export pathway in HL-1 cells, becomes internalized under hypoxia, which leads to a reduction in acid-extrusion capacity. Periods of hypoxia are associated with acidosis, which intuitively merits a stronger acid-extrusion response from cells. However, concurrently, hypoxic cells become more energy-constrained as a result of the switch-over to glycolytic metabolism. Since NHE1 activity can consume a significant fraction of cellular ATP, the internalization process effectively reduces energy consumption by limiting the extent of NHE1-dependent Na<sup>+</sup> loading of cells. This inhibitory effect can minimise the incidence of Ca<sup>2+</sup> overload and arrhythmia (Ford et. al., 2017), which would be a particularly important cardioprotective measure. Other acid-handling mechanisms may become more important in hypoxia, such as MCT driven by lactate production and electrogenic NBC. MCT4 is induced in hypoxia and its activity is not ATP-dependent (i.e. not secondary active). The activity of electrogenic NBC in HL1 cells appears not to be suppressed in hypoxia. Since this transporter has half the coupling ratio of NHE1 (one Na<sup>+</sup> ion for two acid-equivalents), it can be designated a Na<sup>+</sup>-sparing mechanism. Thus, MCT and

NBC may be an energetically efficient alternative to NHE1 activity. NHE1 remains internalized after long-term hypoxia, while the GSH remains depleted (Fig. 8). Thus, the extent of GSH depletion may play a critical role in the balance between sarcolemmal and endocytosed NHE1, and thereby determine the extent of possible cardiac ischemia-reperfusion injury.

### **Acknowledgements**

This study was supported by Ankara University Scientific Research Projects Unit (HBK, 11B3330004), TUBITAK (BT, 109S267), BHF Programme Grant (RG/15/9/31534). We are grateful to Prof. Mehmet Uğur, Prof. Özlem Uğur, and Prof. H. Ongun Onaran for their valuable scientific and technical support on this paper.

### **References**

- Allen DG, Orchard CH. 1983. The effects of changes of pH on intracellular calcium transients in mammalian cardiac muscle. *J Physiol* 335:555-567.
- Ambrose LJ, Abd-Jamil AH, Gomes RS, Carter EE, Carr CA, Clarke K, Heather LC. 2014. Investigating mitochondrial metabolism in contracting HL-1 cardiomyocytes following hypoxia and pharmacological HIF activation identifies HIF-dependent and independent mechanisms of regulation. *J Cardiovasc Pharmacol Ther* 19(6):574-585.
- Andersen AD, Poulsen KA, Lambert IH, Pedersen SF. 2009. HL-1 mouse cardiomyocyte injury and death after simulated ischemia and reperfusion: roles of pH, Ca<sup>2+</sup>-independent phospholipase A<sub>2</sub>, and Na<sup>+</sup>/H<sup>+</sup> exchange. *Am J Physiol Cell Physiol* 296(5):C1227-1242.
- Arsham AM, Howell JJ, Simon MC. 2003. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *J Biol Chem* 278(32):29655-29660.
- Avkiran M, Marber MS. 2002. Na<sup>(+)</sup>/H<sup>(+)</sup> exchange inhibitors for cardioprotective therapy: progress, problems and prospects. *J Am Coll Cardiol* 39(5):747-753.
- Boron WF, De Weer P. 1976. Intracellular pH transients in squid giant axons caused by CO<sub>2</sub>, NH<sub>3</sub>, and metabolic inhibitors. *J Gen Physiol* 67(1):91-112.
- Bountra C, Vaughan-Jones RD. 1989. Effect of intracellular and extracellular pH on contraction in isolated, mammalian cardiac muscle. *J Physiol* 418:163-187.

- Bourseau-Guilmain E, Menard JA, Lindqvist E, Indira Chandran V, Christianson HC, Cerezo Magana M, Lidfeldt J, Marko-Varga G, Welinder C, Belting M. 2016. Hypoxia regulates global membrane protein endocytosis through caveolin-1 in cancer cells. *Nat Commun* 7:11371.
- Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. 2004. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 287(4):C817-833.
- Cao SS, Kaufman RJ. 2014. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxid Redox Signal* 21(3):396-413.
- Chow CW, Khurana S, Woodside M, Grinstein S, Orlowski J. 1999. The epithelial Na(+)/H(+) exchanger, NHE3, is internalized through a clathrin-mediated pathway. *J Biol Chem* 274(53):37551-37558.
- Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ, Jr. 1998. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A* 95(6):2979-2984.
- Crompton M. 1999. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 341 (Pt 2):233-249.
- Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, Schubert D. 2004. Protein disulfide bond formation in the cytoplasm during oxidative stress. *J Biol Chem* 279(21):21749-21758.
- Cutaia M, Parks N. 1994. Oxidant stress decreases Na<sup>+</sup>/H<sup>+</sup> antiport activity in bovine pulmonary artery endothelial cells. *Am J Physiol* 267(6 Pt 1): L649-659.
- Demaurex N, Romanek RR, Orlowski J, Grinstein S. 1997. ATP dependence of Na<sup>+</sup>/H<sup>+</sup> exchange. Nucleotide specificity and assessment of the role of phospholipids. *J Gen Physiol* 109(2):117-128.
- Fliegel L, Haworth RS, Dyck JR. 1993. Characterization of the placental brush border membrane Na<sup>+</sup>/H<sup>+</sup> exchanger: identification of thiol-dependent transitions in apparent molecular size. *Biochem J* 289 (Pt 1):101-107.
- Ford KL, Moorhouse EL, Bortolozzi M, Richards MA, Swietach P, Vaughan-Jones RD. 2017. Regional acidosis locally inhibits but remotely stimulates Ca<sup>2+</sup> waves in ventricular myocytes. *Cardiovasc Res* 113(8): 984-995.
- Ganitkevich V, Reil S, Schwethelm B, Schroeter T, Benndorf K. 2006. Dynamic Responses of Single Cardiomyocytes to Graded Ischemia Studied by Oxygen Clamp in On-Chip Picochambers. *Circ Res* 99:165-171.

- Halestrap AP. 1999. The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. *Biochem Soc Symp* 66:181-203.
- Hisamitsu T, Pang T, Shigekawa M, Wakabayashi S. 2004. Dimeric interaction between the cytoplasmic domains of the Na<sup>+</sup>/H<sup>+</sup> exchanger NHE1 revealed by symmetrical intermolecular cross-linking and selective co-immunoprecipitation. *Biochemistry* 43(34):11135-11143.
- Hisamitsu T, Ben Ammar Y, Nakamura TY, Wakabayashi S. 2006. Dimerization is crucial for the function of the Na<sup>+</sup>/H<sup>+</sup> exchanger NHE1. *Biochemistry* 45(44):13346-13355.
- Hu S, Huang M, Li Z, Jia F, Ghosh Z, Lijkwan MA, Fasanaro P, Sun N, Wang X, Martelli F, Robbins RC, Wu JC. 2010. MicroRNA-210 as a novel therapy for treatment of ischemic heart disease. *Circulation* 122(11 Suppl):S124-131.
- Hu W, Zhang P, Gu J, Yu Q, Zhang D. 2017. NEDD4-1 protects against ischaemia/reperfusion-induced cardiomyocyte apoptosis via the PI3K/Akt pathway. *Apoptosis* 22(3):437-448.
- Hulikova A, Harris AL, Vaughan-Jones RD, Swietach P. 2013. Regulation of intracellular pH in cancer cell lines under normoxia and hypoxia. *J Cell Physiol* 228(4):743-752.
- Kandilci HB, Leem CH, Vaughan-Jones RD. 2007. Acute anoxia attenuates sodium-hydrogen exchange activity in isolated mammalian ventricular myocytes. *Experimental Biology*, Washington D.C., USA. 27 April-02 May 2007. *Faseb Journal* 21(6): A1281-A1282.
- Kandilci HB, Leem CH, Swietach P, Vaughan-Jones RD. 2009. Hypoxic sensitivity of the sodium-hydrogen exchanger in isolated mammalian ventricular myocytes. The role of intracellular ATP. *BPS meeting*, London. 15-17 December 2009. pA2 online 7(4): Abstract No: 48
- Ke Q, Costa M. 2006. Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol* 70(5):1469-1480.
- Kilic A, Huang CX, Rajapurohitam V, Madwed JB, Karmazyn M. 2014. Early and Transient Sodium-Hydrogen Exchanger Isoform 1 Inhibition Attenuates Subsequent Cardiac Hypertrophy and Heart Failure Following Coronary Artery Ligation. *J Pharmacol Exp Ther* 351(3):492-499.
- Kim JS, Jin Y, Lemasters JJ. 2006. Reactive oxygen species, but not Ca<sup>2+</sup> overloading, trigger pH and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. *Am J Physiol Heart Circ Physiol* 290: H2024–H2034.
- Leem CH, Lagadic-Gossmann D, Vaughan-Jones RD. 1999. Characterization of intracellular pH regulation in the guinea-pig ventricular myocyte. *J Physiol* 517 (Pt 1):159-180.

