Regulation Of Apoptotic Alkalinization Through Phosphorylation Of Sodium Hydrogen Exchanger Via P38 Mitogen Activated Protein Kinase

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REGULATION OF APOPTOTIC ALKALINIZATION THROUGH PHOSPHORYLATION OF SODIUM HYDROGEN EXCHANGER VIA P38 MITOGEN ACTIVATED PROTEIN KINASE

by

AMY LYNNE GRENIER
B.S. University of Central Florida, 2002

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Microbiology and Molecular Biology in the Burnett College of Biomedical Sciences at the University of Central Florida Orlando, Florida

Spring Term
2006
ABSTRACT

Regulation of intracellular pH is responsible for many cellular processes, such as metabolism, cell cycle progression, and apoptosis. Many chemotherapeutic agents work by inducing target cells to undergo apoptosis, a cell death process still poorly understood. Previous studies demonstrated that a rise in intracellular pH activated apoptotic proteins leading to cytochrome C release. This “apoptotic alkanization” occurred upon activation of the plasma membrane protein, sodium hydrogen exchanger-1 (NHE1), whose activity is regulated by the stress kinase p38 MAPK. In previous studies, upon cytokine withdrawal from cytokine-dependent lymphocytes induced the activity of the p38 MAP kinase which then phosphorylated the C-terminus of NHE1. To identify the p38 MAPK phosphorylation sites on NHE1, in vitro p38 MAP kinase assays coupled to deletion analysis of NHE1 and mass spectrometry, identified four possible p38 MAPK phosphorylation sites. To establish that NHE1 causes apoptotic alkanization and determine whether the identified phosphorylation sites on NHE1 are functionally significant, we used PCR site directed mutagenesis to mutate T717, S722, S725, and S728 on the C-terminus of NHE1. Stable NHE1 deficient cell lines, expressing wild type (WT) NHE or the four mutated sites (F4MUTNHE), were assessed for apoptotic alkanization using the pH-sensitive fluorescent protein, destabilized YFP. Our results show that NHE1 is required for apoptotic alkanization, since expression of WT NHE restored alkanization in an NHE deficient cell line, and that this process requires the phosphorylation of
the p38 MAPK target sites, since mutation of all four sites prevented the apoptotic alkalinization response.
This thesis is dedicated to all my friends and family that have always believed in me.
ACKNOWLEDGMENTS

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with all the ups and downs of graduate school, I could always count on his love and support to help me through the difficult times. Thank you for helping me to achieve my goals.
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<td>NHE-1</td>
<td>Sodium Hydrogen Exchanger Type 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Minimum Essential Alpha Medium</td>
</tr>
<tr>
<td>BCECF-AM</td>
<td>2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein,-Acetoxymethyl ester</td>
</tr>
<tr>
<td>dEYFP</td>
<td>Destabilized Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>DMA</td>
<td>5-(N, N-Dimethyl) Amiloride Hydrochloride</td>
</tr>
<tr>
<td>PD169316</td>
<td>4-(4-Fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole</td>
</tr>
<tr>
<td>pYN$^+_4$</td>
<td>Human cDNA NHE-1 Vector</td>
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<tr>
<td>AP-1</td>
<td>NHE-1 Deficient CHO cells</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun amino-terminal Kinase</td>
</tr>
<tr>
<td>IL-3</td>
<td>Interleukin-3</td>
</tr>
<tr>
<td>IL-7</td>
<td>Interleukin-7</td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered saline</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethysulphonylfluoride</td>
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1 CHAPTER ONE: INTRODUCTION

1.1 Role of intracellular pH in apoptosis and proliferation

The regulation of intracellular pH is important in maintenance of cellular homeostasis. Oscillations in intracellular pH play a role in cell proliferation and survival but can also promote apoptosis. Apoptosis, a form of programmed cell death, is a process which eliminates cells without an inflammatory response. The events that set the stage for the effector phase of apoptosis are poorly understood. Mitochondrial breakdown, followed by cytochrome C release can be observed at an early stage of apoptosis. Activation of caspase occurs at a late stage. During apoptosis, DNA fragmentation occur during in the late stage of apoptosis. Some of these apoptotic events have been correlated to changes in intracellular pH (Shrode, Tapper, and Grinstein; Coakley et al.). Intracellular acidification is thought to activate DNase II, leading to DNA fragmentation during apoptosis, DNase II becomes active at < 6.0 pH. Conversely, alkaline pH has been shown to activate apoptotic mechanisms, such as mitochondrial translocation of the apoptotic protein, Bax (Tafani et al.; Khaled et al.; Khaled et al.)

Regulating intracellular pH is important in many cellular metabolic processes, such as egg fertilization, DNA replication rates, and regulation of cAMP (Busa and Nuccitelli). One regulator of intracellular pH is NHE1 which effluxes protons in exchange for extracellular sodium when activated. This activation prevents cells from acidifying as a result of metabolic activity. NHE1 can be
activated by many regulatory proteins or kinases, all of which cause intracellular alkalization. Depending on tissue specificity and stimuli, intracellular acidification can either inhibit or activate apoptosis. Previous studies have shown that growth hormone stimulation or acidic conditions can cause Extracellular Signal-Regulated Kinase (ERK) to become phosphorylated, which leads to cell proliferation, cell migration, and survival (Wu et al.). On the other hand, several studies have shown that if p38 MAPK activates NHE1 by stress stimuli, it can lead to apoptosis.

Previous studies have shown that the translocation of Bax in response to a change in intracellular pH, due to the activation of the plasma membrane protein sodium hydrogen exchanger1 (NHE1) (Khaled et al.) Cytokine-withdrawal activates p38 mitogen-activated protein kinase by an unknown mechanism, which then potentially phosphorylates the carboxyl terminus of NHE1. The activation of NHE1 allows for extrusion of protons and influx of sodium leading to a heightened alkaline response (Mahnensmith and Aronson). Cellular stress, i.e. ultraviolet radiation, osmotic stress, heat or cytokine withdrawal can also activate p38 MAPK, which could potentially lead to apoptosis, through the activation of NHE1.

1.2 Structure and function of the sodium hydrogen exchanger

The sodium hydrogen exchanger (NHE) is a family of ubiquitous plasma membrane proteins, which catalyze the exchange of extracellular sodium for
intracellular hydrogen upon activation (Figure 1). NHE is responsible for regulation of intracellular pH, cell volume, cell migration, and apoptosis (Fliegel).

