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**Translocation of the Na<sup>+</sup>-H<sup>+</sup> Exchanger 1 (NHE1) in cardiomyocyte responses to insulin and energy-status signalling.**

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Running title: Regulated NHE1 translocation

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### Summary

The sodium/proton exchanger NHE1 is a highly regulated membrane protein that is required for pH homeostasis in cardiomyocytes. The activation of NHE1 leads to proton extrusion, which is essential for counteracting cellular acidity that occurs following increased metabolic activity or ischemia. The activation of NHE1 intrinsic catalytic activity has been well characterised and established experimentally. However, we have examined here whether a net translocation of NHE1 to the sarcolemma of cardiomyocytes may also be involved in the activation process. We have determined the distribution of NHE1 by means of immunofluorescence microscopy and cell-surface biotinylation. We have discovered changes in the distribution of NHE1 that occur when cardiomyocytes are stimulated with insulin that are PI 3-kinase dependent. Translocation of NHE1 also occurs when cardiomyocytes are challenged by hypoxia, or inhibition of mitochondrial oxidative metabolism, or electrically-induced contraction but these responses occur through a PI 3-kinase independent process. As the proposed additional level of control of NHE1 through translocation was un-expected we have compared this process with the well-established translocation of the glucose transporter GLUT4. In immunofluorescence microscopy comparisons, the translocations of NHE1 and GLUT4 to the sarcolemma that occur in response to insulin appear to be very similar. However, in basal unstimulated cells the two proteins are mainly located, with the exception of some co-localisation in the perinuclear region, in distinct subcellular compartments. We propose that the mechanisms of translocation of NHE1 and GLUT4 are linked such that they provide spatially and temporally co-ordinated responses to cardiac challenges that necessitate re-adjustments in glucose transport, glucose metabolism and cell pH.

**Key words:** NHE1 translocation, Insulin, Cell-energy status, Cardiomyocytes, GLUT4 translocation.

### Abbreviations used:

NHE – Na<sup>+</sup>/H<sup>+</sup> exchanger  
GLUT4 – glucose transporter isoform 4  
PI 3-kinase – phosphatidylinositol 3-kinase  
pH<sub>i</sub> – intracellular pH  
AMPK - AMP dependent protein kinase  
*Lens culinaris* agglutinin – LcH  
Secretory Carrier Associated Membrane Protein 2 - SCAMP2

## INTRODUCTION

The mammalian Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) family of proteins plays an important role in maintenance of intracellular pH (pH<sub>i</sub>)[1]. Nine isoforms have been described in mammalian genomes. NHEs 1 and 6-9 have a ubiquitous expression and NHEs 2-5 have selective tissue expression.[2]. NHE2 and 3 are predominantly expressed in apical membranes of kidney and intestinal epithelial cells[3,4], while NHE4 is highly expressed in the stomach[5]. NHE5 is almost exclusively expressed in the brain, although low levels of expression are also reported in spleen, testes and skeletal muscles[6,7]. In the mammalian myocardium, the only plasma membrane isoform is NHE1 and this exchanger plays a crucial role in the maintenance of pH<sub>i</sub> in healthy cells and impairments in its function is associated with disease state[8]. NHE1 activity is primarily regulated by decreases in pH<sub>i</sub> and maximal catalytic activity of the transporter is achieved at pH<sub>i</sub> 6.5 or less[9,10]. NHE1 has been directly implicated in, and has been found to be associated with, damage caused by ischemia/reperfusion injuries and by hypertrophy[11]. For these reasons NHE1 is a major pharmacological target for development of cardio-protective drugs[12].

NHE1 has been described as a relatively immobile actin-anchored plasma membrane protein[13,14]. In many tissues NHE1 activity is stimulated by growth and hormonal factors, including serum, EGF and insulin which leads to proton extrusion and a transient cell alkalinisation[15-18]. Hormonal stimulations have been linked to direct phosphorylation of NHE1 by protein kinases including Erk 1-2, p160ROCK, p38 MAP kinase, p90 ribosomal S6 kinase (p90rsk), CaM kinase II and Akt and to dephosphorylation dependent upon the protein phosphatases PP1, PP2A and SHP-2. The changes in phosphorylation are thought to modulate the exchanger's catalytic activity, possibly through altered interaction of the C-terminal cytoplasmic tail of NHE1 with the actin cytoskeleton (reviewed[1,8]).

Recent studies on P-type transporters including the Na<sup>+</sup>/K<sup>+</sup> ATPase[19,20] have revealed that, in addition to regulation via changes in intrinsic catalytic activity, changes in activity are also associated with translocation of these proteins from reservoir compartments within the cell to sites where the ion channel activity becomes functionally important. Phosphorylation of aquaporin 2 in the kidney appears to be critical both for initiating translocation and determining the cellular compartment to which translocation occurs[21]. It therefore seems pertinent to determine whether, in addition to the well established control via phosphorylation-dependent changes in catalytic activity, NHE1 activity could also be regulated by translocation.

The translocation process and its underlying mechanisms have been most extensively studied for the glucose transporter isoform 4 (GLUT4) which is regulated by insulin action, and also in response to changes in the cell energy status. Cellular responses to insulin, but not to changes in cell energy status, are mediated through phosphatidylinositol 3-kinase (PI 3-kinase). Cardiac cell stimulation following hypoxia and inhibition of oxidative mitochondrial metabolism culminate in GLUT4 translocation that is initiated by, or dependent upon, signalling-induced changes in the AMP dependent protein kinase AMPK. The GLUT4 translocation mediated through AMPK is functionally important as the resultant increase in cellular glucose transport can lead to the readjustment and replenishment of cell glucose metabolism and ATP levels. Our previous study has shown that insulin action on GLUT4 translocation and NHE1 activity are intricately linked, demonstrated by the finding that NHE1 activity is required for optimal GLUT4 translocation[17]. Cariporide, a NHE inhibitor[22], prevented the insulin induced cytosol alkalinisation and the associated insulin-stimulated GLUT4 translocation and glucose uptake[17]

To explore further the link between NHE1 activity and GLUT4 translocation we have examined here the hypothesis that the NHE1 transporter may also be translocated in response to the same treatments that stimulate GLUT4 translocation. Based on data from use of the complementary techniques of confocal microscopy and cell surface biotinylation, our study has revealed that there is a significant translocation of NHE1 in cardiomyocytes in responses to insulin, hypoxia and electrically-induced contraction that is similar to the established translocation process of GLUT4.

## EXPERIMENTAL

### Reagents

All chemicals, unless otherwise stated, were sourced from Sigma-Aldrich. Type II collagenase was from Worthington Biochemicals (Lakewood, NJ, USA). Fatty acid-free bovine serum albumin was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Insulin was from Novo Nordisk (Bagsværd, Denmark).