- Li X, Liu Y, Kay CM, Muller-Esterl W, Fliegel L. 2003. The Na<sup>+</sup>/H<sup>+</sup> exchanger cytoplasmic tail: structure, function, and interactions with tescalcin. *Biochemistry* 42(24):7448-7456.
- Lluis JM, Morales A, Blasco C, Colell A, Mari M, Garcia-Ruiz C, Fernandez-Checa JC. 2005. Critical role of mitochondrial glutathione in the survival of hepatocytes during hypoxia. *J Biol Chem* 280(5):3224-3232.
- Louch WE, Sheehan KA, Wolska BM. 2011. Methods in cardiomyocyte isolation, culture, and gene transfer. *J Mol Cell Cardiol* 51(3):288-298.
- Lucien F, Brochu-Gaudreau K, Arsenault D, Harper K, Dubois CM. 2011. Hypoxia-induced invadopodia formation involves activation of NHE-1 by the p90 ribosomal S6 kinase (p90RSK). *PLoS One* 6(12): e28851.
- Mailloux RJ. 2016. Application of Mitochondria-Targeted Pharmaceuticals for the Treatment of Heart Disease. *Curr Pharm Des* 22(31):4763-4779.
- Mansfield KD, Simon MC, Keith B. 2004. Hypoxic reduction in cellular glutathione levels requires mitochondrial reactive oxygen species. *J Appl Physiol* (1985) 97(4):1358-1366.
- Mari M, Morales A, Colell A, Garcia-Ruiz C, Fernandez-Checa JC. 2009. Mitochondrial Glutathione, a Key Survival Antioxidant. *Antioxid Redox Sign* 11(11):2685-2700.
- Miao Y, Zhou J, Zhao M, Liu J, Sun L, Yu X, He X, Pan X, Zang W. 2013. Acetylcholine attenuates hypoxia/ reoxygenation-induced mitochondrial and cytosolic ROS formation in H9c2 cells via M2 acetylcholine receptor. *Cell Physiol Biochem* 31(2-3):189-198.
- Moncoq K, Kemp G, Li X, Fliegel L, Young HS. 2008. Dimeric structure of human Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 1 overproduced in *Saccharomyces cerevisiae*. *J Biol Chem* 283(7):4145-4154.
- Neely JR, Whitmer JT, Rovetto MJ. 1975. Effect of coronary blood flow on glycolytic flux and intracellular pH in isolated rat hearts. *Circ Res* 37(6):733-741.
- Nishinaka Y, Kitahara S, Sugiyama S, Yokota M, Saito H, Ozawa T. 1991. The Cardioprotective Effect of Gamma-Glutamylcysteine Ethyl-Ester during Coronary Reperfusion in Canine Hearts. *Brit J Pharmacol* 104(4):805-810.
- Robin E, Guzy RD, Loor G, Iwase H, Waypa GB, Marks JD, Vanden Hoek, TL, Schumacker PT. 2007. Oxidant Stress during Simulated Ischemia Primes Cardiomyocytes for Cell Death during Reperfusion. *J Biol Chem* 282(26):19133–19143.



- Shimoda LA, Fallon M, Pisarcik S, Wang J, Semenza GL. 2006. HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial myocytes. *Am J Physiol Lung Cell Mol Physiol* 291(5): L941-949.
- Simonin A, Fuster D. 2010. Nedd4-1 and beta-arrestin-1 are key regulators of Na<sup>+</sup>/H<sup>+</sup> exchanger 1 ubiquitylation, endocytosis, and function. *J Biol Chem* 285(49):38293-38303.
- Snabaitis AK, Hearse DJ, Avkiran M. 2002. Regulation of sarcolemmal Na<sup>(+)</sup>/H<sup>(+)</sup> exchange by hydrogen peroxide in adult rat ventricular myocytes. *Cardiovasc Res* 53(2):470-480.
- Stone D, Darley-USmar V, Smith DR, O'Leary V. 1989. Hypoxia-reoxygenation induced increase in cellular Ca<sup>2+</sup> in myocytes and perfused hearts: the role of mitochondria. *J Mol Cell Cardiol* 21(10):963-973.
- Tracey WR, Allen MC, Frazier DE, Fossa AA, Johnson CG, Marala RB, Knight DR, Guzman-Perez A. 2003. Zoniporide: A Potent and Selective Inhibitor of the Human Sodium-Hydrogen Exchanger Isoform 1 (NHE-1). *Cardiovascular Drug Reviews*. 21 (1):17–32.
- Vaughan-Jones RD, Spitzer KW, Swietach P. 2009. Intracellular pH regulation in heart. *J Mol Cell Cardiol* 46(3):318-331.
- Williams IA, Xiao XH, Ju YK, Allen DG. 2007. The rise of [Na<sup>+</sup>]<sub>i</sub> during ischemia and reperfusion in the rat heart-underlying mechanisms. *Pflugers Arch* 454: 903–912.

## FIGURE LEGENDS

**Figure 1.** Sarcolemmal acid-extrusion mechanisms in HL-1 cells. A) Expression of genes coding for sarcolemmal NHE isoforms SLC9A1, 2, 3 and 8 and electrogenic SLC4A4, evaluated by PCR (1% agarose gel electrophoresis). B) Time course of  $\text{pH}_i$  in superfused HL-1 cells. Experiments performed in Hepes-buffered Tyrode solution (pH 7.4, 37°C). Ammonium prepulse solution manoeuvre was performed to load cytoplasm with acid. Recovery from low  $\text{pH}_i$  was greatly attenuated in the absence of superfusate  $\text{Na}^+$  (substituted with NMDG) but restored rapidly upon  $\text{Na}^+$  addition, confirming a  $\text{Na}^+$ -dependent (but  $\text{HCO}_3^-$  independent) acid-extrusion mechanism. C)  $\text{pH}_i$  time course in HL-1 cells showing two consecutive ammonium prepulse manoeuvres, the second one performed in the presence of 1  $\mu\text{M}$  (black) or 30  $\mu\text{M}$  (gray) zoniporide, a NHE1 inhibitor. Zoniporide slowed  $\text{pH}_i$  recovery dose-dependently, implicating a role for NHE1. (n=3 clusters of HL-1 cells, on average of 5 cells each seeded and cultured independently).

**Figure 2.** The effect of 10 min (acute) anoxia and 48 hours hypoxia on acid-extrusion in HL-1 cells. A)  $\text{pH}_i$  time course in HL-1 cells, showing three consecutive ammonium prepulses. First  $\text{pH}_i$  recovery was recorded in normoxia (21%  $\text{O}_2$ ), second recovery was recorded under anoxia (1 mM sodium dithionite plus continuously bubbling with 100%  $\text{N}_2$ ), and final recovery was measured in normoxia after wash-out of sodium dithionite. Dashed guide line indicates the  $\text{pH}_i$  at which recovery in anoxia halts ( $\text{pH}_i=6.95$ ). Recovery following anoxia is complete. B) Flux analysis of data from 4 HL-1 clusters (each containing on average 5 cells): acute anoxia (grey) decreased NHE1-flux. C) The effect acute anoxia shown as plot of NHE1-flux in anoxia ( $\text{NHE1-flux}_{\text{anoxia}}$ ) versus control ( $\text{NHE1-flux}_{\text{control}}$ ) plotted at matching  $\text{pH}_i$ . D) HL-1 cells were incubated for 48 hours in 1%  $\text{O}_2$  (hypoxia; grey) or 21%  $\text{O}_2$  (normoxia; black). Ammonium prepulse solution manoeuvres were performed under normoxic conditions to probe for the effects of hypoxia that persist, even after cells are removed from the hypoxic chamber (for up to 2 hours). Recovery of  $\text{pH}_i$  was slower in cells that had been incubated in hypoxia for 48 hrs. E) The experimental protocol shown in the inset. 48 hours of hypoxia (grey) decreased NHE1-flux (n=11 clusters of HL-1 cells, on average of 5 cells each seeded and cultured independently). F) Effect of 48 hours of hypoxia on NHE1-flux plotted as  $\text{NHE1-flux}_{\text{hypoxia}}$  vs  $\text{NHE1-flux}_{\text{control}}$ . \* compared to control  $p<0.05$ . Data are mean  $\pm$  SE.

**Figure 3.** Confocal images showing immunofluorescence staining for HIF-1 $\alpha$  protein in HL-1 cells. A) 5 or 48 hours of hypoxia or DMOG (inhibitor of HIF-1 $\alpha$  degrading enzyme) resulted in a translocation of HIF-1 $\alpha$  to the nucleus (n=3). For masking, nuclei were detected by nucleic acid stain (Yo-pro-1), and converting this into grey scale with white contours. Scale bars: 25  $\mu$ M. Quantification of HIF-1 $\alpha$  levels in B) cytoplasm and C) nucleus, given as fluorescence intensity per unit area and normalized to normoxia. # denotes significant difference compared to 5 hours hypoxia, † compared to 5 hours DMOG incubation. “n” number represents the data obtained from independent group of HL-1 cells grown in different days on coverslips. D) Following a similar protocol to Fig 2E, effect of 48 hours of DMOG incubation on NHE1-flux (n=4 clusters of HL-1 cells, on average of 5 cells each seeded and cultured independently). Experimental protocol is shown in the inset. W/O denotes washout of DMOG. E) Effect of 48 hours of DMOG incubation (in normoxia) on NHE1-flux plotted as NHE1-flux<sub>DMOG</sub> vs NHE1-flux<sub>control</sub>. \* compared to control p<0.05. Data are mean  $\pm$  SE.

**Figure 4.** Western blot analyses of NHE1 (mature form at ~100 kDa). A) Representative Western blots for lysates collected from cells under control conditions, after 48 hours of hypoxia and after 48 hours of DMOG treatment. B) Densitometric analyses of NHE1 were performed and normalized to  $\beta$ -actin. Data normalized to normoxia show no effect of hypoxia or DMOG (n=6-9 blots). C) No difference in expression of SLC9A1, SLC9A8 and SLC4A4 coding genes at mRNA level in 48 hours of hypoxia versus control (n=3). In contrast, SLC9A3 expression was upregulated, albeit from a low baseline level in normoxia. “n” number represents the data obtained from independent group of HL-1 cells grown in different days on coverslips. Data are mean  $\pm$  SE.

**Figure 5.** Hypoxia-induced internalization and reinsertion of NHE1. A) Representative confocal images of surface NHE1 immunofluorescence showing internalization evoked by 10 min acute anoxia, by 48 hours of hypoxia, and 48 hours of DMOG incubation. Membrane reinsertion was observed after wash-out of sodium dithionite after a 10-min exposure (reoxygenation: Reox) and after wash-out of DMOG following a 48-hour treatment period (W/O). In contrast, 2 hours of re-oxygenation in atmospheric O<sub>2</sub> after a 48-hour hypoxic episode was not sufficient to restore NHE1 at the surface membrane. Scale bars: 25  $\mu$ m. B) Quantification of NHE1 internalization (see methods, n=3). The experimental protocols are shown in the inset. “n” number represents the data obtained from independent group of HL-1

cells grown in different days on coverslips. \* compared to control  $p < 0.05$ . Data are mean  $\pm$  SE.