NHE is a family of ten isoforms (Table 2, p 49) which all share a similar topology. Each isoform consists of 815 amino acids, of which 500 make up the amino terminus, which is responsible for ion exchange transport across the plasma membrane. The hydrophilic cytosolic domain consists of 315 amino acid, which is classified as a regulatory domain (Wakabayashi et al.). NHE differs from other proton exchangers such as sodium bicarbonate exchanger (NBC), anion exchangers (AE) and the sodium driven chloride bicarbonate exchanger (NDAE), because it has a short N-terminus and long cytoplasmic domain (Putney, Denker, and Barber).

NHE has a calculated molecular weight of 85-110 kDa, based on sequence. If it is glycosylated (mature) the protein has an approximate molecular weight of 110 kDa whereas if it is unglycosylated (immature) the molecular weight is around 85 KDa (Cavet et al.). The only NHE isoform that possess N-linked and O-linked glycosylation sites is NHE-1 (Counillon, Pouyssegur, and Reithmeier). There are three N-glycosylation sites found in the transmembrane domain, Asn75, 370, and 410, all have Asn-X-Ser/Thr motif. The importance of NHE1 N-linked glycosylation, is not well understood, although it could potentially aid in correct folding conformation and transport to the plasma membrane. N-linked glycosylation occurs co-translationally in the ER, whereas O-linked glycosylation occurs in the trans-golgi network (Counillon, Pouyssegur, and Reithmeier). Sato et. al reported that just after transmembrane 9, loop ten translocates through the
membrane into the lumen, allowing sequestering of modifying enzymes that glycosylate this region after TM9.

There are currently two models that describe the topology of NHE. The model proposed by Wakabayashi et al. consists of both the N-terminus and C-terminus to be located in the cytosol, whereas another model proposes that the N-terminus is located in the extracellular matrix (Counillon, Pouyssegur, and Reithmeier). To date, there is no evidence to support either model. The N-terminus was identified by Kyte-Doolittle hydrophy analysis, to be comprised of 12 alpha helices, which span across the plasma membrane allowing for ion transport. The exact transmembrane domain loop needed for ion transport is unknown. It has been proposed to be on intracellular loop 2 (TM 4 and 5) and intracellular loop 4 (TM 8 and 9) (Wakabayashi et al.). The C-terminus secondary structure was determined to consist of 35% alpha-helix, 17% beta-turn, and 48% random coil, and this was identified by circular dichroism (Gebreselassie, Rajarathnam, and Fliegel). The C-terminus is proposed to function as a regulatory domain that modulates the activity of NHE1 (Wakabayashi et al.). For example, an increase in calcium causes binding of calmodulin which can then functions as a signal transducer for the activation of NHE1 (Wakabayashi et al.). NHE1 is also regulated by several kinases such as Extracellular Signal-Regulated Kinase (ERK), Jun N-terminal kinase (JNK), and p38 MAPK, where all target the c-terminal residues (Khaled et al.; Takahashi et al.; Wang et al.).

The Sodium Hydrogen-1 (NHE1) isoform is of interest, because it is ubiquitously expressed on the basolateral plasma membrane of most epithelial
cells and its main function is regulation of intracellular pH and cell volume. Cell-surface receptors such as tyrosine kinase receptors, G protein coupled receptors, or integrin receptors activate NHE1 through binding of regulatory proteins, phosphorylation, or conformational changes which lead to a change in Hydrogen transport (Putney, Denker, and Barber).

![Diagram](image)

Figure 1: NHE1 proposed regulation

1.3 Activators and inhibitors of NHE

The sodium hydrogen exchanger can be activated by a variety of signaling molecules or regulatory proteins. Signaling molecules become activated upon induction of stress due to serum starvation, ultraviolet radiation, or cytokine
withdrawal. Wang et. al identified that mitogen-activated protein kinase (MAPK) regulates and phosphorylates NHE1 via the cytosolic domain. Several mitogen-activated protein kinases have been observed to phosphorylate NHE1, such as extracellular signal-regulated kinase (ERK), p90RSK, Nck-interacting Kinase (NIK), and p38 MAPK. The phosphorylation of NHE1 leads to activation, which ultimately leads to a change in intracellular pH (Khaled et al.; Wang et al.; Yan et al.; Takahashi et al.; Haworth et al.). NHE1 can also be activated by the binding of regulatory proteins such as Calcineurin B homologous protein (CHP), 14-3-3, and calmodulin.

Even though NHE1 aides in the transport of sodium and hydrogen, calcium is very important for the activation of NHE1. Cellular homeostasis requires regulation of both intracellular pH and intracellular calcium, without one or both many cellular function, (such as proliferation and skeletal movement) will be inhibited. Both regulatory proteins, calmodulin and CHP, are dependent of calcium levels (Wakabayashi et al.; Lehoux et al.). Several diseases have been identified to be caused by activation of NHE1 or MAPK (ERK and p38), such as heart ischemia and hypertrophy (Abe, Baines, and Berk). NHE1, in heart ischemia, can be activated by a decrease or increase of intracellular pH. This causes an intrusion of extracellular sodium, leading to an abundance of intracellular calcium (Masereel, Pochet, and Laeckmann). Thus inhibition of NHE1 has a protective effect during cardiac ischemia –reperfusion injury (Wang et al.).
In addition to the cellular regulatory molecules, there are also chemical compounds which can activate NHE1 directly and indirectly. Phorbol esters, phosphatase inhibitors, staurosporine, or anisomycin can induce the phosphorylation of NHE1 leading to activation (Masereel, Pochet, and Laeckmann). One NHE1 activator, anisomycin, activates it indirectly by inhibiting protein synthesis which causes a stress signal that leads to the phosphorylation of MEK3 and MEK6, leading to p38MAPK activation (Schaeffer and Weber). Staurosporine was previously identified as a protein kinase inhibitor, but Vries-Seimon et al determined that it also activated protein kinases such as p38 MAPK (Vries-Seimon et al.).

In addition to pharmaceutical activators, there are also several classes of inhibitors, such as amilorides and benzoylguanidines (Putney, Denker, and Barber) (Figure 2). Even though NHE isoforms have similar topology, their sensitivity to both classes of inhibitors is greatly different. For example, HOE compounds (benzoylguanidines) are able to inhibit NHE1 but not NHE3. The mechanism by which the inhibitors function is yet to be completely understood. Cardiac vessel damage occurs due to an increase in intracellular calcium. Amilorides may function by blocking sodium transport, which inhibits an increase in intracellular calcium, thus inhibiting cardiac vessel damage (Masereel, Pochet, and Laeckmann)
1.4 Summary of thesis