### Generation and purification of rabbit anti-rat NHE1 antibody.

For the purpose of generating a specific antibody recognising rat cardiomyocyte NHE1 in immunocytochemistry experiments, a 503 bp cDNA fragment corresponding to the last 157 amino acids from rat NHE1 (CT-NHE1) was amplified from a Marathon (Clontech) rat heart cDNA library prepared as described previously [23]. The primers used for the amplification contained restriction sites for BglIII and EcoRI (5'-GTGGCCAGATCTTACGAGGAAGCCTGGAACCAG-3' and 5'-CTAGGAATTCTCTACTGCCCTTTGGGGATG-3') allowing direct sub-cloning into pGEX-2T vector (GE Healthcare). The GST-fusion protein was expressed in BL21 strain of *E. coli*. The GST-NHE1 fusion protein was purified by affinity chromatography using glutathione-Sepharose 4B columns (GE Healthcare) and was then dialysed against phosphate-buffered saline PBS (12.5 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 154 mmol/L NaCl, pH 7.2). Purified protein was used as an antigen for the preparation of a polyclonal rabbit serum. Antiserum was generated in New Zealand White Rabbits at Harlan-Sera (UK) using a standard immunisation protocol.

The specific IgGs were purified from the immune serum using an affinity column with immobilised C-terminal NHE1 protein fragment (as above, but lacking the GST fusion which was removed by thrombin treatment). The specificity of the antibody was tested using whole cell cardiomyocytes lysates. The antibody specifically recognised bands at 110 kDa and the 85 kDa corresponding to the two glycosylated forms of NHE1 described previously in cardiomyocytes [24,25] (Supplementary Figure S1).

### Cardiomyocyte isolation and stimulation

Cardiomyocytes from adult male Wistar rats (260–280 g) were prepared by collagenase digestion by use of a method previously described [17] but with the inclusion of 2 mmol/L inosine in the medium. Cell suspension were adjusted to ≈ 10% cytochrome c in Krebs-Ringer-HEPES (KRH) buffer (128 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 0.2 mmol/L NaHPO<sub>4</sub>, 1.4 mmol/L MgSO<sub>4</sub>, 1 mmol/L CaCl<sub>2</sub>, 10 mmol/L HEPES pH 7.4) supplemented with 0.5% fatty acid-free bovine serum albumin, 2 mmol/L pyruvate and 5.5 mmol/L glucose. With the exception of the hypoxic buffer treatment, cells were continuously gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> to pH 7.4 throughout the treatments. For insulin stimulation cells were incubated with 30 nmol/L insulin for 30 min at 37°C. Where indicated in the figure legends, cardiomyocytes were pre-treated with 100 nmol/L wortmannin or equivalent amount of dimethyl sulfoxide (≤ 0.1%). Stimulation by hypoxia was induced by incubation in KRH buffer with 0.5% (w/v) fatty-acid free BSA that had been deoxygenated for 60 min and pre-gassed with nitrogen for a further 30 min. The nitrogen atmosphere was maintained throughout subsequent incubations. Chemically induced hypoxia was achieved by treating the cells with 5 μmol/L oligomycin for 60 min. For electrical stimulation of contraction, 1 ml aliquots of cell suspensions at 37°C, 10% cytochrome c, were placed in 19 mm diameter polystyrene dishes. A dish lid with attached electrodes was dipped into the cell suspension. Cells were stimulated at exercise levels of contraction for 5 min at 100 V, with pulse duration of 1 ms and frequency of 10 Hz. Contraction was monitored under a microscope. For all the conditions used in this study, no more than 10-20% decrease of the initial cell viability was observed.

### Indirect immunofluorescence microscopy.

Stimulated cardiomyocytes were fixed by incubation with 4% (w/v) paraformaldehyde in KRH buffer for 30 min at room temperature, and washed 3 times with PBS. The cells were then maintained in blocking

buffer (1% (w/v) BSA, 3% (v/v) goat serum in PBS) for 40 min at room temperature. For localisation of the plasma membrane, fixed cells were first incubated with biotinylated *Lens culinaris* agglutinin (LcH) (Sigma) for 30 min at room temperature, washed with PBS to remove unbound LcH, incubated with streptavidin Alexa Fluor 633 conjugate (Molecular Probes) and then washed again with PBS. Cells were then treated with permeabilisation buffer (0.1% saponin, 1% (w/v) BSA, 3% (v/v) goat serum in PBS) for 45 min and incubated with 10 µg/mL of rabbit anti-rat NHE1 antibody overnight at 4°C. The following day, the cells were washed in permeabilisation buffer, incubated with Alexa Fluor 488 goat anti-rabbit IgG (4 µg/mL; Molecular Probes) for 2 h at room temperature and then washed again in permeabilisation buffer. The co-localisation of NHE1 with GLUT4 was determined in cardiomyocytes that were incubated as above for localisation of NHE1 but with the subsequent addition of 0.1 µg/mL mouse monoclonal anti-GLUT4 antibody (Clone 1F8, Biogenesis) overnight at 4°C and then Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes) for 2 h at room temperature with a final wash step in permeabilisation buffer. Cells were mounted onto a glass coverslip with Vectashield mounting medium (Vector Laboratories).

Confocal microscopy was performed on a Zeiss LSM 510 META microscope with 63x 1.4 NA oil-immersion objective and with dual or triple laser excitation at 458–488, 543 and 633 nm. Images were processed using the Zeiss LSM Image analysis software and Adobe Photoshop. For quantification of the changes in NHE1 distribution, the intensities of NHE1 and LcH fluorescence were sampled along radial lines across the perinuclear area to the sarcolemma and intercalated disk surfaces. The proportion of NHE1 signal that overlapped with LcH signal at the cell surfaces was calculated as percent of total NHE1 sampled along the radial line (Figure 2A).

#### Cell surface biotinylation.

Levels of NHE1 at the cell surface were assessed by a surface proteins biotinylation method based on methods described previously [17,26]. Cells were treated under conditions indicated in the figure legends and then cooled to 15°C for 5 min. A cell-impermeable biotin analogue, EZ-Link-Sulfo-NHS-SS-Biotin (Pierce) was added to the cells at a final concentration of 180 µmol/L for 15 min. The reaction was terminated by washing the cells with ice-cold KRH and transferring to ice-cold TES buffer (25 mmol/L Tris-HCl pH 7.4, 1 mmol/L EDTA, 0.25 mol/L sucrose) containing protease inhibitors (antipain, aprotinin, pepstatin A, and leupeptin (each at 1 µg/mL) and 100 µmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride). Membrane pellets were prepared by a modification of a previously described method [27]. Briefly, cells were homogenized in ice-cold TES with 30 strokes of a Potter-Elvehjem homogenizer. Homogenates were then subjected to centrifugation (48,000 g for 30 min at 4°C).