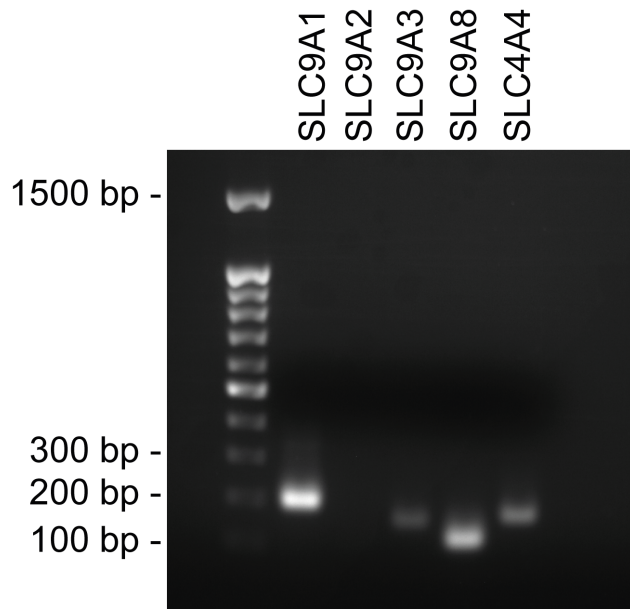
**Figure 6.** Effect on 48 hours of hypoxia on intracellular ATP and GSH. A) Using the same confocal magnification, cell counts were made from DNA staining (Yo-Pro-1 staining). Both DMOG treatment and 48 hour hypoxia decreased cell proliferation ( $n=7$ ). B) ATP levels, measured by luciferase assay, normalized to cell number, after 48 hour of hypoxic and after 2 hours of subsequent reoxygenation ( $n=4$ ). C) Total GSH levels, measured by colorimetric assay, normalized to total protein, after 48 hour hypoxic incubation ( $n=5$ ). The experimental protocols are shown in the inset. “n” number represents the data obtained from independent group of HL-1 cells grown in different days on coverslips. \* compared to control  $p < 0.05$ . Data are mean  $\pm$  SE.

**Figure 7.** Effect of GSH supplementation on NHE1 re-insertion. A) Immediately after removal from the hypoxia chamber, cells were incubated with 1 mM of the cell-permeable glutathione monoethyl ester for 30 min. This GSH supplementation reversed the inhibitory effect of 48-hour hypoxia on NHE1-flux. B) Incubation with the cell-permeable glutathione monoethyl ester (1 mM) in normoxia, without a prior hypoxic episode, did not change NHE1-flux ( $n=4-5$ ). C) Representative images and quantification of data showing the effect of 30 min of GSH incubation on NHE1 protein distribution by immunofluorescence ( $n=3$ ). Scale bars: 25  $\mu$ m. The experimental protocols are shown in the inset. “n” number represents the data obtained from a group of HL-1 cells (average of  $> 5$  Buraks). \* compared to control  $p < 0.05$ . Data are mean  $\pm$  SE.

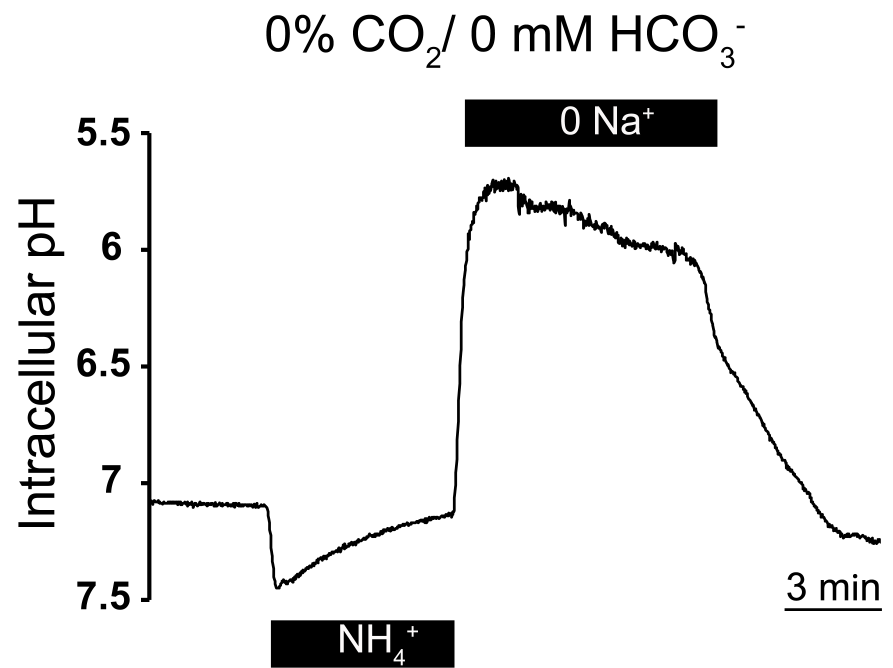
**Figure 8.** Cartoon of proposed model for the mechanism by which hypoxia regulates NHE1 in HL-1 cells. Short deprivation of oxygen is sufficient to internalize NHE1 and reduce sarcolemmal activity. NHE1 internalization, and hence sarcolemmal function, were reversed rapidly upon reoxygenation. Long-term hypoxia triggered internalization that was poorly reversible upon reoxygenation, unless reduced glutathione (GSH) levels were restored.