Regulation of intracellular pH via NHE1 is important for many cellular processes such as proliferation and survival, cell migration, and apoptosis. Previous studies reported that activation of p38MAPK leads to the phosphorylation and activation of NHE1, which ultimately caused apoptosis (Khaled et al.; Khaled et al.; Coakley et al.). To examine the role of p38 MAPK in activation of NHE1, NHE1-GST fusion proteins were constructed with a series of deletions in the c-terminal regulatory domain. Four phosphorylation sites (T717, S722, S725, and S728) on NHE1 were identified by p38 MAPK in vitro kinase
assay and were confirmed by mass spectrometry (Figure 3) (Khaled et al.). Based on these initial findings we hypothesized that not only is NHE1 necessary for alkalization during apoptosis, but that it can be phosphorylated by p38 MAPK within the putative four sites. To determine if NHE1 is the mediator of apoptotic alkalization, site directed mutagenesis was used to construct a mutant (F4MUTNHE) containing alanine residues in place of all four putative p38 MAPK phosphorylation sites. Cells expressing these constructs were then subjected to apoptotic stimuli and changes in intracellular pH were measured using destabilized yellow fluorescent protein (dEYFP) as read out for cytosolic pH. Our results support the hypothesis that p38MAPK activates NHE1 by direct phosphorylation of one or more of the four putative site(s) causing apoptotic alkalization.
Figure 3: NHE1 potential p38 MAPK phosphorylation sites
2 CHAPTER TWO: METHODOLOGY

2.1 Cell Lines

The Chinese hamster ovary cell line, CHO-K1 obtained from American Type Tissue Culture Collections, was cultured in Dulbecco’s Modified Eagle’s Medium with L-Glutamine, Sodium Pyruvate, 4.5 g/L Glucose and supplemented with 10% Fetal Bovine Serum and 1000 U/ml Penicillin/Streptomycin. NHE-1 deficient CHO cells (AP-1) were a kind gift from L. Fliegel from the University of Alberta in Canada. AP-1 cells were cultured in minimum essential alpha medium with L-glutamine, ribonucleoside and deoxyribonucleosides and supplemented with 10% Fetal Bovine Serum, 1000 U/ml Penicillin/Streptomycin, and 25 mM Hepes. AP-1 cells co-transfected with wild type NHE-1 (PYN4) or mutant AP1 (F4MUTNHE) (see below) and destabilized yellow fluorescent protein (dEYFP) were cultured in complete minimum essential alpha medium containing 500 µg/mL geneticin. Corning T25cm² or T75cm² flasks at 37°C with 5% carbon dioxide were used for all cell lines. Cellstripper, a non-enzymatic cell dissociation solution, was added to dissociate adherent cells from flasks, and then incubated for 5 minutes at 37°C and 5% CO₂.
2.2 Plasmid construction and site direct mutagenesis

Site directed mutagenesis was performed using the pYN4+ plasmid to construct wild type (WT) and mutant NHE1 (F4MUTNHE). The plasmid pYN4+ contains cDNA encoding for the human sodium hydrogen exchanger-1 isoform with a hemagglutinin (HA) tag on the C-terminus of the protein (Figure). It also contains a Rous sarcoma virus (RSV) promoter, thymidine kinase poly (A) signal and neomycin resistance gene. Through fluorescent automated DNA sequence analysis, our pYN4+ plasmid was found to contain an additional Apal restriction site, than published sequence (see table1 for primers).

Figure 4: Pyn4+ plasmid
To construct F4MUTNHE mutant, PCR site directed mutagenesis was performed targeting residues T718, S723, S726, S729, by changing serines to alanines. An *ApaI* restriction digestion site was also added to facilitate ligation reactions (Table 1). The PCR reaction contained 5 µl of 10x PCR buffer, 1.5 µl of 10 mM dNTP, 1 µl of 50 mM MgSO$_4$, 1.5 µl of 10 mM Forward primer, 1.5 µl of Reverse primer, 1 µl of 100 ng/µl pYN4$^+$, 1 µl of pfX DNA polymerase, and 37.5 µl water. PCR conditions for all amplifications were 94°C for 3 minutes for one cycle; the next 25 cycles were as followed: 94°C for 30 seconds, 65°C for 30 seconds, and then 72°C for 30 seconds. The last cycle was 94°C for 7 minutes, and samples were then placed at 4°C. PCR products were analyzed on 1% Agarose gel. Bands corresponding to our product (757 bp) were excised and purified with a gel extraction kit following manufacturer’s protocol (QIAGEN gel extraction kit). Cloning and transformation was performed following manufacturer’s protocol for Zero blunt TOPO PCR cloning kit (Invitrogen). After adding 4 µl PCR product, with 1µl salt solution, and 1 µl TOPO vector, the mixture was incubated at room temperature for 5 minutes. Two microliters of TOPO reaction mixture was added to 1 vial of competent, and incubated on ice for 15 minutes. The competent cells were then incubated at 42°C for 30 seconds followed by 2 minutes on ice. Fifty microliters of competent cells were added to 250 µl of SOC media, the culture was then placed at 37°C for 1 hour with constant agitation. After the incubation,Luria-Bertani (LB) medium with Kanamycin (50 µg/ml) were inoculated with 100 µl, 50 µl, 25 µl of competent cell
mixture. Clones were isolated. DNA was extracted using a DNA maxi prep isolation kit following manufacturer’s protocol (QIAGEN Maxi prep).

To subclone the F4MUTNHE into the pYN4 vector, the Zero blunt TOPO vector with F4MUTNHE PCR and pYN4+ vector were both digested using Apal restriction enzyme, both plasmids were then analyzed on 1% agarose gel. Corresponding bands were extracted using QIAGEN gel extraction. For ligation, 1.4 µl pYN4+ (17 ng/µl) and 9 µl mutant PCR (8 ng/µl) was added to master mix containing 1 µl T4 Ligase, 4 µl 5X Ligase buffer, and 4.6 µl deionized water. After, the ligation mixture was incubated at 22°C for 5 minutes, it was transformed into competent cells following previous protocol.

To determine whether a clone had the F4MUTNHE inserted in the correct orientation, they were digested with Apal (previous conditions). Correct orientation was identified by restriction digestion with Afl II and Nhel. After orientation was identified, the corresponding clones were sent for automated DNA sequence analysis to confirm the sequence. See table 1 for primers used for sequencing.
Table 1: Primers used for sequencing and the construction of NHE1 mutant

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Type of mutation</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Wild-type Forward</td>
<td>Sequencing primer</td>
<td>GCAGCTGGAGCAGAAGATCAAC</td>
</tr>
<tr>
<td>Wild-type Reverse</td>
<td>Sequencing primer</td>
<td>CGAGTCAGTGGAGGAGGAAGCG</td>
</tr>
<tr>
<td>F4MUTNHE Forward</td>
<td>Mutation of sites:</td>
<td>CgggccGCATCGGCTCAGACCCACT</td>
</tr>
<tr>
<td></td>
<td>T718,S723,S726,S729</td>
<td>GGCCTATGAGCCGAAGGGAGGCC</td>
</tr>
<tr>
<td></td>
<td>From Ser/Thr to Ala</td>
<td>TGCCTGTATCGCCATCGACCCGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTGCCCGCGAGCGACCCGAGCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGACC</td>
</tr>
<tr>
<td>RAp1NHE</td>
<td>Reverse primer for site directed mutagenesis</td>
<td>CTAACCCACGgggccGTGGCTAT</td>
</tr>
</tbody>
</table>

* All primers start at the 5’ end. Lower case residues indicate mutations; italic residues indicate Apal restriction sites.