The cell pellets were resuspended in TBS (25 mmol/L Tris-HCl pH 7.4, 140 mmol/L NaCl) and solubilised in lysis buffer (1% (w/v) triton, 1% (w/v) sodium deoxycholate, 0.2% (w/v) SDS, 25 mmol/L Tris-HCl pH 7.4, 140 mmol/L NaCl) containing protease inhibitors for 2 h at 4°C. The solubilised membranes were adjusted for equal protein content (625 µg/mL) and incubated overnight with 50 µL of 50% slurry of immobilised streptavidin on agarose beads (Pierce). After the pull-down, beads were washed twice with lysis buffer, twice with TBS and once with TE buffer (10 mmol/L Tris-HCl pH 7.4, 5 mmol/L EDTA). Proteins were eluted in SDS-PAGE sample buffer containing 100 mmol/L dithiothreitol, separated by SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis of samples precipitated with streptavidin-agarose was performed using a monoclonal anti-NHE1 antibody (Chemicon, MAB3140 clone 4E9) and a peroxidase conjugated secondary antibody (Pierce). Signals were detected by ECL and were quantified using Epi-Chemi II Dark Room equipment and LabWorks analysis software (UVP).

A control biotinylation experiment was performed to confirm the specificity of the biotinylation of NHE1 by processing the cardiomyocytes in the same way as above with the following modifications. At the end of the treatments, the cardiomyocytes were labelled with EZ-Link-Sulfo-NHS-LC-Biotin (Pierce) instead of EZ-Link-Sulfo-NHS-SS-Biotin to avoid cleavage of the biotin moiety during the processing of the samples. The cardiomyocytes were then processed as described above but the detergent solubilised membrane samples were immunoprecipitated with 8 µg of rabbit anti-rat NHE1 antibody overnight. The following day, 20 µL of 50% slurry of immobilised Protein A (Pierce) were added to the

samples and incubated for further 3 h at 4°C. The immunoprecipitated NHE1 were then resolved by SDS-PAGE, transferred to nitrocellulose for blotting using Extravidin-peroxidase conjugate (Sigma) to detect the biotinylated proteins.

## RESULTS

### Subcellular localisation of NHE1 in cardiomyocytes

In order to investigate the subcellular localisation of NHE1 in cardiomyocytes a rat NHE1 specific antibody was developed. This antibody was then used to study the subcellular localisation of NHE1 in rat cardiomyocytes by immunofluorescence and confocal microscopy. In agreement with a previous study[28] immunofluorescent detection of endogenous NHE1 in non-stimulated cardiomyocyte membranes revealed that NHE1 preferentially localised at the intercalated disks and the sarcolemma. NHE1 was also localised in t-tubule regions identified as distinctive lines running transversely across the cell and at 90° to the longitudinal region of sarcolemma (Figure 1, left panel). At the sarcolemma and t-tubules of unstimulated basal cardiomyocytes, NHE1 was localised in punctate spots of varying intensity. NHE1 was also seen at deeper perinuclear locations.

In insulin-stimulated cardiomyocytes, NHE1 distribution at the sarcolemma was more uniform and homogenous compared with the punctate sarcolemma distribution observed in basal cells (Figure 1, left panel). One of the problems associated with use of confocal microscopy to demonstrate protein translocation to the limiting cell membranes is that, on fusion with the limiting membrane, any signal becomes more delocalised and indistinct. The signal from proteins inserted into the limiting membrane is difficult to detect unless the extent of colocalisation with a plasma membrane marker protein is determined. This phenomenon is documented in numerous studies examining the translocation of GLUT4 in cardiomyocytes and skeletal muscle cells and cell lines. We have previously quantified the translocation of endogenous GLUT4 in cardiomyocytes using immunocytochemical techniques by comparing the GLUT4 and syntaxin4 colocalisation at the sarcolemma[17]. To quantitatively evaluate the extent of NHE1 translocation to the sarcolemma we labelled the cell surface with a biotinylated *Lens culinaris* agglutinin (LcH) in conjunction with an Alexa Fluor 633 streptavidin conjugate. LcH labels the whole of the sarcolemma but not the t-tubules (Figure 1, middle panel). We determined the level of NHE1 signal that overlapped with that of LcH at the intercalated discs and the longitudinal edge regions of the sarcolemma and expressed this level as a percentage of the total NHE1 sampled (Figure 2A and 2B). We did not observe any difference in the pattern of NHE1 redistribution along the longitudinal edge of the sarcolemma when compared to the intercalated discs so the data from these two regions of the sarcolemma were combined. The NHE1 levels at the sarcolemma were found to increase in response to insulin, to oxidative metabolism stress induced by hypoxia or oligomycin treatment, and after electrical stimulation to mimic contraction. For all the stimuli, an increase of  $\approx 2$ -fold in the amount of NHE1 at the sarcolemma was observed (Figure 2B).

### Comparison of NHE1 and GLUT4 translocation

NHE1 has been primarily considered to be a plasma membrane resident protein and has not previously been considered to be regulated by translocation in cardiomyocytes. We therefore compared the traffic of NHE1 with that of the well studied and established process of translocation of the glucose transporter GLUT4 using comparable immunofluorescent microscopy techniques. NHE1 and GLUT4 have remarkably similar patterns of distribution throughout the cardiomyocytes (Figure 3). In the basal state both proteins are present at high levels deep within the cell in the perinuclear region and within punctuate structures throughout the cell interior. On insulin action, translocation of both proteins occurs. Close examination of images suggests that there is less punctuate NHE1 and GLUT4 in the region of the sarcolemma following stimulation with insulin, but this change in distribution is not immediately obvious for either transporter protein unless plasma membrane markers are used. The translocation of GLUT4 in

cardiomyocytes is much more visually apparent using a HA-GLUT4-GFP construct and detection with a fluorescent anti-HA antibody that recognises only cell surface exposed proteins[29].

Both NHE1 (Figure 1) and GLUT4[30] translocate to the limiting membranes of cardiomyocytes in response to insulin action and stimuli (including hypoxia and contraction) that impinge upon the energy-status signalling pathway. Both transporters show a similar pattern of subcellular distribution amongst intracellular compartments both in the perinuclear and t-tubule regions. However, there is surprisingly little overlap between the localisation of NHE1 and GLUT4 (Figure 3; merged image, third panel, and zoomed image, fourth panel) particularly in the basal state. There is some co-localisation or overlap of signals in the perinuclear region but the distribution of punctuate structures throughout the rest of the cell is distinct and non-overlapping. It appears that red (GLUT4) and green (NHE1) localisation signals alternate along radial axis that lead to the sarcolemma (Figure 3, zoomed images).