**FIG 1**

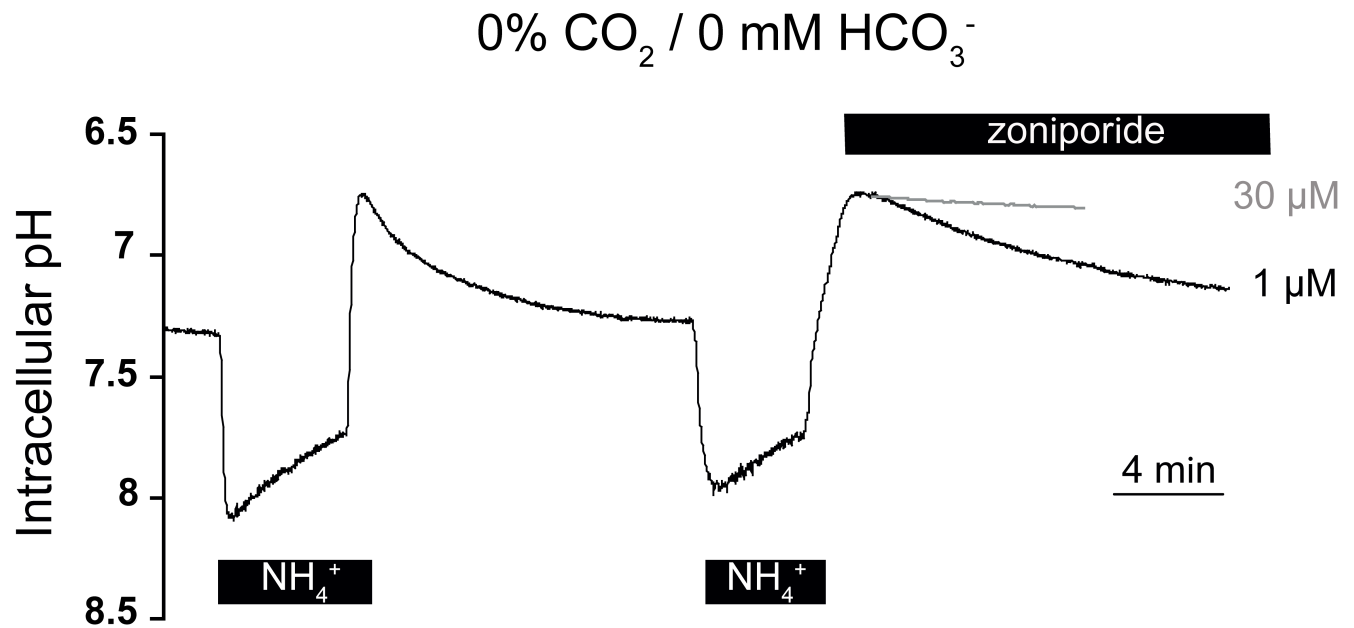
**A**



**B**



**C**



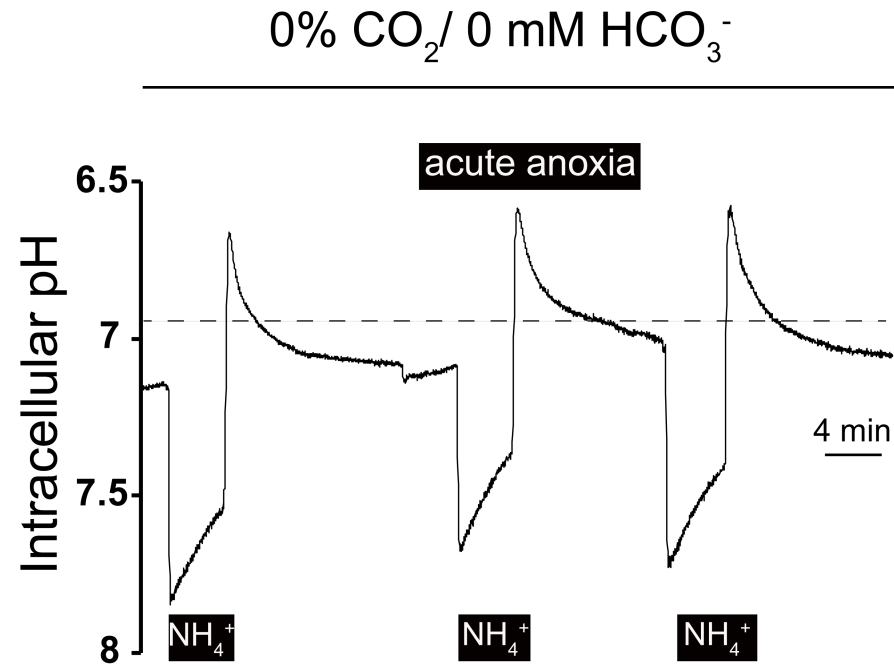
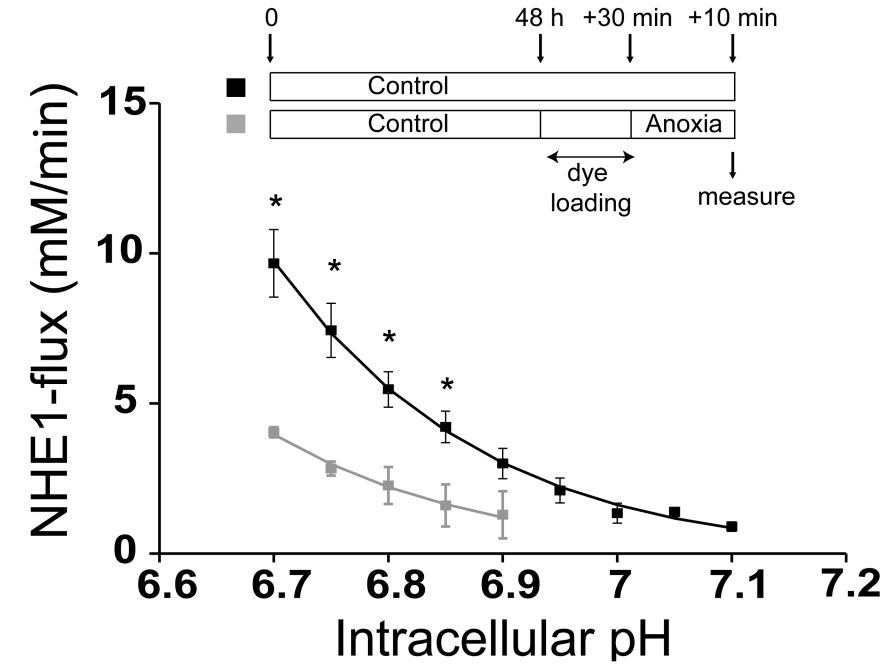
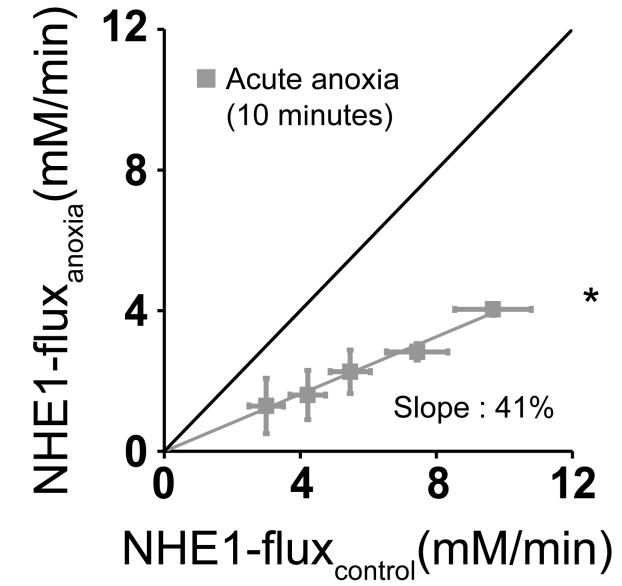
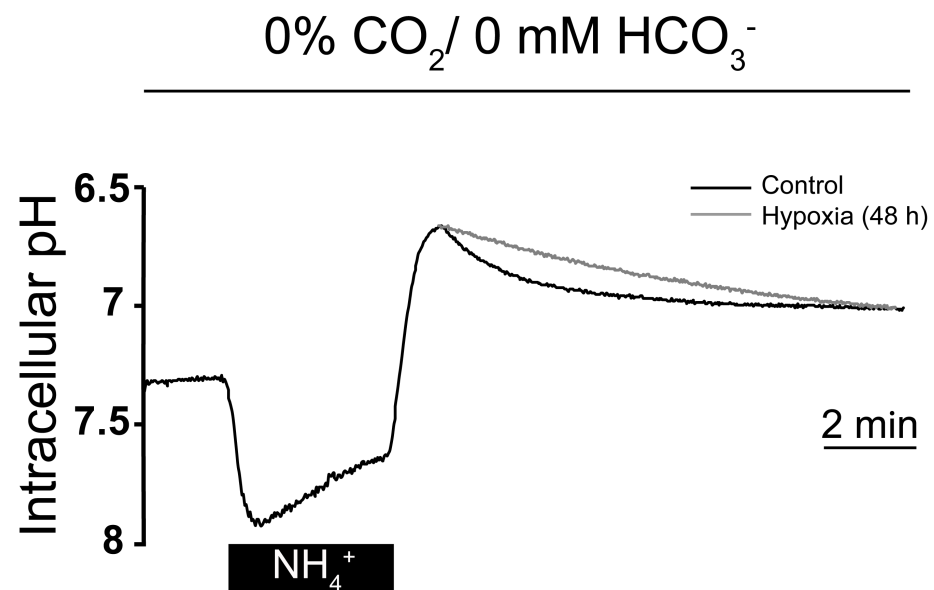
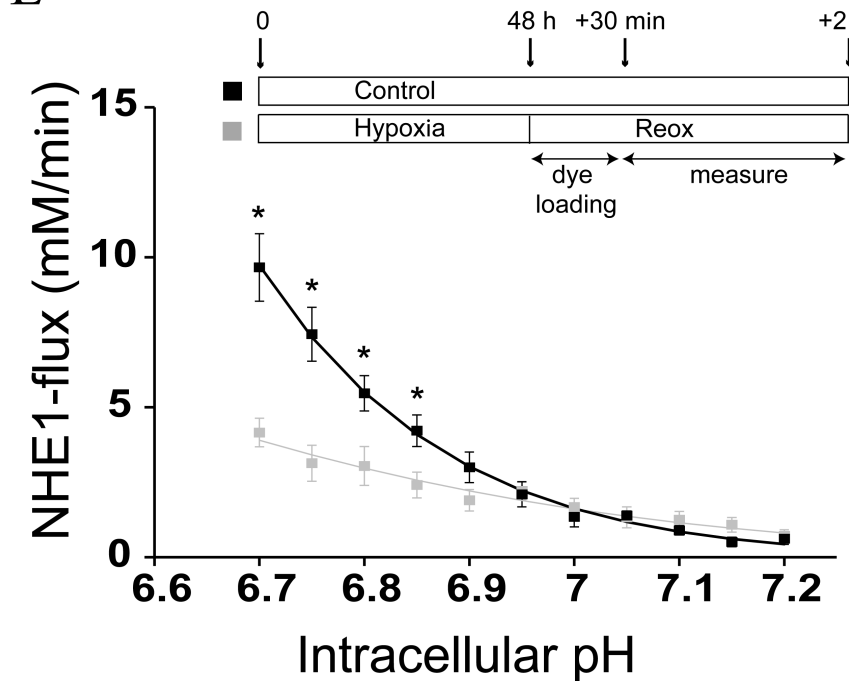