2.3 Co-Transfection of AP-1 with dEYFP and either wild-type NHE1 or F4MUTNHE

To establish a stable NHE1 expressing cell line, AP-1 cells (0.5x10⁶) were plated in Corning six well tissue culture plates in 2 mL of complete minimum essential alpha medium overnight at 37°C with 5% CO₂. When 75-80% confluence was met, the medium was removed and 2 mL of fresh medium without fetal bovine serum was added, and then, incubate for four hours at 37°C with 5% CO₂. Lipofectamine 2000 was used to transfect AP-1 cells with either human cDNA wild type or F4MUTNHE, following a similar protocol to the
manufacturers protocol. Briefly, two polystyrene cell culture tubes were setup with 250 mL of Minimum Essential Alpha medium (α-MEM) without FBS. To one tube, 4 µg of DNA from Wild type or F4MUTNHE and 4 µg of dEYFP (destabilized yellow fluorescent protein vector Pd2EYFP-N1) were added; the other tube had 10 µl Lipofectamine 2000 added to medium. After both tubes received the corresponding DNA they were incubated at room temperature for four minutes, then the two tubes were mixed together. Five hundred microliters of mixed DNA in medium was added to each well, and then incubated at 37°C with 5% CO₂ for four to six hours. Subsequently, 10% fetal bovine serum was added and then incubated overnight at 37°C with 5% CO₂. After twenty-four hours, media was removed and 2 ml of fresh medium was added. After thirty-six hours, cells were split and resuspended in complete medium with 800 µg/mL geneticin (Invitrogen); optimal geneticin concentrations were previously determined. For the first three days medium was changed daily using complete medium with 800 µg/ml Geneticin. After that, cells were changed every two days until they reached confluence with medium containing 500 µg/ml geneticin. Once theses cultures were confluent, they were maintained in corning T25 or T75 flask with complete minimum essential alpha medium with 500 µg/ml geneticin.

2.4 Detection of dEYFP

To identify if co-transfected cells were expressing dEYFP, cells were analyzed by flow cytometry using a BD FACSCalibur. Cells were grown on T25
corning flasks overnight at 37°C with 5% CO₂, at 75% confluence, cells were stripped and resuspended in 1 ml of sorting buffer (1X PBS, 25 mM Hepes pH 7.0, 5 mM EDTA, and 1% FBS). Cells were then analyzed by flow cytometry equipped with a 488 nm Argon laser and analyzed with an emission of 530 nm (Green fluorescence).

2.4.1 Cell sorting

To separate the high expressing dEYFP cells from the low expressing dEYFP cells, cell sorting was performed on a BD FACS Aria. All transfected AP-1 cells were plated in a T75 corning flask overnight at 37°C with 5% CO₂. At optimal confluence, 70-80%, cells were stripped and resuspended in 3 ml of sorting buffer and placed in 12x75 mm cell culture tubes. Cells were then sorted per protocol on BD FACS aria using 488 nm argon laser with an emission of 530 nm (green fluorescence). Parameters for sorting were set to acquire 50% of the fluorescent population. After cells were sorted, they were plated in a T25 corning flask with 10 ml fresh complete media containing 25000 ug/mL of penicillin/streptomycin to prevent contamination. After 4 hours, media was removed and 10 mL of fresh complete media containing 500 ug/mL Geneticin was added per flask.
2.5 Detection of NHE-1

2.5.1 Detection of NHE1 by immunoblot

To identify if NHE1 expression was present, immunoblots were performed. Whole-cell lysates were made using approximately $1 \times 10^6$ cells in a modified RIPA buffer (Khaled et al.) (50 mM Tris, 150 mM NaCl, 100\% [wt/vol] NP40, 10\% [wt/vol] Sodium deoxycholate, 10\% [wt/vol] Triton X-100, 0.5M EGTA, 0.1M PMSF, 0.1M Benzamidine, 1 complete mini EDTA-free protease inhibitor cocktail tablet) (ROCHE). Cells were incubated with 50-100\µl RIPA buffer on ice for five minutes, followed by centrifugation at 13,200 RPM at 4°C for 6.5 minutes. Lysates were removed and placed in new tubes. Approximately 100 \µg protein was loaded on to 8-16\% Tris-Glycine SDS gradient gels (invitrogen) and then transferred on to 0.2 \µM Polyvinylidene fluoride (PVDF) membrane by Tris-Glycine transfer buffer (Invitrogen). Membranes were then incubated for 2 hours in phosphate buffer with 0.1\% Tween (PBSt) with 10 \% Milk. Membranes were then washed three times for five minutes with PBSt, and then probed with (1:1000 dilution) mouse monoclonal NHE-1 antibody (Chemicon) in PBSt with 5 \% milk and incubated overnight at 4°C. Membranes were then washed three times for five minutes each, followed by incubation with (1:2000 dilution) secondary antibody, goat anti-mouse conjugated to HRP (Santa Cruz). The membrane was developed using Supersignal West Pico Chemiluminescent Substrate (Pierce) following
manufacturer’s protocol. To identify equal loading, GAPDH was probed using (1:1000 dilution) Anti-GAPDH mouse antibody (Ambion) in PBSt with 5% milk, and then with (1:2000 dilution) goat anti-mouse antibody in PBSt with 5% milk (Cell signaling). The blot was developed using Pierce chemiluminescence as discussed methods. Genesnap and Genetool from Syngene were used to determine density.