#### **Modulation of NHE1 levels at the cell surface of rat cardiomyocytes in response to insulin.**

We found that subcellular fractionation of membranes from cardiomyocytes could not resolve translocation of neither NHE1 nor GLUT4 (Supplementary Figure S2). In order to confirm the immunocytochemical evidence for regulated translocation of NHE1 we therefore applied a cell surface biotinylation technique and used an impermeable cleavable biotin succinimide reagent Sulfo-NHS-SS-Biotin to covalently tag the cell surface protein. Following the preincubation treatments described in the figure legends, cardiomyocytes were maintained with these treatments and in the presence of the biotinylating reagent. After washing away the unbound biotinylation reagent, membranes were prepared and solubilised and this material was subjected to streptavidin precipitation and SDS-PAGE resolution of the precipitated proteins. Western blot analysis with anti-NHE1 antibody revealed that insulin treatment induced an increase of the amount of biotinylated NHE1 at the cell surface, thus confirming the results observed by confocal microscopy (Figure 4A). The quantification of the blotting data indicated that insulin induced a 2.8-fold increase in the level of NHE1 present in the cell surface of cardiomyocytes. This increase was complete within 30 min and occurred without any change in the total level of NHE1 present in the cells as detected, using the same NHE1 antibody, when blotting the detergent solubilised cell membrane control (Figure 4A, lower blot). Insulin is known to activate multiple signalling pathways in cardiomyocytes. However, one of the major limbs in insulin signalling is through the activation of PI 3-kinase. To investigate whether PI 3-kinase is involved in the insulin-stimulated increase in cell surface levels of NHE1, the biotinylation experiment was performed in presence of wortmannin, a PI 3-kinase inhibitor. Wortmannin pre-treatment blocked the insulin effect but did not affect the level of biotinylation of NHE1 in the basal cells (Figure 4A and 4B).

An alternative method for processing and analysing the extent of cell surface biotinylation of NHE1 was also examined. After labelling with the impermeant biotinylating reagent, solubilised membranes were subjected to immunoprecipitation with rabbit anti-rat NHE1 antibody. Control samples were subjected to the same procedure but using a non-specific IgG pull-down. After resolution of the NHE1 by electrophoresis, the levels of biotinylated NHE1 were detected using Extravidin-peroxidase. These data (Figure 4C) confirmed the insulin-stimulated increase of NHE1 at the cell surface and also the specificity of the biotinylation technique in detecting variations in NHE1 levels.

#### **Modulation of NHE1 levels at the cell surface of rat cardiomyocytes in response to oxidative metabolism stress and contraction.**

Disrupted oxidative metabolism and stimulated contraction alter the energy status of the cells. Readjustments of the cell glucose metabolism, the AMP/ATP ratio and intracellular pH are necessary after such treatments. In the case of GLUT4 translocation the signalling that follows these stimuli is independent of PI 3-kinase and is instead correlatively associated with increases in AMPK[30]. Treatment of the cardiomyocytes under hypoxic conditions led to a 3-fold increase in cell surface biotinylation of NHE1 (Figure 5A). Quantification of these data (Figure 5B) revealed that this change was not additive to the insulin effect. When the cells were pre-treated with insulin prior to hypoxia stimulation, no further increase in the amount of biotinylated NHE1 was observed. Similar results were obtained following

disruption of the energy status of the cardiomyocytes by treatment with the mitochondrial ATPase (F1) inhibitor oligomycin (Figure 5A and 5B). Electrical stimulation of contraction led to a large 4-fold increase in NHE1 levels at the cell surface. Wortmannin inhibition of PI 3-kinase activity did not affect the redistribution of NHE1 to the cell surface that occurred in response to hypoxia, oligomycin or electrical stimulation of contraction (Figure 5A and 5B) suggesting that these stimuli affect NHE1 by PI 3-kinase independent processes. Treatment of cardiomyocytes with compound C gave a partial inhibition of the contraction response consistent with an AMPK involvement (Supplementary Figure S3). However, compound C is now known to have quite poor specificity for selective inhibition of AMPK signalling[31].

## DISCUSSION

In this study, we have shown for the first time that NHE1 levels at the sarcolemma of cardiomyocytes are acutely modulated in response to stimulation by insulin or through changes in cell-energy status. Since regulation of NHE1 translocation in cardiomyocytes is somewhat unexpected, we confirmed the occurrence of this process using the complementary techniques of immunofluorescence microscopy and cell surface biotinylation. The microscopy is most useful for determining the changes in localisation of NHE1 but is limited by quite poor resolution of the NHE1 that has become incorporated into the limiting sarcolemma membrane unless this distribution is compared with that of a limiting membrane marker such as LcH. The cell surface biotinylation technique is more useful for quantification of a range of responses to modulators and inhibitors.

Other NHE isoforms have been shown to redistribute in response to signalling. The translocation of NHE3, the apical epithelial isoform of kidney and intestine has been most studied. In the proximal tubule of kidneys NHE3 redistributes from the apical plasma membrane to a sub-apical endomembrane location in response to hypertension or increased intervascular pressure[32,33]. Increased apical cell surface expression of NHE3 in epithelial tissues occurs in response to angiotensin II, glucocorticoids and insulin but this may be partly due to increased cellular NHE3 levels (reviewed[34]). NHE5 is almost exclusively expressed in the brain and translocates between the cell surface and recycling endosomes. This process has been reported to be mediated by PI 3-kinase and to be dependent on intact actin cytoskeleton[35]. Recently, the small GTPase Arf6 together with the secretory carrier associated membrane protein 2 (SCAMP2) was reported to positively regulate the NHE5 recycling process in neurones[36]. The recruitment of NHE1 may be cell type specific and dependent on cell-specific demands for regulation of pHi. In platelets there is no detectable translocation of NHE1 and all the available NHE1 is present at the cell membrane in the unstimulated basal state[37].

Insulin is known to induce cell alkalinisation in cultured muscle and adipose cell lines[38,39], adipocytes[18], cardiomyocytes[17], and erythrocytes[40]. The insulin-induced pH changes have been linked to activation of the PI 3-kinase signalling pathway. We have previously found that the insulin action on cell alkalinisation in adipocytes[18] and cardiomyocytes[17] is associated with increased PI 3-kinase dependent GLUT4 translocation. Here we have demonstrated that the insulin effect on NHE1 translocation is also mediated through the PI 3-kinase pathway, as wortmannin completely abolished the observed increase in NHE1 levels at the cell surface.