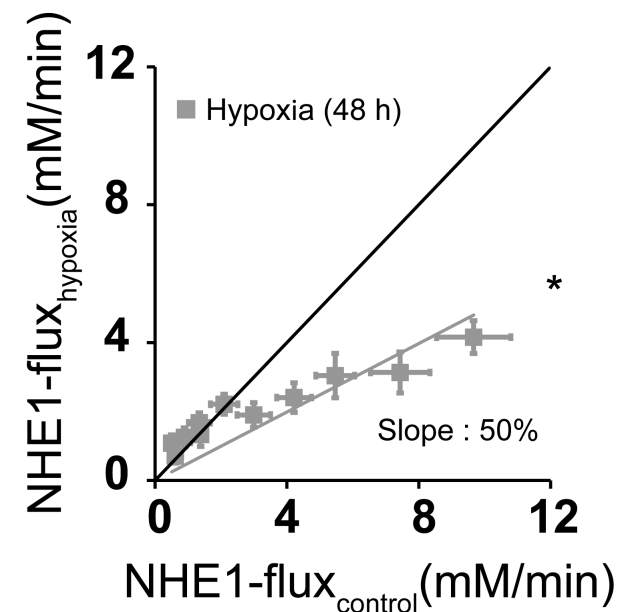
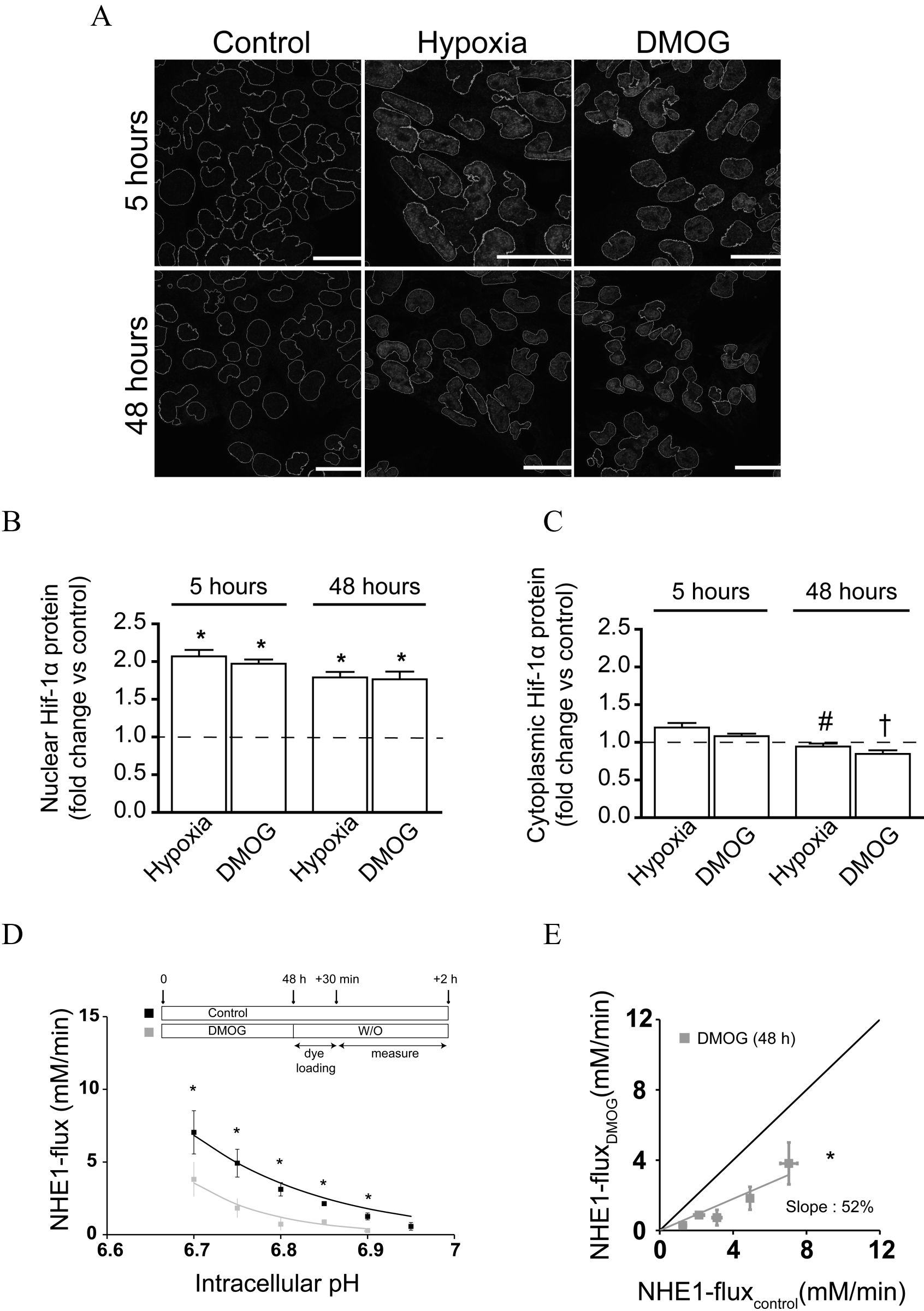
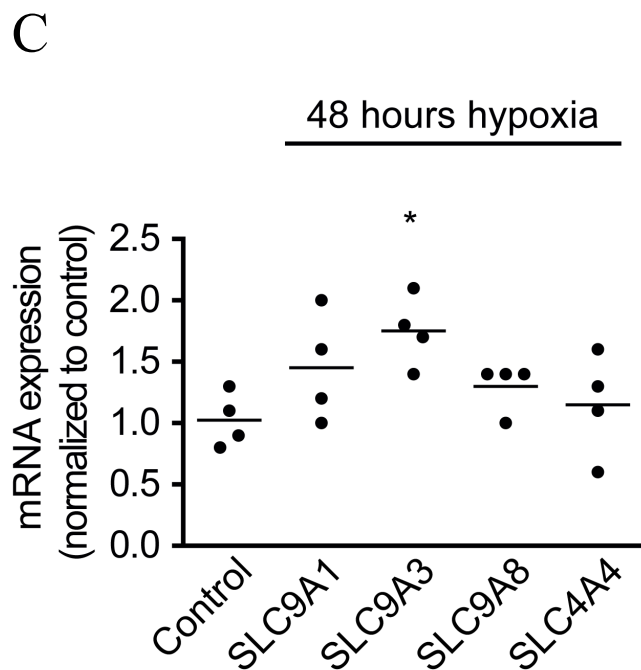
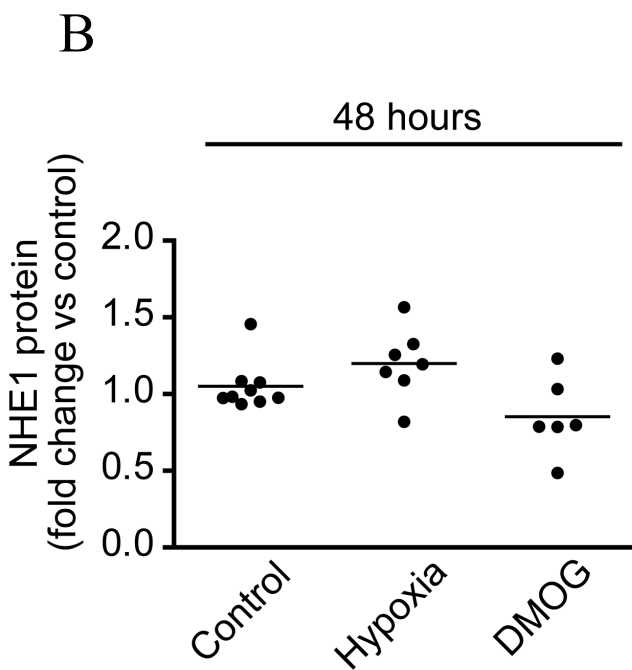
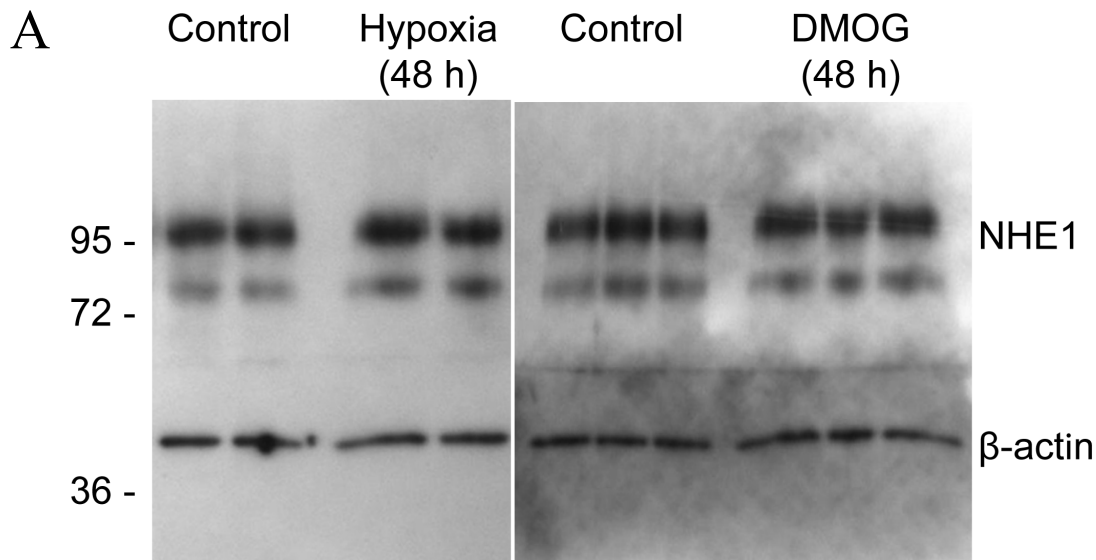
**FIG 2****A****B****C****D****E****F**