2.5.2 Detection of NHE1 surface expression

To identify if the transfected wild-type and F4MUTNHE cells were being expressing on the cell surface, transfected cells were treated with Proteinase K. All AP-1 transfected cell lines were plated in 6 well plates (1 x10^6 / ml) overnight at 37°C with 5% CO2. Media was removed when confluence was 70-80%, then 100 µg/mL of proteinase K (invitrogen) in Tris-HCL (pH 7.4) and 1 M CaCl\textsubscript{2} was added and incubated at 37°C for 5 minutes. PMSF (5 mM) was added and incubated for 2 minutes to stop the reaction. Cells were then stripped and centrifuged for 10 seconds at 13,200 RPM on a tabletop centrifuge. Cells were resuspended in cold PBS, and then centrifuged again. The cells were then lysed in 25 µL of RIPA buffer and previously described immunoblot methods were followed (section 2.5.1).
2.5.3 Detection of NHE1 by confocal microscopy

To identify if NHE1 was expressing on plasma membrane, confocal microscopy was performed. Before preparing 25 mm glass coverslips for confocal microscopy, they were washed three times with 70% ethanol. Once dry, 200 ug/mL of poly-L-lysine was added to each coverslip and incubated for 1 to 2 hours at room temperature. The coverslips were rinsed 3 times with PBS, then each was placed in a well of a 6 well corning plate. AP-1 cells (0.5x10^6/ml) were added to each well containing a total of 2mL of complete media. Coverslips were incubated overnight at 37°C with 5% CO₂, and grown until 70% confluence was met. Then glass coverslips were removed from the media and washed three times with PBS. The cells were then fixed and permeabilized with cold methanol for 5 minutes. Coverslips were then incubated in blocking buffer for 1 hour, followed by incubation with 1:25 dilution of HA antibody (Santa Cruz) in blocking buffer. Glass coverslips were then washed 3 times with PBS. Cells were then incubated for 1 hour with (1:200) donkey anti-mouse Cy-5 (Jackson immunological) in blocking solution. The coverslips were then washed three times with PBS. A drop of Prolong anti-fade solution was placed on glass slides; coverslips were placed on top face down and incubated at room temperature in a dark area for 1 to 2 hours to allow complete dryness. The slides were then placed in the dark at 4°C for storage. Slides were analyzed using ZEISS confocal microscope LSM 510 NLO at 100X using immersion oil.
2.6 P38 MAPK in vitro activity

For the detection of phosphorylated p38MAPK, a modified p38 mitogen
activated protein kinase non-radioactive kit from Cell signaling was used. AP-1
cells were plated (0.5 x10^6) in six well plates overnight at 37°C with 5% CO₂. At
75-80 % confluence, 1 µM staurosporine was added to corresponding wells. Cells
were then harvested and resuspended in 100 µl 1X ice-cold lysis buffer plus 1mM
PMSF, then incubated on ice for 10 minutes. Cells were then centrifuged at
13,200 RPM for 15 minutes at 4°C. Supernatant was transferred into new
centrifuge tubes, and kept at -80°C for later use. Ten microliters of resuspended
immobilized phospho-p38 MAP kinase monoclonal Antibody was added to 100µl
of cell lysate and was then gently rocked overnight at 4°C. After samples were
washed, the pellets were resuspended in 25µl of Master mix constituting 1X
kinase buffer supplemented with 200 µM ATP and 2µg ATF-2 fusion protein
substrate with and without 20µM PD169316 (p38 MAPK inhibitor) and 200µM
DMA (NHE1 inhibitor) inhibitors. Next, the samples were incubated for thirty
minutes at 30°C, followed by the addition of 25 µl 3X SDS with 2X Tris-glycine
SDS sample buffer to terminate the reaction. Samples were then run on 8-16%
Tris-Glycine SDS gel, and then transferred to 2.0 µM Nitrocellulose membrane.
The membrane was washed 3 times in PBSt, and then incubated in 5% milk in
PBSt for one hour. It was then washed 3 times in PBSt, and then incubated in
phospho-ATF2 (thr76) (1:1000 dilution) in 5% BSA in PBSt overnight at 37°C.
The membrane was washed 3 times with PBSt, and then incubated for 1 hour in
5% BSA with (1:2000 dilution) HRP-conjugated secondary antibody and (1:1000 dilution) HRP-conjugated anti-biotin. The membrane was washed 3 times with PBSt, and then developed following manufacturers protocol.

2.7 Detection of intracellular pH

2.7.1 Detection of intracellular pH BCECF-AM

BCECF-AM (Molecular probe) was used for the detection of intracellular pH. Cells (0.5 x 10^6) were plated and incubated at 37°C with 5% CO₂ overnight in tissue culture six well plates. At 70-80 % confluence, cells were harvested and resuspended in Heps pH buffer (25 mM Hepes, 140 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂, and 5.0 mM glucose) with 1 µM BCECF-AM. The cells were incubated for 20 minutes at 37°C without CO₂. For calibration, 1 x 10^6 cells were resuspended in 1ml high potassium Hepes buffer (25 mM Hepes, 145 mM KCl, 0.8 mM MgCl₂, and 5.5 mM Glucose) at specific pH standards and supplemented with 13.8 mM Nigericin. They were then incubated for 20 minutes at 37°C without CO₂. For samples, 1x 10^6 cells were resuspended in Hank’s balanced salt solution, with an addition of 10 ug/ul Anisomycin (ICN) to corresponding wells. Sample cells were incubated for 30 minutes at 37°C without CO₂. Calibration and experiment samples were read on BD FACS Calibur excited with a 488nm argon laser, with an emission of 530 nm. The pH values were determined by a standard pH calibration curve, which was determined by taking the absorbance
ratio between FL1H and FL3H. Actual pH values were determined by taking the ratio between FL1-H and FL3-H of experiment samples then correlating them to a pH value on the standard calibration curve.

2.7.2 Detection of intracellular pH using dEYFP

Cell lines stably expressing dEYFP, were used to determine pH within varying environments. Cells were plated in T75 tissue culture flasks overnight at 37°C with 5% CO₂. Calibration curve was determined by first harvesting cells at 70-80% confluence. Cells were centrifuged at 18,000 g for 3 minutes, then resuspended in 1 mL high potassium Hepes buffer at specific pH values and supplemented with 10 µM Nigericin. Incubated for 20 minutes at room temperature in the dark, cells were then analyzed with BD FACS Calibur with a 488nm argon laser with emission of 530 nm (green fluorescence). Cells were centrifuged at 18,000 g for 3 minutes, then resuspended in 1 ml PBS pH Hepes Buffer (1X PBS, 1% Fetal bovine serum, and 25mM Hepes buffer) in cell culture tubes. Twenty micromolar of PD169316 (p38 MAPK inhibitor), 200 µM 5, N-N-Dimethyl-amiloride (NHE-1 inhibitor), or staurosporine (p38 activator) (0, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10µM) was added to corresponding cell culture tubes. After thirty minutes the negative control and staurosporine sample was analyzed. After one hour 1 µM staurosporine was added to the inhibitors tubes and incubated again for thirty minutes at 37°C with 5% CO₂, and
then analyzed. Analysis was performed on BD FACS Calibur with a 488nm Argon laser with 530 nm emission (green fluorescence).
3 CHAPTER THREE: RESULTS

Previous studies showed that withdrawal of cytokines from cytokine-dependent lymphocytes induced the activity of the p38 MAP kinase, which in turn phosphorylated the C-terminus of NHE1. Four p38 MAPK phosphorylation sites on NHE1 were identified by deletion analysis and confirmed by mass spectrometry. Our hypothesis is that activation of key sites on NHE1 by p38 MAPK is necessary to induce apoptotic alkalinization.