Alterations in the oxidative metabolism of cardiomyocytes induced by hypoxia or by treatment with the mitochondrial inhibitor oligomycin also led to elevated levels of NHE1 at the cell surface. However, the mechanism involved in this stimulation is different from the insulin activation process, as wortmannin did not modify the levels of NHE1 at the cell surface of cardiomyocytes incubated in hypoxic conditions or treated with oligomycin. Changes in the AMP/ATP ratio occur in response to these treatments and are thought, at least in the case of GLUT4 traffic, to be associated with altered net translocation and retention at the cell surface. Contraction-induced NHE1 redistribution was also observed here to be independent of PI 3-kinase signalling and insensitive to wortmannin inhibition. Contraction induced signalling in cardiomyocytes and skeletal muscle only partly correlates with

activation of AMPK[30,41] and the details of the signalling mechanism by which increased contraction signals to GLUT4 traffic are currently unclear. No additivity in the combined responses of NHE1 translocation to insulin and changes in cell-energy status were observed in cardiomyocytes studied here. This contrasts with effects of these stimuli on GLUT4 translocation. It is possible that only a single limited population of NHE1 exchangers are available for translocation and that all available NHE1 in this compartment is released in response to activation of either the PI 3-kinase or the AMPK signalling pathways.

The regulated changes in NHE1 translocation may reflect the need for alkalinisation of the cells when increased anaerobic glucose metabolism and an associated cell acidosis occurs[1]. The processes by which increased translocation of NHE1 occurs may utilise the same signalling machinery that is present in cardiomyocytes for generating increased GLUT4 translocation. Such a linkage would ensure that the increased glucose metabolism is associated with restoration of the cell AMP/ATP ratio and at the same time restoration of normal cellular  $pH_i$ . Although the GLUT4 and NHE1 translocations appear to be triggered by common signalling steps, there must be some divergence in the respective links between signalling and traffic as intracellular NHE1 and GLUT4 vesicle locations are distinct over large parts of the cell interior. However, there is some overlap between intracellular GLUT4 and NHE1 in the perinuclear regions of the cardiomyocytes and this may be a site at which signals to the NHE1 and GLUT4 translocation systems converge.

The role of NHE1 translocation and the relative importance of altered translocation *versus* changes in intrinsic catalytic activity will need further investigation. Phosphorylation of NHE1 downstream of Akt has been reported to lead to both an increase [16] in catalytic activity and a decrease[25]. Phosphorylation of NHE1 downstream of protein kinase A and protein kinase G lead to decreased catalytic activity while activation of Erk downstream of protein kinase C and reactive oxygen species leads to stimulation of catalytic activity[1,42]. Increases in cell calcium also lead to activation of NHE1 intrinsic catalytic activity via calmodulin- and CaM kinase II-dependent mechanisms[1,43]. Modulation of activity also occurs through alteration of the extent of interaction with the actin cytoskeleton[13,14]. With such fine control of catalytic activity, the translocation may appear to be redundant, superfluous and un-necessary. However, there is a very clear need for multiple mechanisms for control of  $pH_i$  and NHE1 activity in cardiomyocytes[1]. There are requirements for very localised changes in  $pH_i$ [44]. It would be of interest to determine the extent to which translocation of NHE1 is associated with altered phosphorylation. Mathematical models have been used to analyse experimental kinetic data on NHE1 activity in cardiomyocytes and have suggested that catalytic activity alone may not fully account for the observed changes[45,46]. It would therefore be useful to incorporate regulated NHE1 translocation into models of control of total cellular and spatially localised NHE1 activity.

In addition, phosphorylation dependent changes in catalytic activity may exert different temporal control of NHE1 activity in comparison with translocation. Prolonged treatment with interferon- $\gamma$ , in addition to the already reported inhibition of NHE1 activity, increased NHE1 localisation to the cell surface of human intestinal epithelial cells without affecting the total amount of NHE1 in the cell[47,48]. The observed increase in the cell surface localisation of NHE1 was obtained after a prolonged 24 h treatment with the interferon- $\gamma$ . By contrast, in cardiomyocytes we observed translocation changes in NHE1 within 15-30 min of treatments but this change could be associated with even more immediate changes in catalytic activity. The combination of a range of control mechanisms would allow temporal versatility in response to challenges to cell pH homeostasis.

Perturbed integration of control of insulin action on GLUT4 translocation, glucose transport, glucose metabolism and NHE1 activity may be important in disease states including type 2 diabetes. In the Goto-Kakizaki rat model for type 2 diabetes cellular NHE1 levels and activity are activated[49]. However, decreases in NHE1 activity are associated with decreased pancreatic insulin secretion occurring in the streptozotocin model of type 1 diabetes[50]. Examination of the role of the newly discovered process of insulin and energy-status control of NHE1 translocation in these disease processes seems warranted. Such studies may help further elucidate how GLUT4 translocation, glucose metabolism,  $pH_i$ ,

NHE1 activity and NHE1 translocation are linked and may provide insights into how metabolic disease, including insulin resistance, affects cardiac function.

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## FIGURE LEGENDS

**Figure 1.** Immunocytochemical analysis of NHE1 subcellular distribution in insulin-, hypoxia- and contraction-stimulated cardiomyocytes.

Isolated cardiomyocytes were maintained in a basal un-stimulated state or incubated in one of the following conditions: with 30 nmol/L insulin for 30 min, in a hypoxic buffer for 15 min, with 5  $\mu$ mol/L oligomycin for 60 min, or electrically stimulated to contract for 5 min. After the treatments, cells were washed briefly and fixed with 4% (w/v) paraformaldehyde. After permeabilisation with 0.1% saponin cells were incubated with rabbit anti-rat NHE1 antibody and Alexa Fluor 488 conjugated anti-rabbit IgG (green) (left panel) and visualised by confocal microscopy. All cells were viewed in approximately the same focal plane. For quantification of the changes in the NHE1 levels at the sarcolemma, un-permeabilised cells were labelled with *Lens culinaris* agglutinin (LcH) and streptavidin – Alexa Fluor 633 conjugate (red) prior to proceeding to the immuno-labelling of NHE1 (middle panel). Merged images are shown in the right panel. Control cells were incubated with rabbit anti-rat NHE1 antibody that had been pre-incubated with the purified NHE1 fragment that was used as the antigen. Immunofluorescent images shown are representative from 8 to 31 cells examined per condition from 3 to 5 independent experiments. Bars 20  $\mu$ m.

**Figure 2.** Quantification of the extent of redistribution of NHE1 along radial lines from the perinuclear area to the sarcolemmal surface (SM).

Representative intensity distribution profiles of NHE1 along radial lines across the perinuclear area to the sarcolemma. **B.** The proportion of NHE1 that co-localised with LcH (red) at the sarcolemmal surface was calculated as percent of the total NHE1 sampled. Results are means from 8 to 31 cells selected at random per condition per experiment and from 3 to 5 independent experiments. The asterisk denotes statistical significance ( $p < 0.05$ ).