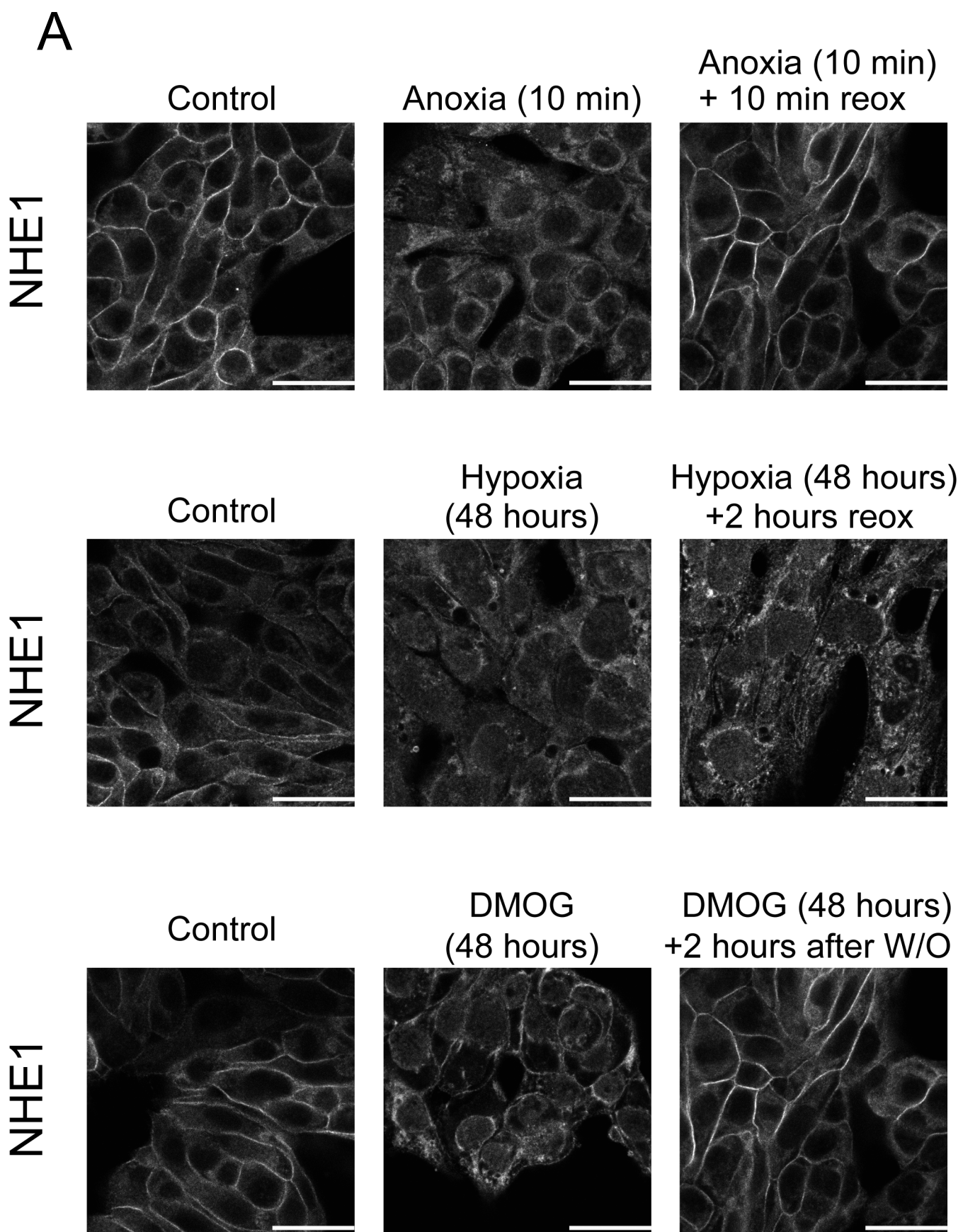
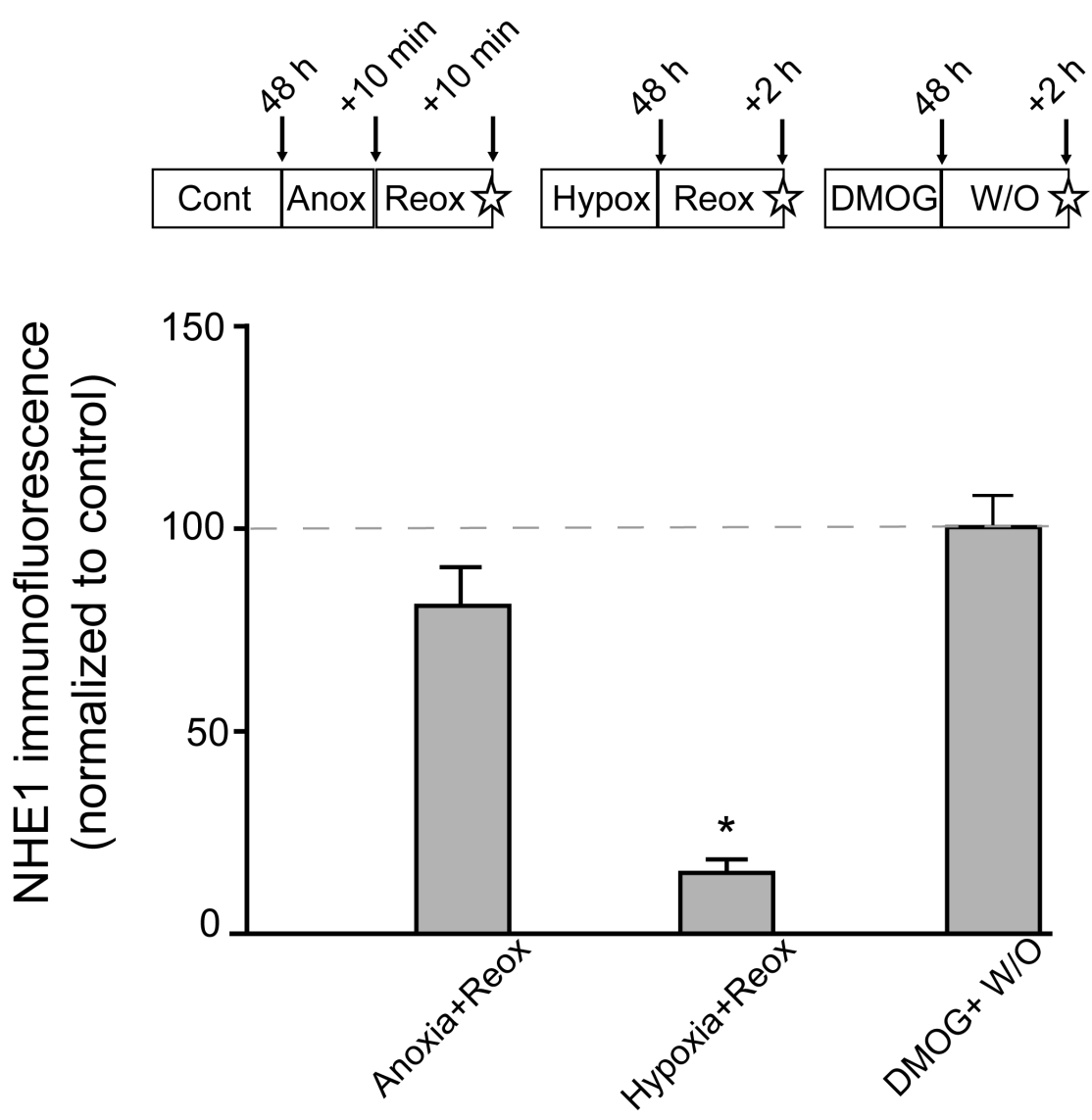
FIG 3