3.1 NHE1 protein expression

To establish that NHE1 causes apoptotic alkalinization and identify the regulatory phosphorylation sites on NHE1, we used PCR site directed mutagenesis to mutate the four potential phosphorylation sites (T717, S722, S725, and S728) in the C-terminus of NHE1 from serine or threonine to alanine. NHE1 deficient cells (AP-1) were co-transfected with dEYFP and wild type NHE1 or F4MUTNHE constructs. After the cells were stably transfected, protein expression detected by immunoblot using an anti-NHE1 monoclonal antibody (Chemicon). In Figure 3, NHE1 protein expression was observed in all 3 lanes, at approximately 100kDa.

Since NHE1 is a 12 transmembrane protein, we next want to determine if NHE1 was being expressed on the cell surface. The molecular mass of NHE1 varies from approximately 100 kDa (mature) to 75 kDa (immature). This variability is due to the glycosylation state. The mature form occurs when the
protein is processed in the golgi and ER and then expressed on the cell surface. When an immature state is observed, one can conclude that it was not processed and exported to the plasma membrane. The mature state has two glycosylation sites N-linked and O-linked on the transmembrane domain, by incubating with proteinase K at 100µg/mL, the NHE1 surface protein was digested leaving only the immature state. Figure 5 shows AP-1 (NHE1 deficient cells), wild-type NHE1 expressing AP-1 cells, and F4MUTNHE expressing AP-1 cells. The A and B refer to different clones. Lanes 5-9 are untreated; therefore both mature and immature forms can be visualized. When the samples were treated with proteinase K (Lanes 1-4), the mature form of NHE was digested leaving only an immature state, indicating that NHE1 is being expressed on the cell surface.

We next confirmed that NHE1 was being expressed on the plasma membrane by confocal microscopy. Both the wild-type NHE1 and F4MUTNHE contain a triple HA tag at the carboxyl terminus of NHE1 protein, therefore we are able to use a hemagglutinin (HA) antibody as a probe to determine NHE1 localization. Cells were prepared on coverslips following the protocol described in the Materials and Methods. For wild-type NHE1 expressing cells (Figure 6A), a HA-probe conjugated to Rhodamine was used for labeling, whereas for F4MUTNHE cells (Figure 6B) a HA probe was followed by donkey anti-mouse CY7 (Jackson immunological). In both figures, a distinct red labeled punctuation can be observed around the plasma membrane, thus NHE1 is being expressed on the surface of the plasma membrane.
To confirm NHE1 protein expression, transfected AP-1 cells were lysed, and electrophoresed on a SDS 8%-16% gradient gel. The PVDF membrane was incubated with mouse anti-Na/H exchanger monoclonal antibody followed by autoradiography as described in materials and methods. Lane 1 AP-1 cells (NHE1 -/-), lane 2 Wild-type NHE1, and lane 3 F4MUTNHE all are expressing NHE1 at approximately 100 kDa.

Figure 5: Detection of NHE1 protein expression
To confirm protein surface expression, proteinase K was used to digest surface proteins. AP-1, Wild-type NHE1, and F4MUTNHE expressing cells were incubated with Proteinase K (lane 1-4) to degrade the NHE1 surface protein. Lanes 5-9 were not incubated with proteinase K. The two bands observed in lanes 5-9 show a mature glycosylated form (undigested) of NHE1, whereas in lane 1-4, after digestion, only show the lower band consistent with the internal (unglycosylated) or immature form. This confirms NHE1 protein surface expression.
Figure 7: Conformation of surface expression of NHE1

To confirm NHE1 was being expressed on the cell surface, wild-type NHE1 and F4MUTNHE expressing cells were subjected to confocal imaging. (A) AP-1 cells transfected with wild-type NHE1 has NHE1 localized on the plasma membrane. (B) AP-1 cells were transfected with F4MUTNHE1 cells are expressing NHE1 on the plasma membrane. Cells were processed and analyzed as described in Materials and Methods for HA-probe.
3.2 Destabilized YFP expression

After determining NHE1 protein expression; dEYFP expression also had to be confirmed in cells stably co-transfected with destabilized YFP (dEYFP) and either wild-type NHE1 or F4MUTNHE1. Destabilized YFP is a variant of the green fluorescent protein. The significance of YFP versus GFP is that it can be used as an indicator of intracellular pH at pH > 6.0, whereas GFP detects acidic pH changes. dEYFP expression was determined by BD FACS, with an excitation of 488 nm, and an emission of 530nm. Figure 7 shows presort expression and post sort expression of dEYFP in AP-1, wild-type NHE1, and F4MUTNHE cells. The purpose of sorting the high expressers was to obtain a single peak, which could be monitored for shifts in response to pH change. Multiple populations can be seen in figure 7 A-D, because it is a presorted population, whereas after sorting only a single peak is observed in E-G. This allows for accurate detection of spectral shifts in accordance to intracellular pH.
Figure 8: Stable dEYFP expression in transfected AP-1 cell lines

Destabilized YFP expression was determined by excitation with 488 nm argon laser, and excited at 530 nm. The X axis is dEYFP expression emission; the Y axis signifies cell count number. Figure A-D shows the dEYFP expression before sorting, whereas Figures E-G, are representative of the sorted population of dEYFP expressing cells.
3.3 Intracellular pH calibration

Different intracellular pH indicators have been used by others. Our initial studies were performed using the pH-sensitive fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM). Shown in Figure 8, are several examples of calibration samples using BCECF. Consistent results were not achievable when determining the ratio between the 535 nm and 610 nm. Such variability did not occur with the use of dEYFP. Due to this inconsistency, destabilized YFP was used as our indicator of intracellular pH changes.