**Figure 3.** Comparison of NHE1 and GLUT4 subcellular distribution in cardiomyocytes.

Isolated cardiomyocytes were maintained in the basal un-stimulated state or were incubated with 30 nmol/L insulin for 30 min. Cells were then washed, fixed and permeabilised as described in the Material and Method section. The cardiomyocytes were incubated with rabbit anti-rat NHE1 antibody and Alexa Fluor 488 conjugated anti-rabbit IgG (green) (first panel) and mouse anti-GLUT4 antibody and Alexa Fluor 568 conjugated anti-mouse IgG (red) (second panel). Immuno-labelled cells were visualised by confocal microscopy. All cells were viewed in approximately the same focal plane. The fourth panel represents zoomed images of square delimited areas indicated in the merged images (third panel). Bars 10  $\mu$ m. Immunofluorescent images shown are representative from 5 to 10 cells examined per condition from 3 to 5 independent experiments.

**Figure 4.** Treatment with insulin increases the abundance of NHE1 at the cell surface through a PI 3-kinase dependent mechanism.

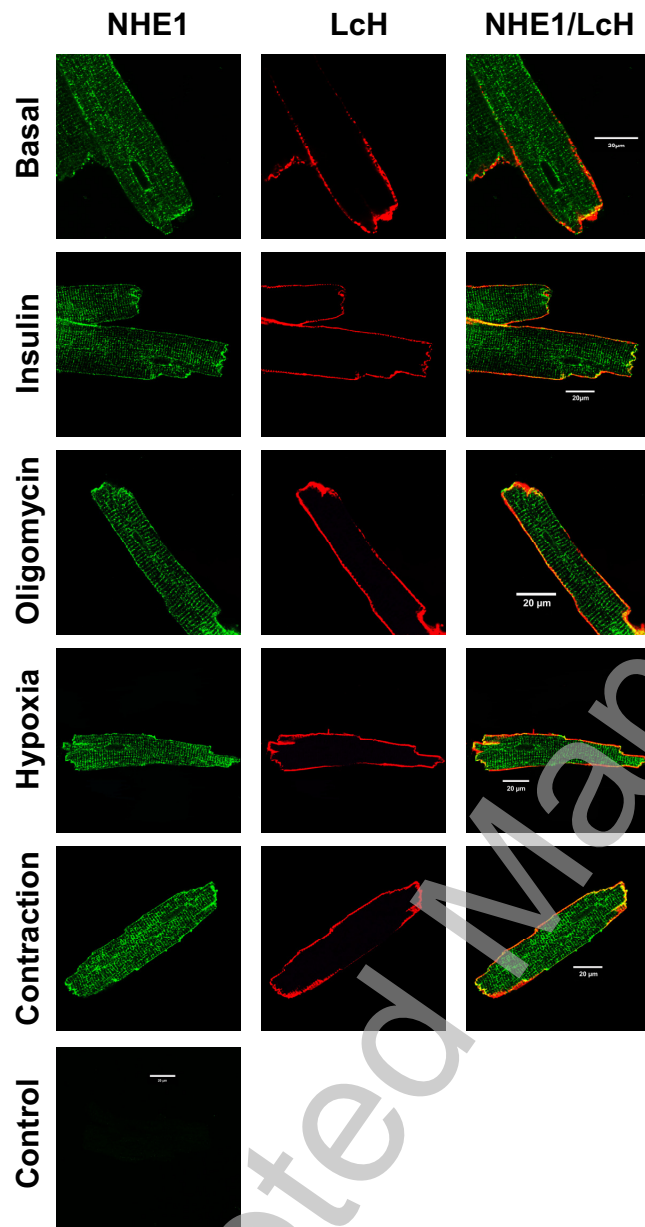
**A.** Isolated cardiomyocytes were incubated for 30 min with or without 30 nmol/L insulin. Where indicated cells were pre-treated for 15 min with 100 nmol/L wortmannin before the addition of insulin. Cardiomyocytes were then cooled to 15°C, labelled with 180  $\mu$ mol/L Sulfo-NHS-SS-Biotin, washed, and membranes prepared. Membranes were solubilised (20  $\mu$ g of protein was taken for loading control), biotinylated protein was precipitated with immobilised streptavidin, the precipitated proteins were separated by SDS-PAGE and NHE1 detected by Western blot analysis. The blots are representative of 5 independent experiments. **B.** Quantification data from Western blot results. The graph represents the mean and S.E.M. from 5 independent experiments. The asterisk denotes statistical significance ( $p < 0.05$ ). **C.** Isolated cardiomyocytes were treated as in **A** above but solubilised membrane proteins were immunoprecipitated either with rabbit anti-rat NHE1 antibody or non-specific IgG and protein A. The precipitated proteins were separated by SDS-PAGE and biotinylated proteins were detected by Western

blot analysis using Extravidin-peroxidase conjugate. The blot represents a typical result from at least 3 independent experiments.

**Figure 5.** Effects of hypoxia, oligomycin treatment and contraction on the abundance of NHE1 levels at the cell surface.

**A.** Isolated cardiomyocytes were incubated in hypoxic buffer for 15 min, or treated with 5  $\mu\text{mol/L}$  oligomycin for 60 min or were electrically stimulated to contract for 5 min. When indicated, cells were pre-incubated with 100 nmol/L wortmannin for 15 min. When the combined effect of insulin stimulation and hypoxia was studied, cardiomyocytes were first incubated with 30 nmol/L insulin for 30 min and insulin was then maintained throughout the hypoxic treatment. When the combined effect of insulin and oligomycin treatment was examined, after 30 min of oligomycin treatment 30 nmol/L insulin was added for the remaining 30 min of the oligomycin treatment. In each experiment, control cells were left untreated or incubated with 30 nmol/L insulin for 30 min. After the treatments, cardiomyocytes were cooled to 15°C, labelled without (No Biotin Control - NBC), or with 180  $\mu\text{mol/L}$  Sulfo-NHS-SS-Biotin. Membrane samples were solubilised (20  $\mu\text{g}$  taken for loading control, bottom panel), biotinylated protein was precipitated with immobilised streptavidin, the precipitated proteins were separated by SDS-PAGE and NHE1 detected by Western blot analysis (top panel). The blots are representative of 3 to 5 independent experiments. **B.** Quantification data from Western blot results. The graph represents the mean and S.E.M from 3 to 5 independent experiments. The asterisk denotes statistical significance ( $p < 0.05$ ).

Figure 1.



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Figure 2.