# FIG 4

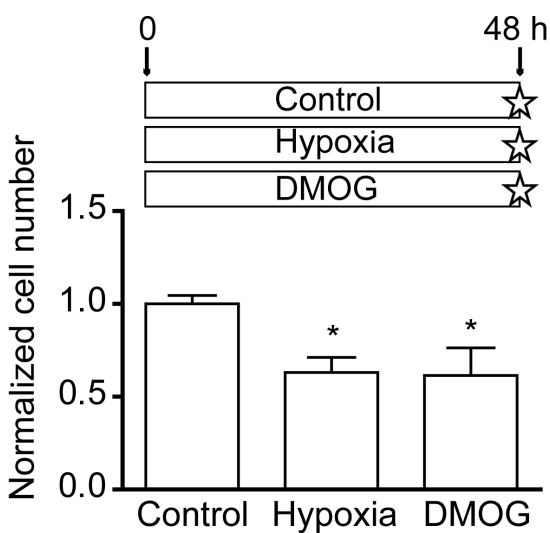




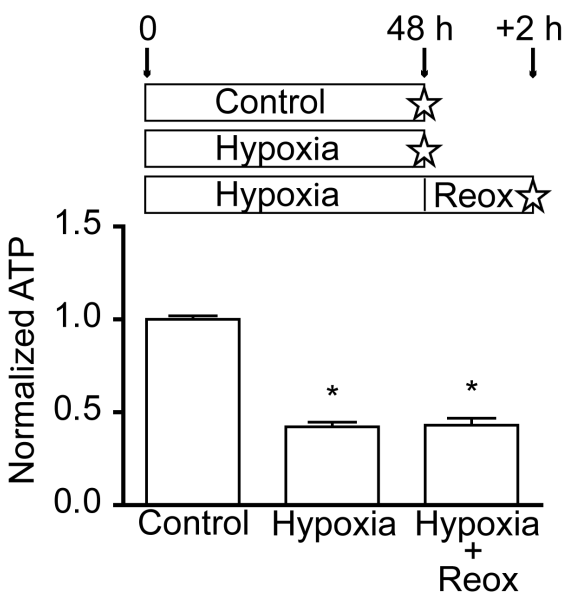
**FIG 5****B**

# FIG 6

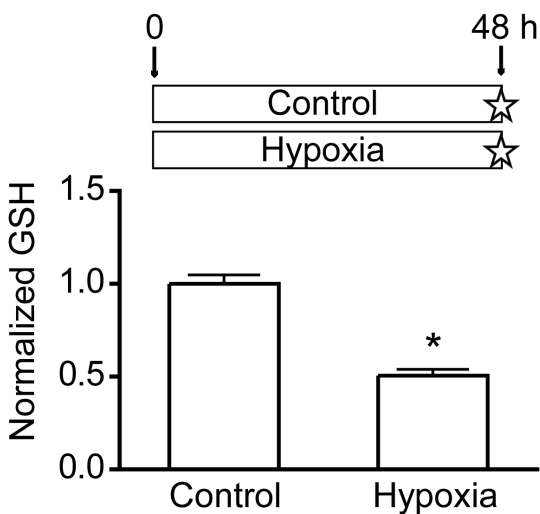
A

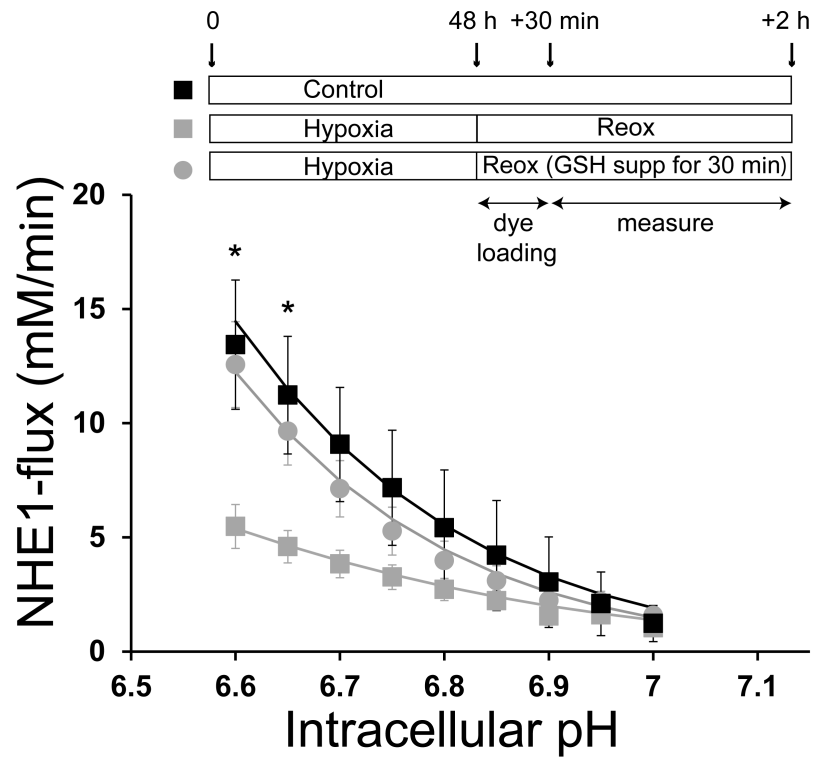
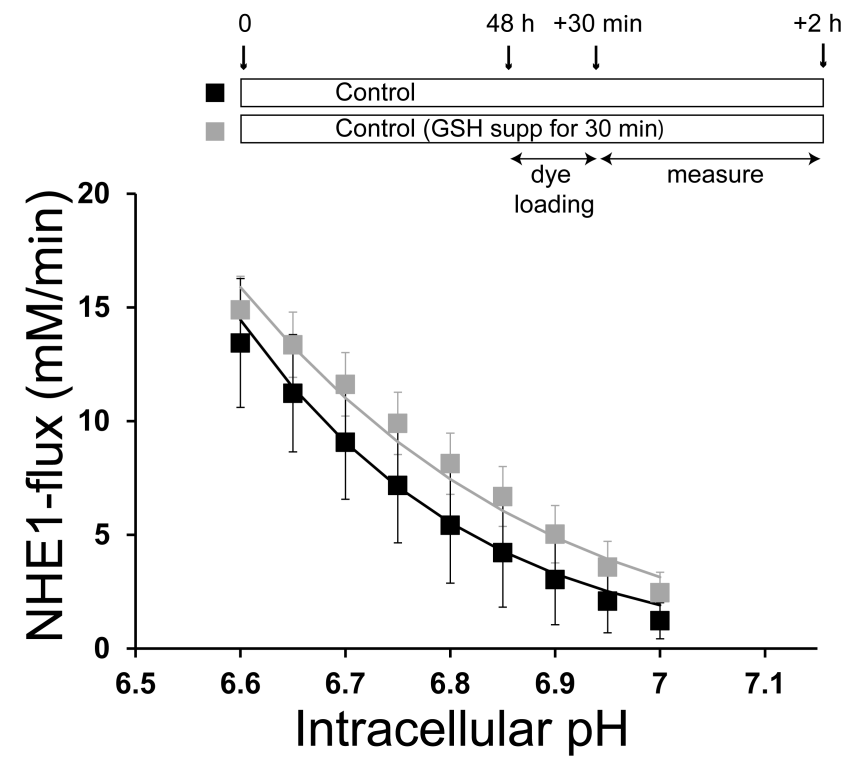
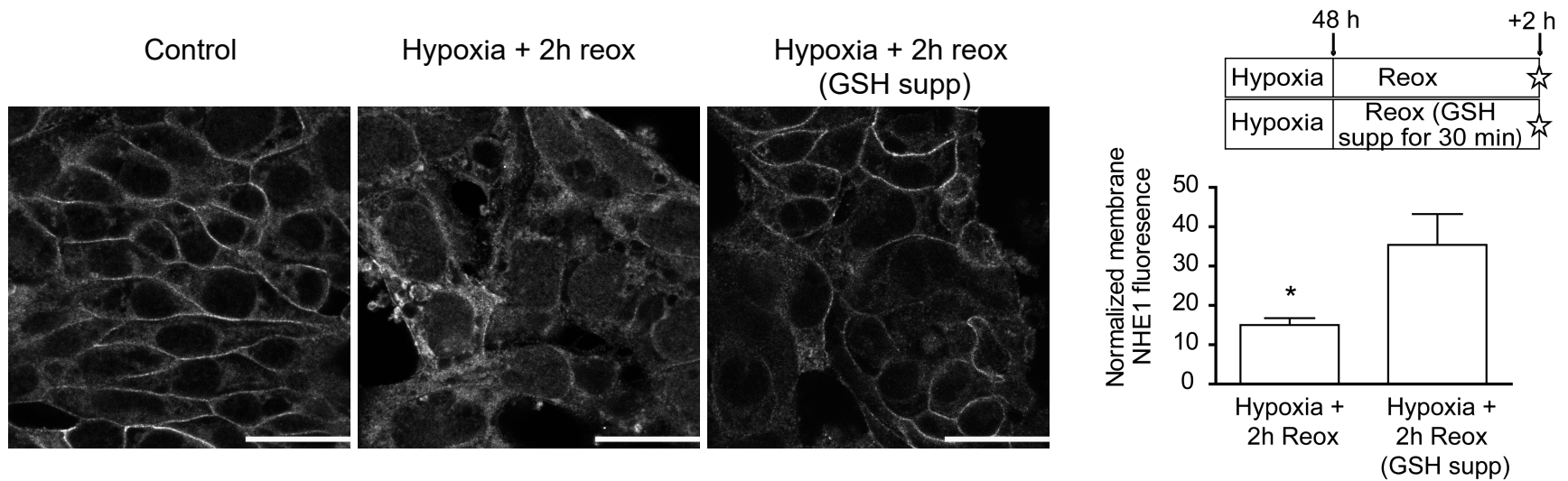