To identify if dEYFP could be calibrated in AP-1 cell lines, cells were transfected with dEYFP (Figure 9). AP-1, wild-type NHE1, and F4MUTNHE cells were harvested and resuspended in a high potassium buffers and calibrated to different ranges of pH (7.0 - 8.0) along with 10 µM of Nigericin and incubated at room temperature for 30 minutes in the dark. Nigericin is an ionophore, which exchanges hydrogen for potassium, allowing for the cytosol to become equivalent to the pH buffer. After 30 minutes, samples were analyzed using a 488 nm Argon laser, with an emission of 530 nm. Unlike BCECF, destabilized YFP does allow for accurate intracellular pH calibrating. Figure 10 is representative of greater than 11 experiments performed.
To generate a calibration curve, Wild type NHE1 expressing cells were incubated with BCECF for 5 minutes at 37°C, and then analyzed on BD FACS Calibur which is excited with a 488 Argon laser. The fluorescence of BCECF was determined by taking the ratio between the emissions (485 nm)/ (430 nm). Each series represents a different experiment, thus showing the irreproducibility of BCECF.
Figure 10: Wild-type cells calibrated using dEYFP

Wild type NHE1 expressing AP-1 cells were incubated with 10 μM of Nigericin in different pH high potassium buffers for 20 minutes at room temperature, and then excited by 488 nm Argon laser. The Y-axis is emission of dEYFP, whereas the X-axis represents of the different pH buffers used to calibrate the cells. This figure is representative of > 50 similar experiments performed.
3.4 Effect of staurosporine on p38 MAPK activity and alkalinization

We next needed to determine if staurosporine, a known protease inhibitor, could activate p38 MAPK and thereby activate NHE1 (Figure 11). Wild-type NHE1 expressing AP-1 cells were incubated with varying concentrations of staurosporine (0 pm, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM) at 37°C with 5% CO₂ for 30 minutes. Each sample was then lysed and the cytosolic extracts were immunoprecipitated with phosphorylated p38 MAPK antibody. p38 MAPK activity was determined by use of an in vitro kinase assay as described in the materials and methods (Section 2.6). As shown in Figure 10, p38 MAPK is activated by staurosporine, with an optimal activity greater than 1nM.

Because p38 MAPK can be phosphorylated by staurosporine, we next wanted to determine if the addition of staurosporine could cause alkalinization (Figure 11). Wild-type NHE1 expressing cells were harvested and either prepared for calibration or for the pH determination experiment following previously described methods. After the cells were incubated in staurosporine for 30 minutes, they were analyzed by BD FACS Calibur with an excitation of 488 nm and an emission of 530 nm. When wild-type NHE1 expressing cells were exposed to different staurosporine concentrations, only 1 µM and 10 µM doses elicited a rise in intracellular pH. This data does not parallel with the optimal concentrations that induced p38 MAPK activity in figure 11. A possible explanation is that only cytosolic p38 MAPK was isolated and used in the in vitro kinase assay. The
higher concentrations of staurosporine may induce change in the intracellular localization of p38MAPK, such that the kinase may have translocated to the nucleus, or associated with the membrane complexes. This may indicate that high concentrations of staurosporine can activate p38 MAPK in a manner that enables it to translocate to membrane compartments associated with NHE1.
Figure 11: p38 MAPK activation via staurosporine stimulation.

Wild-type NHE1 expressing AP-1 cells were incubated with staurosporine for 30 minutes, followed by cell lysis. Cytosolic protein extracts were assayed for p38 MAPK activity using an in vitro kinase assay (Cell Signaling) following manufacture’s protocol.
Figure 12: Wild-type NHE1 cells responded to varying staurosporine concentrations

Wild type NHE1 expressing cells were incubated with varying staurosporine concentrations from 0 pM to 10 µM for 30 minutes. Intracellular pH was determined by dEYFP as described in the Materials and Methods.
3.5 NHE1 effect on apoptotic alkalization

We next wanted to determine if activated p38 MAPK could phosphorylate key site(s) on NHE1 to cause apoptotic alkalization. In figures 12-14, all cell lines were assayed using the same parameters. AP-1, wild-type NHE1, and F4MUTNHE expressing AP-1 cells were harvested, and then resuspended in either pH buffers containing 10 µM Nigericin or with a PBS pH buffer (1X PBS, 1% FBS, and 25 mM Hepes. Both NHE1 inhibitor (DMA) and p38 MAPK inhibitor (PD169316) were added to corresponding tubes, after the addition of staurosporine, samples were analyzed with an excitation of 488 nm and emission of 530 nm. The reason DMA and PD169316 were used, was to determine if the apoptotic alkalization was due to the activation of NHE1 and if it was regulated by p38 MAPK.

AP-1 cells which are devoid of NHE1 activity were treated with staurosporine or staurosporine with inhibitors. There was no visible effect on intracellular (Figure 12). In the absence of NHE1, AP-1 cells do not alkalinize, and these are not inhibited by DMA or PD169316.

Expressing wild-type NHE1 in AP-1 cells restored normal NHE1 activity, when treated with staurosporine. After the addition of both inhibitors, a decrease in alkalinization was observed. This indicated that not only is NHE1 necessary for alkalinization, but also that NHE1 was regulated p38 MAPK (Figure 13).
After demonstrating restoration of NHE1 activity by expression of wild-type NHE1, we next wanted to determine if the four putative p38 MAPK phosphorylation sites, previously identified by in vitro kinase assay, were necessary for activity. F4MUTNHE cells, which contain Alanines in place of threonine 717, serine 722, serine 725, and serine 728, were assayed using the same method discussed above. Alkalization in response to staurosporine was not restored by expression of F4MUTNHE1 in AP-1. Like the AP-1 cells, F4MUTNHE expressing cells were not able to alkalinize through NHE1 in response to apoptotic stimuli (Figure 14).

Wild-type NHE1 expressing cells displayed the only statistically significant difference (p< .00000136) between the negative control and staurosporine treatment. Because F4MUTNHE1 expressing cells did not alkalinize, we conclude that induction of apoptotic alkalization in response to staurosporine is due to the activation of p38 MAPK phosphorylation the putative site(s) of NHE1 cytosolic tail.
Figure 13: Apoptotic alkalization assay of AP-1

AP-1 cells transfected with dEYFP were incubated with 1 µM staurosporine (STS) with and without 200 µM DMA (NHE1 inhibitor) or 20 µM PD169316 (p38 MAPK inhibitor) and then excited with a 488 nm argon laser, with an emission of 530 nm. This is representative of n=11.
Figure 14: Apoptotic alkalinization of wild-type NHE1

Wild-type NHE1 expressing AP-1 cells, transfected with dEYFP were incubated with 1 μM staurosporine (STS) with and without 200 μM DMA (NHE1 inhibitor) or 20 μM PD169316 (p38 MAPK inhibitor) and then excited with a 488 nm argon laser, with an emission of 530 nm. The P values were calculated using Microsoft Excel T-test. The * symbolizes the P value was significant with < 0.05. For all samples n=11.
Figure 15: Apoptotic alkalinization of mutant NHE1

F4MUTNHE expressing AP-1 cells transfected with dEYFP, were incubated with 1µM staurosporine (STS) with and without 200µM DMA (NHE1 inhibitor) or 20 µM PD169316 (p38 MAPK inhibitor) and then excited with a 488 nm argon laser, with an emission of 530 nm. This is represented by n=11.
4 CHAPTER FOUR: DISCUSSION

The sodium hydrogen exchanger is a family of proteins with 10 different isoforms. NHE1 is important because it is ubiquitously expressed on the plasma membrane of most epithelial cells, and its primary role, is the regulation of intracellular pH. Activated NHE1 regulates intracellular pH by extruding protons and importing extracellular sodium ions. NHE1 is regulated by mitogenic stimuli such as growth hormone, osmotic stress, ultraviolet radiation, and cytokine withdrawal, which can activate several kinase pathways such as p38 MAPK, ERK, JNK, and NIK (Wang et al.; Abe, Baines, and Berk; Yan et al.). Our data suggests NHE1 has a normal function in response to growth signals to maintain intracellular pH and during apoptosis its function is subverted to promote cell death.