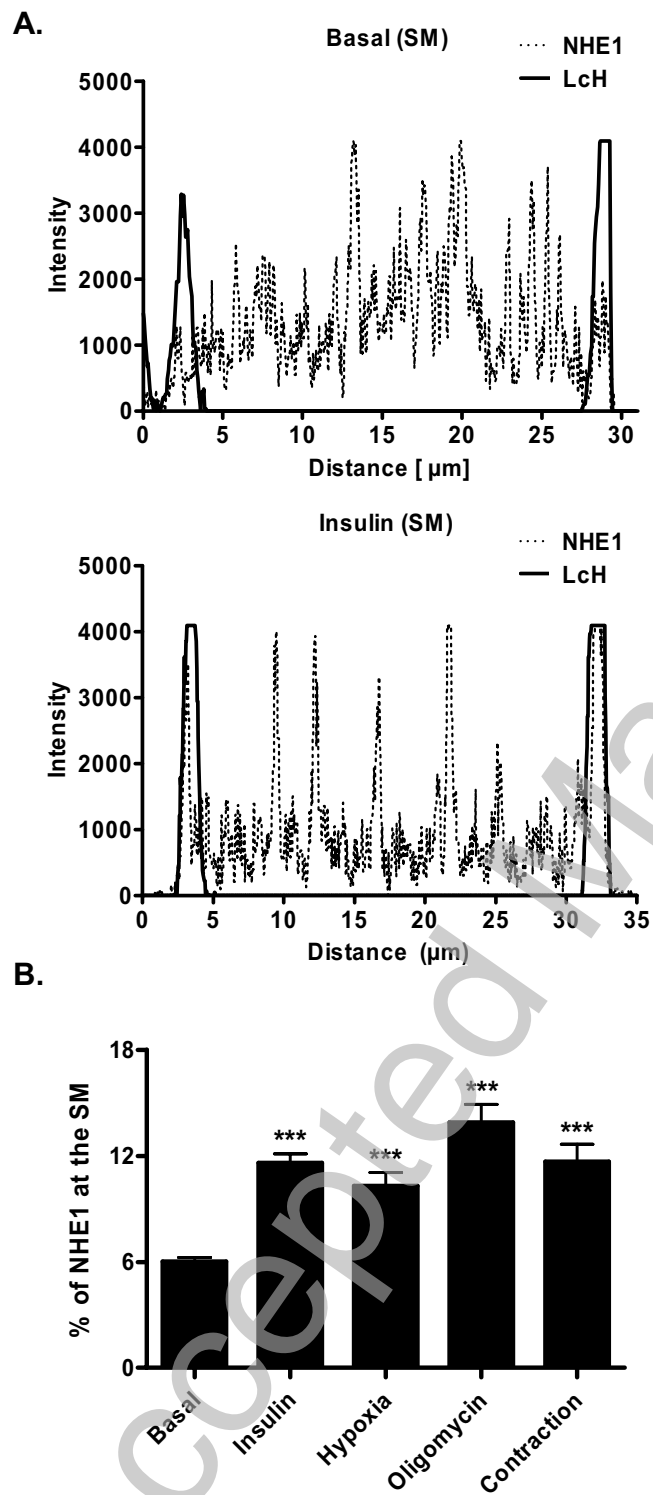
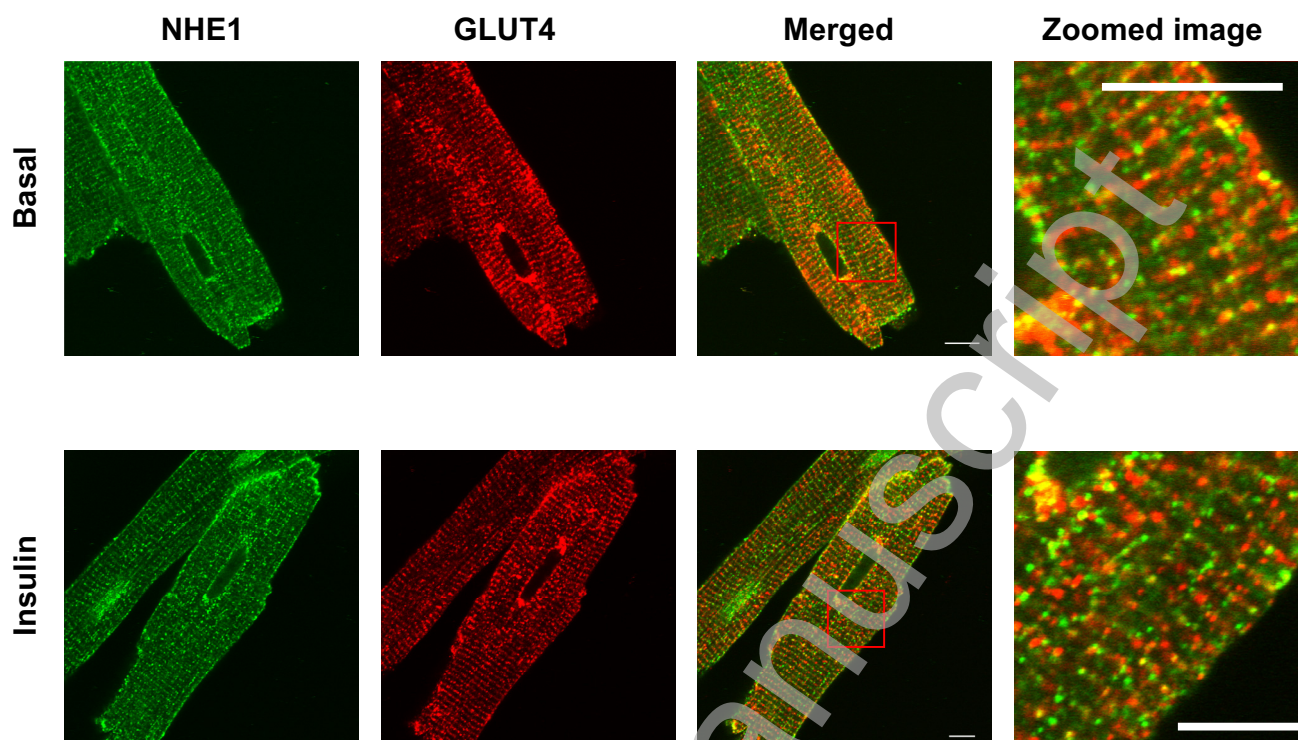
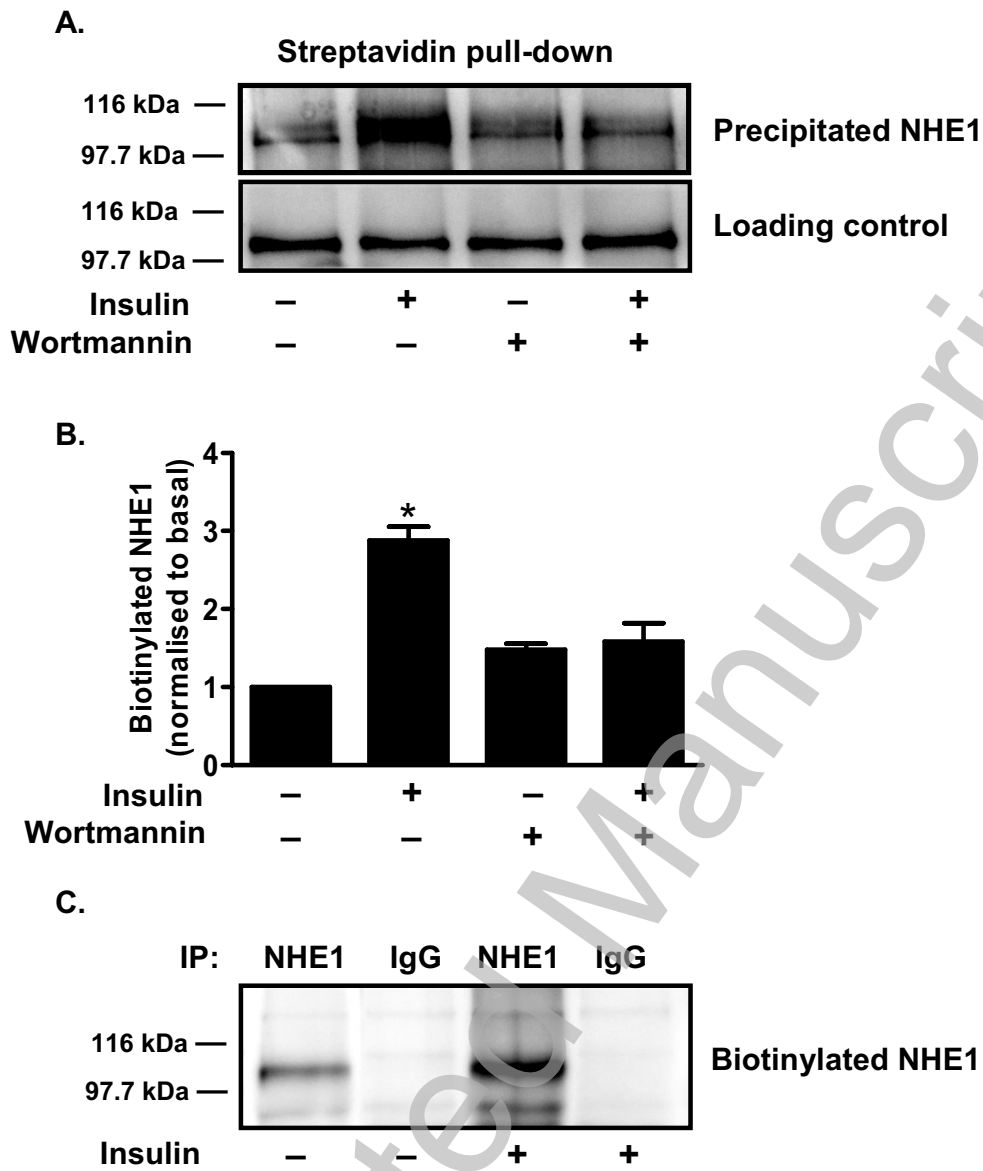