B

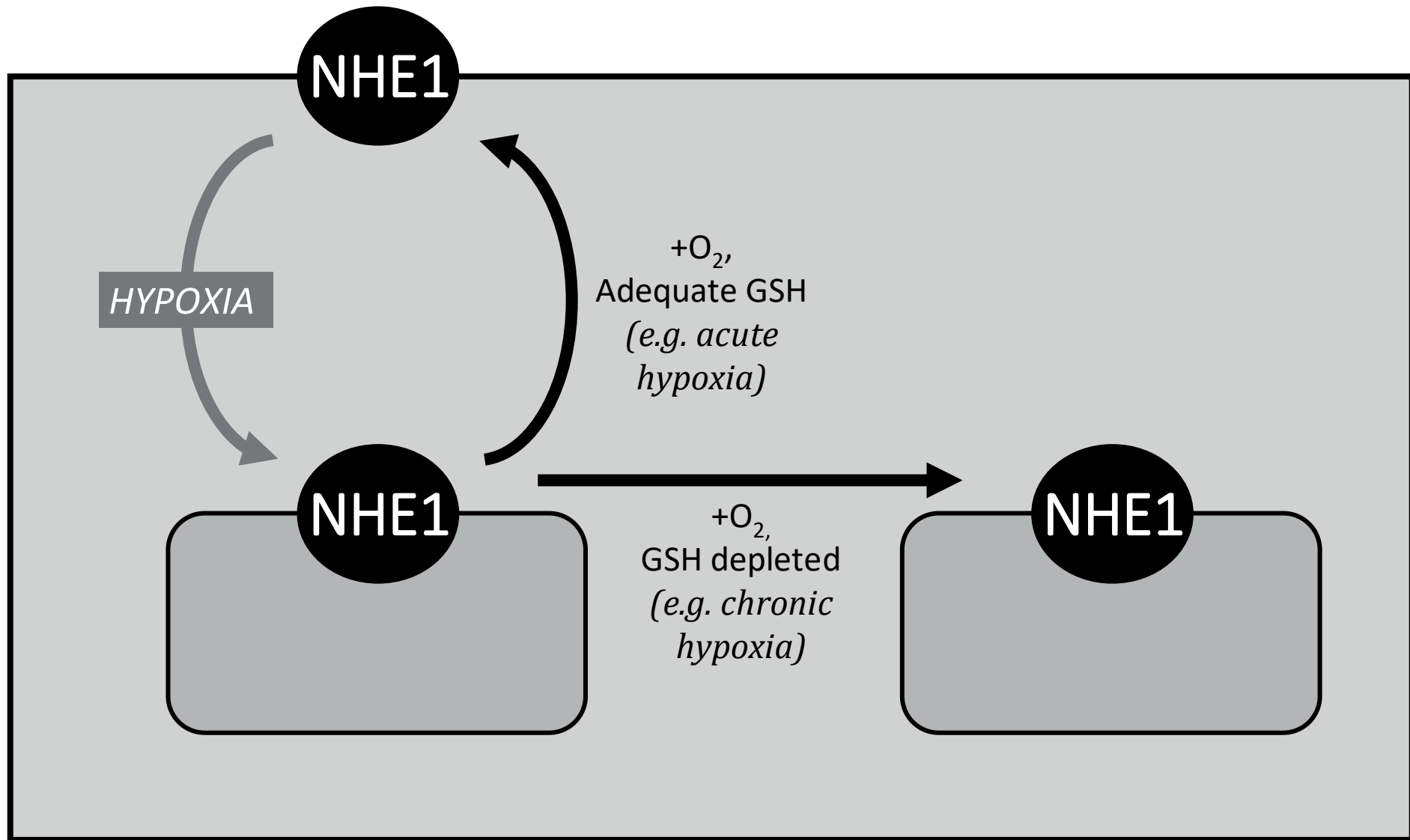


C



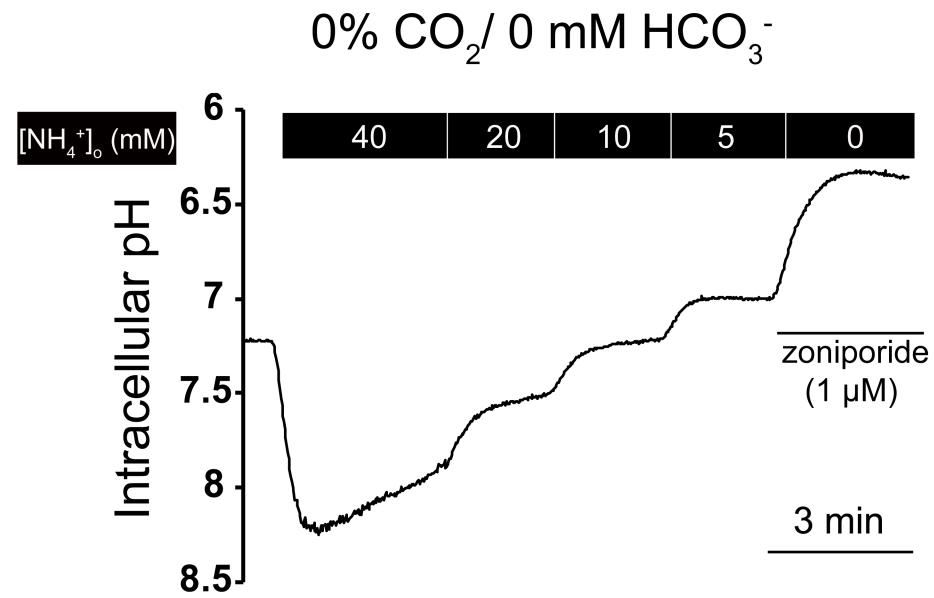
**FIG 7****A****B****C**

**FIG 8**

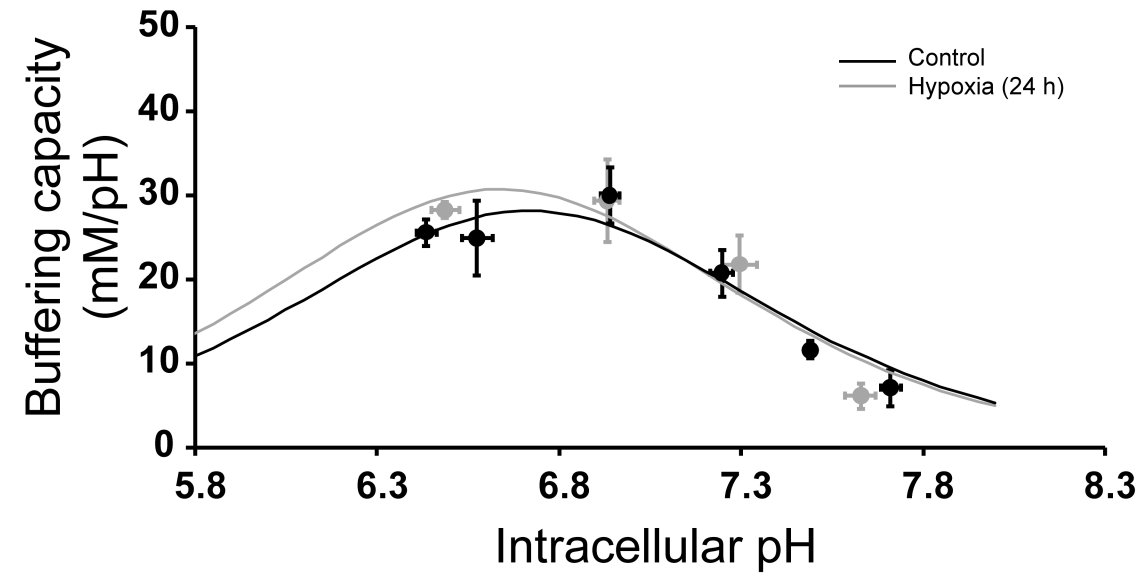


# FIG SUPP 1

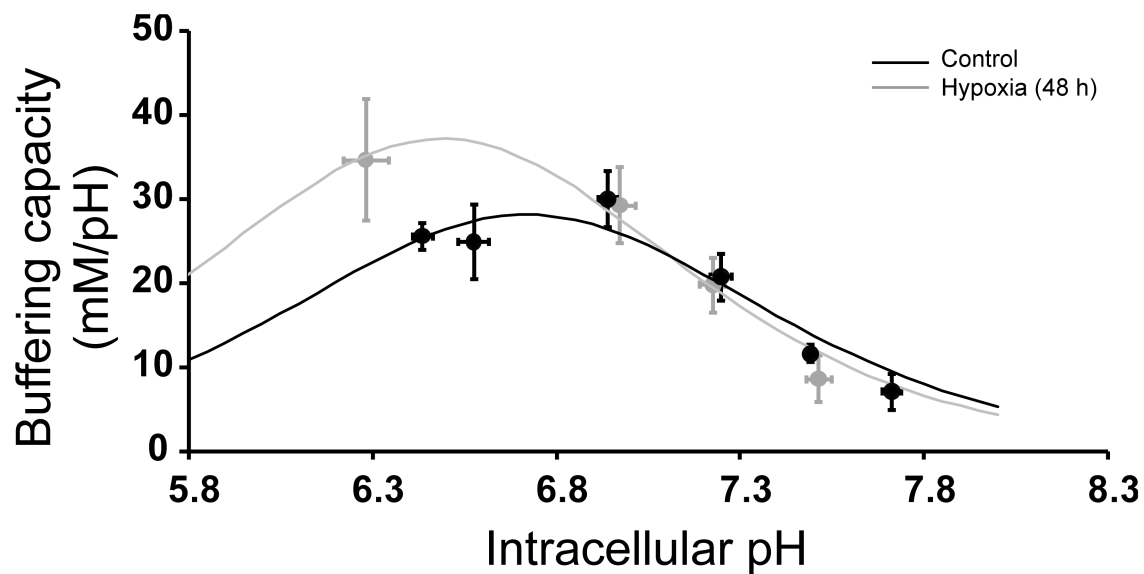
A



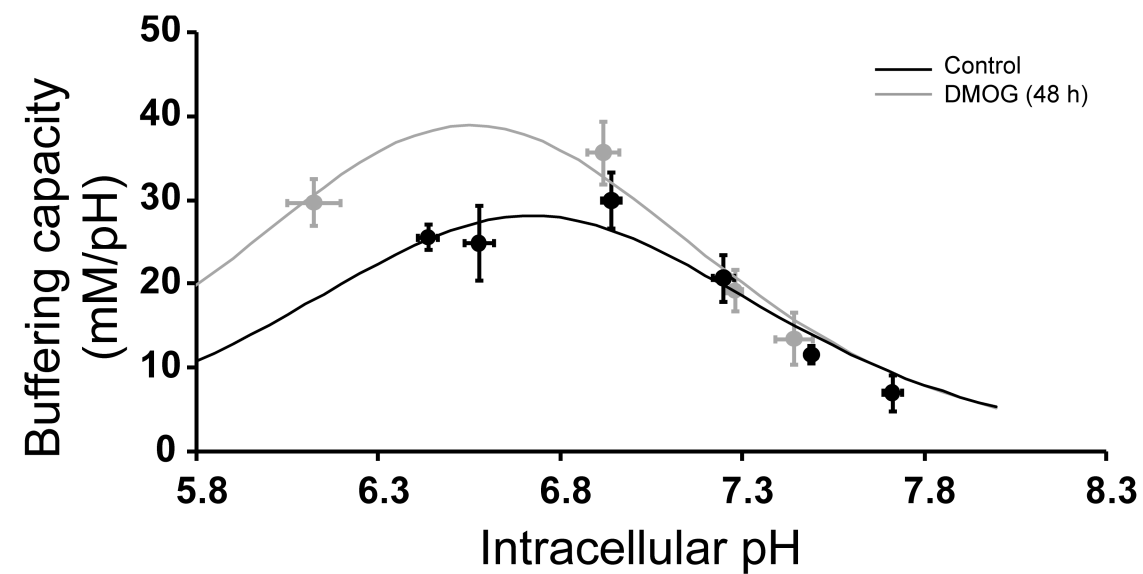
B



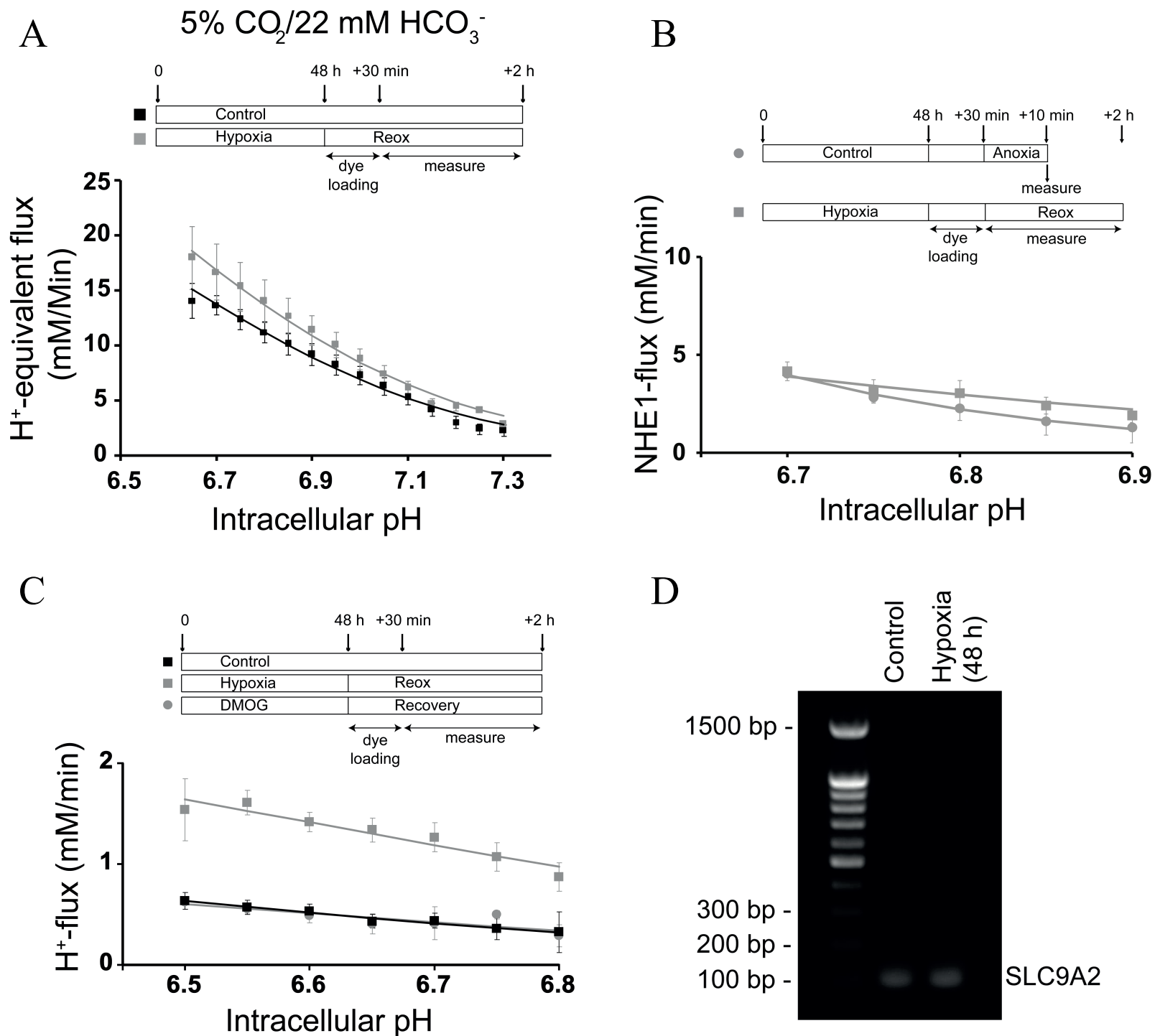
C



D

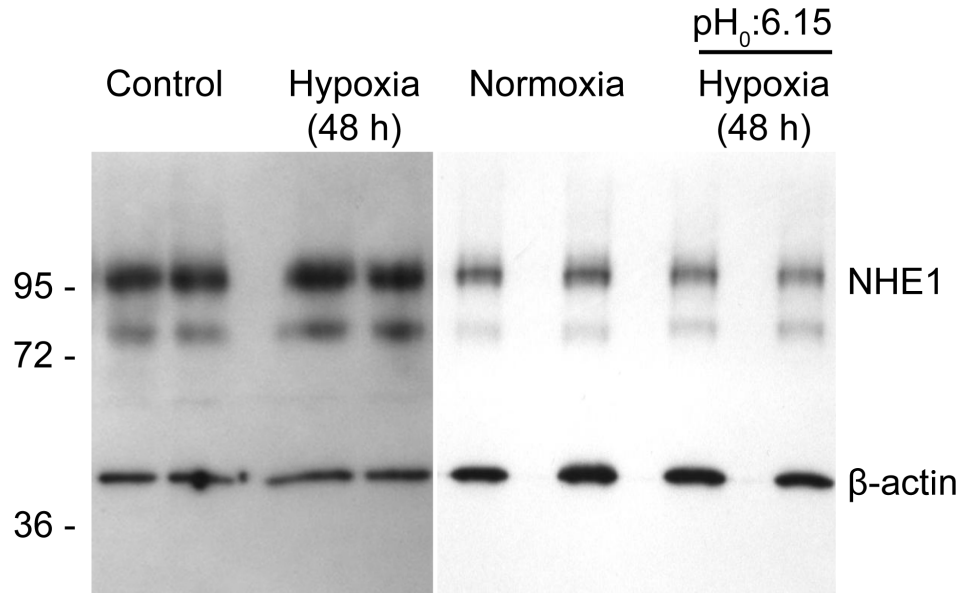


# FIG SUPP 2

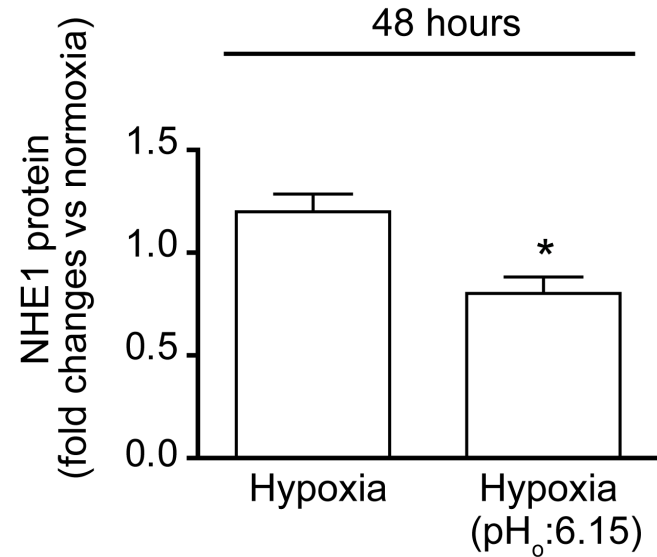


# FIG SUPP 3

A



B



**FIG SUPP 4**