Normal function of NHE1 activity is regulated by mitogenic stimulation such as growth factors. The presence of growth factors has been determined to directly activate NHE1 by activating ERK which then phosphorylates the downstream kinase p90RSK. Phosphorylation of p90 ribosomal S6 kinase then in turn phosphorylates Serine 703 on the carboxyl terminus of NHE1 (Takahashi et al.). It was recently shown, that the phosphorylation of S703 creates a docking site for â14-3-3 protein, this binding allows for NHE1 regulation (Lehoux et al.). Proliferation and cell survival in other models have also been linked to 14-3-3 binding. ERK activation has been identified as a dependent mechanism for survival and proliferation. The proposed model described by Wu et al, shows phosphorylated ERM can bind to the carboxyl terminus (S703) of NHE1, which
act as a scaffolding protein to recruit other signaling complexes leading to activation of AKT, a known anti-apoptotic protein. In contrast, the role of activated NHE1 in apoptosis is far different.

NHE1 has always been associated with cell survival, because of its established role in cell proliferation, but recently it was identified also as a regulator of apoptosis (Khaled et al.; Wu et al.). Many recent publications in literature support the hypothesis that NHE1 activation leads to intracellular alkalinization, which activates downstream regulators such as AKT leading to an anti-apoptotic effect. In contrast, recent studies also determined that NHE1 activation also leads to apoptosis. NHE1 activation was found to induce the translocation of Bax to the mitochondria, which activates cytochrome c release causing apoptosis (Khaled et al.; Khaled et al.; Coakley et al.). The discovery that NHE1 is phosphorylated by p38 MAPK identified a new method to identify an early stage of apoptosis that takes place before mitochondrial breakdown during cytochrome C a caspase activator during late stage apoptosis.

In our studies we determined that p38 MAPK activates NHE1 by phosphorylation of one or more of the four putative sites on NHE1. The exact phosphorylation site(s) is not known and one or more sites may be involved. However, the established phosphorylation motif of p38MAPK is a serine-proline motif, so we can propose that two sites, Serine 723 and Serine 726 followed by prolines, could be involved in regulation of apoptotic alkalinization. The putative p38MAPK sites on NHE1 may also have additional functions essential for pH homeostasis.
In addition to p38MAPK, other kinases activated by diverse signaling pathways may also target NHE1. Figure 15 represents the current model for the role of NHE1 in cell growth and apoptosis. The difference between the two pathways is that ERK activation of NHE1 through phosphorylation of Serine 703 leads to cell proliferation and survival, whereas stress stimuli causes p38 MAPK activation phosphorylating NHE1 at a different site(s), causing apoptotic alkalization. The different outcome of the two pathways may be due to the magnitude of the change in pH through NHE1 activation. Phosphorylation of NHE1 through ERK in response to growth factors results in a pH change of less than 0.2 pH units, while stress/apoptotic stimuli activates p38MAPK resulting in a pH change of greater than 0.2 pH unit (Wu et al.).

The significance of these findings is the potential to develop a novel way to detect early apoptosis through stress stimulated phosphorylation of NHE1 through p38MAPK activation. NHE1 phosphorylated at one or more of the punitive p38MAPK sites could be used as a read out to characterize the effect of pharmaceuticals upon disease parameters regulated by NHE1 activity. Future studies will be focused on identifying the exact p38 MAPK phosphorylation site(s) on NHE1 carboxyl domain, determining the functional consequences of phosphorylation of the sites on NHE1 in healthy and apoptotic cells, and finally producing an antibody for detection of p38 MAPK phosphorylated form of NHE1.
Figure 16: Model of regulation of apoptosis via p38 MAPK activated NHE1

(A) Normal NHE1 function: In the presence of growth factors, ERK is upregulated leading to both 14-3-3 binding, an also scaffolding of signaling complex needed for AKT activation, both of which promote cell survival. (B) Apoptotic NHE1 function: In the presence of stress stimuli such as cytokine withdrawal, p38 MAPK becomes activated which then phosphorylates NHE1 causing apoptotic alkalinization. This alkalinization allows for BAX to translocate to the mitochondria, which causes cytochrome c release leading to apoptosis.
APPENDIX A: NHE family of isoforms
Table 2: NHE family of isoforms

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Distribution</th>
<th>Location</th>
<th>Function</th>
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</thead>
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<tr>
<td>NHE 1</td>
<td>Ubiquitous</td>
<td>Plasma membrane</td>
<td>Intracellular pH, cell volume, proliferation</td>
</tr>
<tr>
<td>NHE 2</td>
<td>Kidney, Gl tract</td>
<td>Apical membrane</td>
<td>Re-absorption of Sodium</td>
</tr>
<tr>
<td>NHE 3</td>
<td>Brush border, Renal Epithelia</td>
<td>Apical membrane</td>
<td>Sodium bicarbonate re-absorption</td>
</tr>
<tr>
<td>NHE 4</td>
<td>Kidney, Gl tract</td>
<td>Basolateral membrane</td>
<td>Promotes osmoregularity</td>
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<tr>
<td>NHE 5</td>
<td>Brain</td>
<td>Recycling endosomal pathway</td>
<td>Intracellular pH in neurons</td>
</tr>
<tr>
<td>NHE 6</td>
<td>Heart, Brain, and Skeletal Muscles</td>
<td>Intracellular in Mitochondria</td>
<td>Lysozomal biogenesis</td>
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<td>NHE 7</td>
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<td>Exchanges Sodium or Potassium for Hydrogen</td>
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<tr>
<td>NHE 8</td>
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<td>Apical membrane</td>
<td>May interact with NHE3</td>
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<td>NHE 9</td>
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<td>Late recycling endosome</td>
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<td>NHE 10</td>
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5 REFERENCES


3 Cavet, Megan E., et al. "Na+/H+ exchangers (NHE1-3) have similar turnover numbers but different percentages on the cell surface." AJP - Cell Physiology 277.6 (1999): C1111-C1121.