Figure 3.

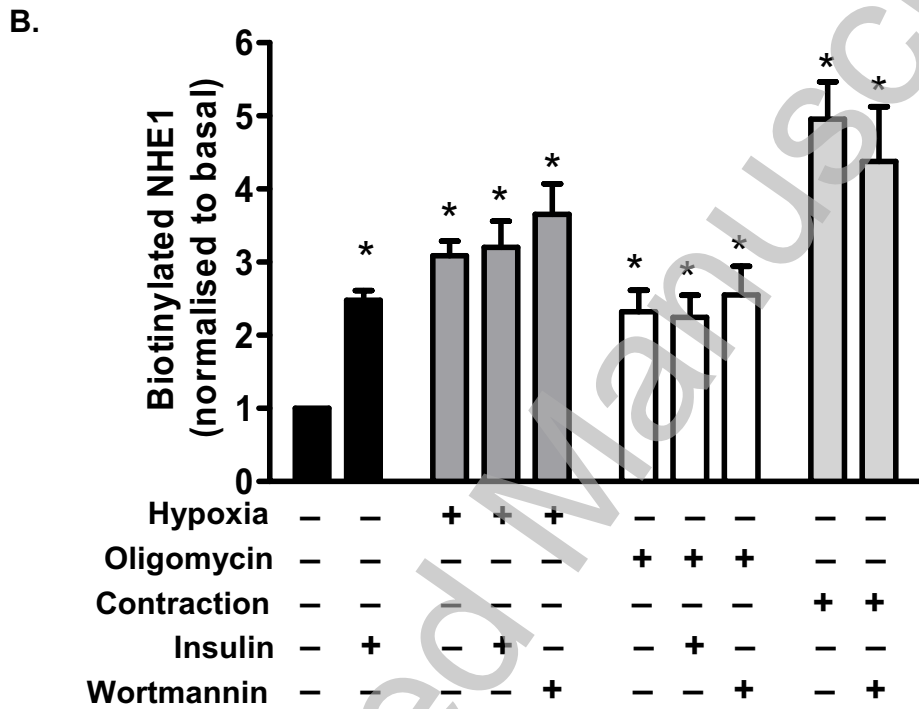
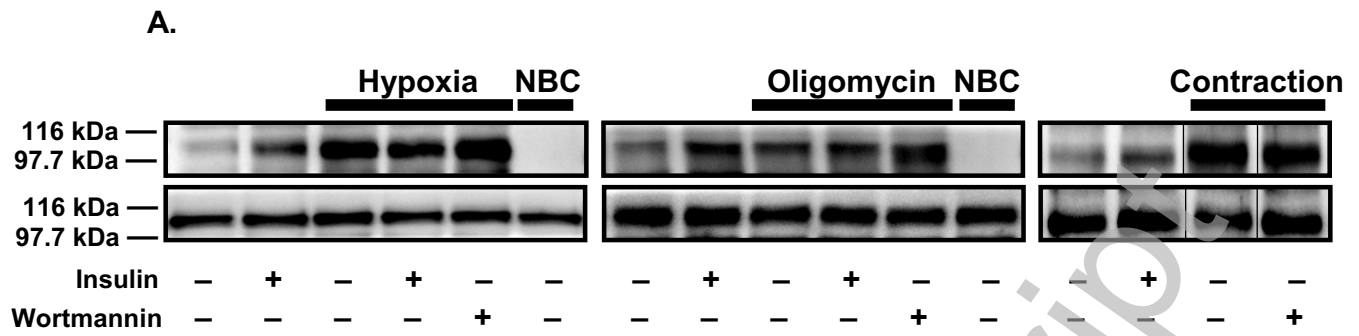


**Figure 4.**



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**Figure 5.**



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